FRDC
FISHERIES RESEARCH & DEVELOPMENT CORPORATION

Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry:

Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)




DAJ Stone, MA Booth and SM Clarke (editors)

June 2019

FRDC Project No. 2016-200.30 & NSW DPI. 2016-200.20

Government of South Australia
Primary Industries and Regions SA

SARDI
SOUTH AUSTRALIAN RESEARCH AND DEVELOPMENT INSTITUTE

NSW GOVERNMENT Department of Primary Industries

SKRETTING a Nutreco company

CLEANSEAS Australian Kingfish

RIDLEY

HUON AQUACULTURE

ISBN: 978-1-876007-17-1

Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027).

FRDC Project No. 2016-200.30 & NSW DPI. 2016-200.20

2019

Ownership of Intellectual property rights

This publication (and any information sourced from it) should be attributed to Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June.

Creative Commons licence

Disclaimer

Researcher Contact Details

Name:	David Stone
Address:	2 Hamra Avenue West Beach SA 5024
Phone:	08 8207 5400
Fax:	08 8207 5415
Email:	david.stone@sa.gov.au

FRDC Contact Details

Address:	25 Geils Court
	Deakin ACT 2600
Phone:	02 6285 0400
Fax:	02 6285 0499
Email:	frdc@frdc.com.au
Web:	www.frdc.com.au

In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

Cover Photograph Acknowledgements: clockwise from top left - Dr Craig Foster, Clean Seas Seafood; Dr Mark Booth, NSW DPI; Mr Matthew Whittle, Huon Aquaculture; and Dr Matthew Bansemer, SARDI.

Contents

Acknowledgments

The editors of this report would like to acknowledge that this project was supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia also contributed actively to the project through the participation of their representatives as members of the Steering Committee, Research Advisory Group, Technical Working Group meetings, attendance and presentation at annual Research Workshops, critique and input of technical information, and the provision and manufacture of experimental feeds.

The editors would especially like to acknowledge:

- The key executives that drove the development of the project: Prof Gavin Begg, Research Director, SARDI Aquatic Sciences; Dr Craig Foster, former Chief Executive Officer, Clean Seas Seafood; Dr Wayne O'Connor, Research Leader, DPI NSW; Dr Patrick Hone, Managing Director, FRDC.

- The members of the Steering Committee, some who changed over the course of the project: Dr Patrick Hone (Chair), Dr Jenny Cobcroft, Mr Josh Fielding and Mr Wayne Hutchinson, FRDC; Dr Craig Foster, Mr David Head, Dr Trent D'Antigana, Mr Dan Fisk and Mr Jay Dent, Clean Seas Seafood; Mr David Whyte and Mr Matthew Whittle, Huon Aquaculture; Dr Richard Smullen and his proxy Dr Michael Salini, Ridley; Dr Leo Nankervis, Skretting Australia; Ms Erica Starling, Indian Ocean Fresh Australia; Mr Greg Jenkins, South Metropolitan TAFE, WA; Prof Gavin Begg (deputy Chair) and Steven Clarke (Executive Officer), SARDI; and Dr Wayne O'Connor and Dr Mark Booth, NSW DPI.

- The three Senior Research Fellows or equivalents without whose contributions it would not have been possible: Dr Matthew Bansemer and Dr Sarah Catalano, SARDI Aquatic Sciences and Dr Igor Pirozzi, NSW DPI (and James Cook University), as well as Dr Stewart Fielder, Senior Research Scientist involved in the design and management of the broodstock research at the Port Stephens Fisheries Institute, NSW DPI.

- The key Technical Officers that supported the researchers; in particular, Mr Paul Skordas, SARDI Aquatic Sciences and Dr Basseer Codabaccus, Mr Ian Russell, Mr Justin Tierney and Mr Luke Vandenberg, NSW DPI.

- The Honours, Masters and six PhD students and their university co-supervisors that participated in this project (the PhD students were: through SARDI, Mr Benjamin Crowe, Flinders University; and Ms Samantha Chown and Mr Thibault Legrand, University of Adelaide; and through NSW DPI, Ms Caroline Candebat, James Cook University; Ms Angela Liu, University of New South Wales; and Ms Dam Thi My Chinh, University of Sunshine Coast.

Below is a list of all project participants (RAC = Research Advisory Committee member; SC = Steering Committee member; SS = Student Supervisor; TG = Technical Group member; TL = Theme Leader):

Australian Genome Research Faclity Ltd, SA	
Dr Naga Kasinadhuni	Bioinformatics Officer
Dr Paul Gooding	Senior Scientist
Dr Leanne McGrath	South Australian Accounts Manager
Dr Sonika Tyagi	Bioinformatics Officer
Clean Seas Seafood (SA)	
Dr Erin Bubner	R&D Coordinator

Dr Trent D'Antignana (SC, RAC, TG)	R&D Manager
Mr Jay Dent (RAC, TG)	R&D Manager
Dr James Fensham	Veterinarian
Mr Dan Fisk (RAC, SC)	General Manager - Aquaculture
Dr Craig Foster (RAC, SC, TG)	Former Chief Executive Officer
Mr Toby Fox	Health Team
Mr David Head (SC)	Chief Executive Officer
Dr Matt Landos	Veterinarian (Future Fisheries Veterinary Service Pty Ltd., NSW)
Mr Evan Reeves	Health Team
Mr Chester Wilkes	Feed Manager

Fisheries Research and Development Corporation

Dr Jennifer Cobcroft	NEAO Subprogram Leader
Mr Joshua Fielding	Senior Portfolio Manager – Research/ NEAO Subprogram Leader
Dr Patrick Hone (SC)	Managing Director
Mr Wayne Hutchinson	Portfolio Manager – Research/ NEAO Subprogram Leader

Huon Aquaculture (NSW)

Mr Trevor Chalker	Farm Assistant
Mr Jason Clark	Marine Operations Manager
Mr Charlie Coulson	Central Support Manager
Mr Daniel Creevey	Farm Assistant
Mr Phillip Dayton	Zone Manager
Mr Mark Dunkley	Farm Assistant
Ms Elisha Lovell	Farm Assistant
Mr Jayson Maartensz	Farm Assistant
Mr David McGough	Farm Assistant
Mr David Mitchell	Hatchery Manager
Mr Rhys Mitchell	Assistant Zone Manager
Mr David Morehead	General Manager Marine Operations
Mr Georgie Pember	Farm Assistant
Mr Jon Rowlings	Farm Assistant
Mr Matthew Whittle (RAC, SC, TG)	Group Development Manager
Mr David Whyte (RAC, SC, TG)	Group Technical Manager

New South Wales Department of Primary Industries

Mr Alex Armstrong	Fish Technician
Dr Mark Booth (RAC, SC, SS, TG)	Senior Research Scientist
Mr Luke Cheviot	Hatchery Manager
Dr Basseer Codabaccus	Technical Supervisor
Ms Emily Collier	Fish Technician
Dr Stewart Fielder	Senior Research Scientist
Mr Brendan Findlay	Fish Technician
Mr Steve Gamble	Fish Technician
Mr Nicholas Gibson	Fish Technician
Mr Martijn Johnson	Fish Technician

Ms Brooke McCartin	Fish Technician
Mr Dylan Nash	Fish Technician
Dr Wayne O'Connor (SC)	Research Leader
Dr Igor Pirozzi (SS, TG)	Senior Research Fellow
Mr Ian Russell	Fish Technician
Mr Justin Tierney	Fish Technician
Mr Luke Vandenberg	Fish Technician
Mr Daniel Yee	Fish Technician

Other project participants

Assoc. Prof. Cathy Abbott (SS)	Lecturer/ Research Scientist (Flinders University, SA)
Assoc. Prof. Andrew Barnes	(University of Queensland, QLD)
Mr David Blyth	Agriculture and Food (CSIRO, Bribie Island, QLD)
Mr Nicholas Bourne	Chemist, CSIRO Agriculture and Food (CSIRO, St Lucia, QLD)
Ms Susan Cheers	Chemist, CSIRO Agriculture and Food (CSIRO, St Lucia, QLD)
Ms Cheryl Day	Laboratory technician (University of Adelaide, SA)
Prof. Steve Donnellan (SS)	Affiliate Professor (University of Adelaide, SA)
Prof. Abigail Elizur (SS)	Research Scientist (University of the Sunshine Coast, QLD)
Dr Rebecca Forder	Lecturer (University of Adelaide, SA)
Prof. Robert Gibson (SS)	Senior Research Fellow (University of Adelaide, SA)
Assoc. Prof. James Harris (SS)	Lecturer/ Research Scientist (Flinders University, SA)
Mr Barney Hines	Chemist, CSIRO Agriculture and Food (CSIRO, St Lucia, QLD)
Mr Greg Jenkins (SC)	Director (South Metropolitan TAFE, WA)
Mr Ken Lee	Microbiology Technician (University of Adelaide, SA)
Prof. Graham Mair (SS)	Lecturer/ Research Scientist (Flinders University, SA)
Dr Debashish Mazumder (SS)	Senior Research Scientist (Australian Nuclear Science and Technology Organisation, NSW)
Dr Todd McWhorter (SS)	Senior Lecturer (University of Adelaide, SA)
Dr James Munro	Senior Lecturer/ Research Scientist (University of Adelaide, SA)
Assoc. Prof. Gavin Partridge (RAC, SC, TG)	Research Scientist (South Metropolitan TAFE, WA)
Dr Stephen Pyecroft	Senior Lecturer Veterinary Pathology (University of Adelaide, SA)
Prof. Jian Qin (SS)	Lecturer/ Research Scientist (Flinders University, SA)
Assoc. Prof. Jess Sammut (SS)	Lecturer/ Research Scientist (University of New South Wales, NSW)
Mr Joel Scanlon	Feed manufacturer (Aquafeeds Australia, SA)
Assoc. Prof. Kathryn Schuller (SS)	Senior Lecturer/ Research Scientist (Flinders University, SA)
Dr Cedric Simon	Team Leader, Aquaculture Nutrition and Aquaculture, CSIRO Agriculture and Food (CSIRO, St Lucia, QLD)
Dr Erica Starling (SC)	Director/ Owner (Indian Ocean Fresh Australia, WA)
Dr Fran Stephens	Director/ Owner (Aquatilia Healthcare, WA
Dr Amber Thomas	Director/Owner (Educational Statistical Consultancy, SA)
Dr Tomer Ventura (SS)	Research Scientist (University of the Sunshine Coast, QLD)
Prof. Johan Verreth (SS)	Research Scientist (Wageningn University and Research Centre, Holland)
Dr Laura Weyrich (SS)	ARC Future Fellow (University of Adelaide, SA)
Assoc. Prof. Jane Williamson (SS)	Lecturer/ Research Scientist (Macquarie University, NSW)
Dr Lindsey Wooley	Research Scientist (South Metropolitan TAFE, WA)
Dr Melissa Wos-Oxley	Honorary Associate (Museum of South Australia, SA)

Dr James Wynne (SS)	Senior Research Scientist (CSIRO, TAS)
Dr Emmanuelle Zoccola	Researcher (The University of Queensland, QLD)

Ridley (Narangba, QLD)

Dr Michael Salini (RAC, TG)	Product Developer
Dr Richard Smullen (SC)	Manager
Dr Simon Tabrett	Nutritionist

Skretting Australia (Cambridge, TAS)

Dr Matthew Bransden	Technical Account Manager
Dr Leo Nankervis	Marketing Manager
Dr Nicole Ruff	Product Manager

South Australian Research and Development Institute (SA)

Dr Matthew Bansemer (SS, TG)	Research Scientist
Prof. Gavin Begg (SC)	Research Chief
Ms Jessica Buss	Technical Officer
Dr Sarah Catalano (SS, TG)	Research Scientist
Mr Steven Clarke (SC)	Senior Research Scientist (Executive Officer)
Ms Krishna-Lee Currie	Technical Officer
Mr Paul Czechowski	Technical Officer
Dr Marty Deveney (TL)	Senior Research Scientist
Ms Filipa Duarte	Technical Officer
Mr Leigh Keurschner	Technical Officer
Dr Andrew Oxley (RAC, SS, TG, TL)	Senior Research Scientist
Mr Mark Purvis	Technical Officer
Mr Paul Skordas	Research Services Officer
Assoc. Prof. David Stone (RAC, SS, TG, TL)	Senior Research Scientist
Ms Nichole Thomson	Technical Officer

Students

Ms Caroline Candebat	PhD student (James Cook University, QLD)
Ms Dam Thi My Chinh	PhD student (University of the Sunshine Coast, QLD)
Ms Samantha Chown	PhD student (University of Adelaide, SA)
Mr Ben Crowe	PhD student (Flinders University, SA)
Mr Jackson Doherty	Honours student, 1st class (Flinders University, SA)
Mr Leigh Kuerschner	Honours student, 2A (Flinders University, SA)
Mr Thibault Legrand	Honours student 1st class and PhD student (University of Adelaide, SA)
Ms Angela Liu	PhD student (University of New South Wales, NSW)
Ms Marina Rubio	Masters research intern (Wageningen University and Research Centre, Holland)
Mr Aaron Teoh	Honours student, 1st class (Flinders University, SA)

Plain English Summary

Introduction

The project "Growing a profitable, innovative and collaborative Australian Yellowtail Kingfish aquaculture industry: bringing 'white' fish to the market' was undertaken from 2015-2019 as part of the Rural R&D for Profit Programme, Department of Agriculture and Water Resources (DAWR), Australian Government.

Yellowtail Kingfish (*Seriola lalandi* - YTK) farming was identified by the Fisheries Research and Development Corporation (FRDC) as the greatest opportunity for new aquaculture development in Australia in the next few decades through substantial increases in farmed area and product, and the use of aquafeeds, resulting in growth in regional economies and employment. The key challenge to achieving this growth for YTK was for industry to diversify its focus from supplying only the relatively small volume, high price sashimi market to the larger volume, lower price Australian 'white fish' market, while enhancing farm productivity and reducing operating costs to maintain profitability and improve sustainability. Feed and feeding strategies comprise 60% of YTK farming operating costs and reducing these costs were the industry's highest common research and development (R&D) priorities.

More specifically, this project was designed to provide new information to assist industry to grow its position by developing more cost effective, sustainable feeds and feeding strategies to enhance YTK health and production.

Benefits to Producers

At completion of the project, the independent project impact assessment identified economically quantifiable benefits to the Australian YTK industry in the areas of increased productivity and profitability, which flowed from:

- improvements to YTK specific growth rates and food conversion ratios as a result of improved feed composition and/or adoption of optimal feeding strategies for different water temperatures and YTK size/age classes;
- reduced production losses because of improved management of YTK nutritional health (i.e. reduced incidence of disease); and
- reduced input costs along the supply chain, particularly for aquafeeds, because of optimised more sustainable feed formulations and use of cheaper sources of protein to replace fish meal.

The unquantified project benefits were identified as increased efficiency and capacity of future YTK R&D and enhanced community well-being, which flowed from:

- strengthened Australian YTK R&D networks across regions and between industry participants and greater knowledge of YTK R&D, including methodologies; and
- flow-on effects of a more productive, profitable and sustainable YTK aquaculture industry on the social 'fabric' of Australian regional population centres and the environment where improved marine biodiversity and water quality was likely as a result of reduced nutrient loadings through improved feed composition, feeding strategies and feed assimilation.

The independent project impact assessment conservatively estimated that DAWR's $3.65 investment in this project produced, in present value terms, an estimated total benefit of $126.63 million, net present value of $119.26 million, benefit-cost ratio of 17.2:1, internal rate of return of 46.5%, and a modified internal rate of return of 16.1%, all based on a medium coverage of benefits and confidence in assumptions. However, it was also noted that these figures were highly dependent on the underlying YTK aquaculture production data, which included recently announced expected future production from Western Australia and that if these production figures were excluded the benefit to-cost ratio would still be 3:1.

Objectives

The project's focus was on growing the production and profitability of the Australian YTK aquaculture industry.

To achieve this the project had three primary research aims:

Nutrition

Identify economically sustainable feeds and improved diet formulations to reduce the costs of commercially available YTK feeds by:

- evaluating alternative Australian farm protein and oil sources and the levels that they should be added in YTK diets so as to reduce the necessity for using more costly and potentially less sustainable wild derived fish meal and fish oil;
- investigating the potential to reduce the level of protein that is required in YTK feeds by using higher energy and lower protein diets;
- developing YTK summer and winter diet formulations that use optimal oil types and levels;
- identifying the dietary needs of YTK for select essential dietary nutrients, particularly amino acids and fatty acids; and
- investigating the costs and benefits of dietary supplements.

Feeding Strategies

Develop improved YTK feeding strategies that can improve producers' profits by:

- comparing the effects of optimal feed formulations and feeding strategies developed as a result of this project's R&D with a commercially produced and fed YTK feed;
- evaluating the costs and benefits of using high as compared to low energy feeds at warm and cool water temperatures to determine the optimum number of times YTK should be fed;
- evaluating the most advantageous combination of diet and feeding frequency for YTK;
- evaluating the effect of feeding strategy and diet on the health and capacity of YTK broodstock to be successfully bred and produce juveniles; and
- developing an improved feeding schedule for YTK based on the incorporation of more information on water oxygen levels and temperature on fish nutrient and energy utilisation.

Nutritional Health

Evaluate methods to determine the health of YTK though the analysis of the gut bacteria community (microbiome), immune system and blood chemistry and understand how different diets and feeding strategies can affect fish health and production by:

- developing a method to better evaluate fish health when undertaking nutrition and feeding strategy R&D;
- collecting data on the health of the blood and key organs of YTK used in this project's R&D to ensure the nutrition and feeding strategy outcomes are for healthy fish and commercially meaningful;
- characterising the type and abundance of the microbiome within the digestive system of YTK so that they might be managed to enhance YTK production in the future; and

- collecting information to understand how the YTK environment, growth, farm management procedures, disease and parental stock influence the microbiome of the YTK digestive system. The project also had the aim to:

Enhance the capability, knowledge and networking of project participants and the broader Australian aquaculture industry by:

- disseminating project information through the holding of workshops, provision of presentations at conferences, and the publication of popular and scientific articles; and
- training the students engaged in the project so as to develop the next generation of industry R&D providers; and
- incorporating the outcomes of the project more broadly so as to allow the extension and translation outputs of this project to other Australian YTK producers, and Australian aquaculturists producing other 'white flesh' fish such as Cobia and Mulloway.

Methods and Outputs

The research in this project involved juvenile through to broodstock YTK, primarily held in tanks in the onshore environmentally controlled facilities of the two key research providers, the South Australian Research and Development Institute (SARDI), Adelaide, South Australia and the New South Wales Department of Primary Industries (NSW DPI), Port Stephens, New South Wales.

The key outputs were:

Nutrition

Nutrition experiments were undertaken using a series of treatments and a control against which they could be compared, typically with 3-4 replicate tanks holding multiple YTK for each experimental treatment. The research demonstrated the relative capacity of YTK to digest nutrients and energy from a wide range of marine and land animal and plant protein and oil sources, and that the use of the optimum ones evaluated can be incorporated into commercial diets to reduce dependence on wild fish meal and oil sources, thereby increasing diversity of choice, reducing costs and enhancing sustainability. The optimum levels of protein, oil and energy, as well as select essential amino acids and dietary supplements to use in feeds were also defined, including in some instances when fed at summer and winter water temperatures.

Feeding Strategies

Feed strategy experiments were typically undertaken using a series of treatments and a control, in replicated tanks holding multiple fish, although in two instances they involved using YTK in replicated cages in ponds.

The optimal feeding frequency was defined for two sizes of juvenile YTK under particular environmental conditions (e.g. summer as compared to winter water temperatures), as was the relationship between dissolved oxygen in the water and the health and performance of YTK. A traditional 'best practice' Sardine and Squid broodstock feeding strategy was also compared to a more convenient manufactured pellet feed one and the benefits and costs of each determined.

Nutritional Health

In general, it was demonstrated from digestive tract histology, blood haematology and biochemistry that the YTK used in the nutrition and feeding strategy experiments were healthy, and the results of these experiments were not impacted by the presence of unhealthy fish. A 'challenge test' was also developed and validated that could be used to characterise the health ("robustness") of YTK used in nutrition and feeding strategy experiments in tanks.

The experiments used to evaluate the microbiome (bacteria community) associated with the digestive system and skin of YTK were either achieved by sampling nutrition and feeding strategy experiments in tanks; sea-cages on a commercial farm where differences existed in various farm management practices, including feed type, disease status and genetic stock; or wild sourced YTK. The project provided the first detailed descriptions of the microbiome in Australian YTK in relation to a wide range of different factors. It was found that in general, an increase in dominance by some bacterial species and a significant reduction in the diversity of other bacterial species occurred as a result of disease, farming and the feeding of a specific diet. However, this was not always clear, suggesting that many interacting factors contribute to the nature of the microbiome in the YTK digestive system.

A novel manipulation experiment undertaken at the end of the project showed that the gut and skin microbiome of YTK can be modified, important if this research is to lead to the development of proactive techniques to manage YTK health. This experiment also demonstrated that the application of an antibiotic to control a common YTK disease may exacerbate the issue by allowing the proliferation of other bacteria species.

Outcomes

The key outputs were:

Nutrition

The results of the nutrition research have provided aquafeed manufacturing companies with information to improve YTK feed formulations and the cost effectiveness of commercial feeds. The provision of these improved commercial feeds will increase on-farm productivity, profitability and/or reduced operating costs, as well as enhanced the on-farm environment through improved nutrient utilisation.

Feeding Strategies

The results of the feeding strategy research has enabled YTK aquaculturists to optimise the frequency and amount of feed fed on-farm to closer align with the nutritional requirements of YTK and the environment in which they are farmed. This has already reduced on-farm operating costs, increased profitability and enhanced the farm environment.

Nutritional Health

Sampling methodologies, such as a challenge test, parasite monitoring and treatment, digestive tract histology, blood haematology and biochemistry, and microbiome assessment, were advanced for determining YTK nutritional health. A large reference data set was also established and early detection markers of changing health status proposed for two on-farm health issues. These advances will lead to increased on-farm productivity and profitability.

Collaboration

As a result of the project, strong relationships were built between researchers from the two research organisations involved, researchers and the four industry participants, and the six supporting universities. The four Honours, two Masters and six PhD students, and three postdoctoral fellows (or equivalent), benefited greatly from the applied research and opportunity for close interaction with industry. Students, researchers and project technical staff, both from research organisations and industry, also participated in a number of training sessions, with the ‘YTK Health Training Workshop’ the most substantial.

Extension

A wide range of extension activities were undertaken as part of the project, with the annual 'K4P Research Workshop' a highlight. Many workshop and conference presentations (verbal and posters) were given, and a number of popular and scientific publications produced, with more underway. As a final project extension activity a presentation was given as part of the 'Fisheries - making the most from a renewable resource' session at the ABARES Outlook 2019 conference, held in Canberra on the 5th and 6th March 2019.

1. Executive Summary

1.1. What this report is about

This project (Growing a profitable, innovative and collaborative Australian Yellowtail Kingfish Aquaculture Industry: bringing 'white' fish to market. RnD4Profit-14-01-027) was part of the Rural R&D for Profit Programme, Department of Agriculture and Water Resources, Australian Government and ran from 1st May 2015 to the 31st March 2019. It focused on growing the production and profitability of the key existing Australian Yellowtail Kingfish (YTK) industry participants, as well as the industry as a whole, and directly addresses FRDC's new strategic plan to build Australian sustainable aquaculture development through the activities of the new 'New and Emerging Aquaculture Opportunities' (NEAO) Subprogram. The project also aligns with the National Marine Science Plan to grow the blue economy and the national Aquaculture Statement and Strategy to grow Australian aquaculture production, and the national Research Providers Network (RPN) to better coordinate fisheries and aquaculture R&D resources nationally.

This project builds on earlier nutrition research on YTK undertaken through the Fisheries Research and Development Corporation (FRDC) and the Australian Seafood Cooperative Research Centre (ASCRC) and delivers specific outcomes for the YTK industry partners of this project. It will also provide benefits to the broader finfish aquaculture industry, particularly the sectors targeting the production of 'white' fish (e.g. Barramundi, Cobia and Mulloway). Some indirect benefits will also flow to the community and the environment through the development and assimilation of better more sustainable feeds and feeding strategies. The project centred around two key Australian YTK aquaculture companies; Clean Seas Seafood (South Australia - SA) and Huon Aquaculture (New South Wales - NSW), but also interacted with Indian Ocean Fresh Australia, the only other Australian company developing YTK farming. The SA and NSW YTK producers were, respectively, aligned with the South Australian Research and Development Institute (SARDI) and the New South Wales Department of Primary Industries (NSW DPI), two major Australian research institutions, and Australia's two largest aquafeed companies (Ridley and Skretting Australia). The project provided all participating partners the opportunity to work closely together enabling farm production of Australian YTK to expand and with this expansion broaden its marketing from the high value, lower volume sashimi market to the lower value, higher volume 'white fish' whole fish and fillet market in Australia.

1.2. Background

Yellowtail Kingfish (*Seriola lalandi*; YTK) farming was identified nationally by the FDRC and Industry participants as the greatest opportunity for new aquaculture development in Australia in the next few decades through substantial increases in farmed area and product, and use of aquafeeds, resulting in growth in regional economies and employment. At the start of the project it was predicted that within 10 years, Australian YTK production is expected to increase by 34,000 tonnes, worth $440 million, and using 68,000 tonnes of aquafeed worth $136 million (estimate based on collective inputs of initial project participants), although FRDC, in documenting its NEAO Subprogram objectives, indicated a more conservative level of 2,500 tonnes within 5 years for all Australian white fish. The key challenge to achieving this growth for YTK is for industry to diversify its focus from supplying only the relatively small volume, high price sashimi market to the larger volume, lower price Australian 'white fish' market, while enhancing farm productivity and reducing operating costs to maintain profitability and improve sustainability. More specifically, this project was designed to provide new information to assist industry to grow its position by developing more cost effective, sustainable feeds and feeding strategies to enhance YTK production and health; the industry's highest common R&D priorities as feed and feeding strategies comprise 60% of operating costs.

1.3. Aims/objectives

This project was part of the Rural R&D for Profit Programme, Department of Agriculture and Water Resources, Australian Government. It aligned with the Round 1 Programmes priorities:

1. Increase the profitability and productivity of primary industries
    - Help producers increase yields and/ or reduce costs by applying innovative technologies and/ or technologies from other industries.
    - Help producers manage natural resources in an integrated way at enterprise or regional level for long-term use and profit.
2. Strengthen primary producers' ability to adapt to opportunities and threats
    - Integrate data and deliver information to help producers manage risk, benchmark performance and make production decisions for greatest profit.
3. Strengthen on-farm adoption and improve information flows
    - Consolidate knowledge of extension and adoption to better deliver practical results to primary producers, founded on what producers want from extension services.
    - Identify practical proposals to stimulate private sector extension services, particularly to fill current gaps.
    - Identify practical means to co-ordinate extension services for producers, including the development of tools and/or platforms.

To meet with these Rural R&D for Profit Programme priorities, the project addressed the Australian YTK industry's key common R&D priorities, both at conception during 2014/15, and throughout the course of the project, through three key themes and their associated activities and outputs. The three key themes were:

Theme 1 Nutrition;

Theme 2 Feeding Strategies; and

Theme 3 Nutritional Health.

The key activities of this project central to the efficient and effective delivery of its objectives/outputs were:

1. Project initiation and management;
2. Identify economically sustainable feeds and improve diet formulation;
3. Improve feeding strategies to increase profit;
4. Improve nutritional health to boost productivity; and
5. Extending YTK capability.

The project activities were:

Activity 1. Project initiation and management

Output 1(a) Establish steering and research advisory committees and provide their terms of reference

Output 1(b) Execute agreements and contracts with partner organisations and service delivery agents as needed

Output 1(c) Finalise an extension and communication strategy. The strategy must include communications and extension activities including, but not limited to publications, workshops and newsletters

Output 1(d) Create a monitoring and evaluation plan for the project

Output 1(e) Undergo end of project evaluation in accordance with output 1(d) and provide a report to the department. The evaluation must report on the projects outcomes against the program objective, including quantitative information on the outcomes achieved and independent expert analysis of expected and/or demonstrated quantifiable returns on investment

Activity 2. Identify economically sustainable feeds and improved diet formulations (Nutrition theme)

Output 2(a) Evaluate alternative Australian farm protein and oil sources and identify their ideal inclusion levels in juvenile and sub-adult production diets to reduce dependence on fishmeal and fish oil

- Determine the apparent digestibility coefficients (ADC) of common raw materials by sub-adult YTK

Output 2(b) Investigate protein sparing effect of using higher energy and lower protein diets

Output 2(c) Develop winter diet formulations that use ideal lipid types and levels for less than two kilogram YTK during periods of suboptimal water temperatures

Output 2(d) Determine dietary requirements of selected essential nutrients for juvenile and sub-adult YTK

- Determine the histidine requirements of juvenile YTK
- Determine the choline requirement of juvenile YTK
- Determine the taurine requirement of juvenile YTK
- Determine the methionine requirement of juvenile YTK

Output 2(e) Investigate the cost-benefit of using dietary supplements to improve the production of juvenile and sub-adult YTK

- Investigate the use of prebiotic and probiotic bioactive supplements on growth, digestibility and gut health in sub-adult YTK

Activity 3. Improve feeding strategies to increase profit (Feeding strategies theme)

Output 3(a) Evaluate optimal feeding strategies for juvenile and sub-adult YTK, including but not limited to comparing experimental nutrient-dense and commercially available feeds, floating versus sinking feeds, feed sizes and feeding strategies

- Validation trial in pond cages to asses growth and FCR on newly developed feeds and feeding strategies for juvenile and sub-adult YTK (fishmeal origin)
- Benchmark study in pond cages of a commercial diet and feeding strategies for sub-adult YTK on the NSW DPI – Huon Aquaculture Marine Aquaculture Research Lease (MARL) (fishmeal reduction)

Output 3(b) Evaluate the cost-benefit of using high versus low energy feeds for juvenile and sub-adult YTK at varying water temperatures

- Determine optimum feeding frequencies in warm water (24 °C) with sub-adult YTK grown towards market size
- Determine optimum feeding frequencies in cool water (16 °C) with sub-adult YTK
- Evaluate the effects of feeding strategy and diet specification on performance of sub-adult YTK

- Evaluate impacts of dietary shift on reproductive output and health of YTK broodstock (3 feeding experiments)

Output 3(c) Develop an improved feed ration model for on-farm YTK feed management

- Critical oxygen threshold and hypoxia tolerance in juvenile YTK
- Utilisation and maintenance requirements of juvenile YTK; quantifying abiotic factors (temperature and dissolved oxygen)
- Refine bioenergetic model for YTK and develop a predictive farm-based management tool for YTK

Activity 4. Improve nutritional health to boost productivity (Health theme)

Output 4(a) Develop a challenge test method for fish health evaluations associated with tank based nutrition and feeding strategy R&D
- Further refine the challenge model by better understanding the YTK immune system

Output 4(b) Collect histopathology and blood chemistry data of diseased and healthy YTK to characterise the general health of YTK used in tank based nutrition and feeding strategy R&D
- Further refine the role of the gut microbiome in YTK gastrointestinal health by sampling additional wild fish in SA for subsequent histological and microbiomic evaluations

Output 4(c) Characterise and understand the microbiome of the digestive system of YTK in particular in relation to different diets and feeding strategies, and how this might be managed to enhance YTK health, diets or food conversion ratios
- New health theme activity - manipulation of the microbiome of diseased YTK

Output 4(d) Collect baseline data to differentiate the effects of the environment, YTK growth and farm production cycle, disease and different genetic cohorts on the microbiome

Activity 5. Extending YTK capability

Output 5(a) Conduct workshops and provide publications to extend the outputs from the project to industry participants, and the broader aquaculture industry, scientific community and public in line with output 1(c)

Output 5(b) Student training to develop the next generation of industry R&D providers including up to three postdoctoral research fellows, up to six PhD students and up to 12 Honours students

Output 5(c) Incorporate the outcomes of the project into the new subprogram established by the FRDC or the development of new and emerging aquaculture growth opportunities to allow the direct extension and translation of outputs to potential 'white' fish and other new and emerging aquaculture opportunities

Performance indicators

The key performance indicators for this project for the scientifically and technically orientated Activities 2, 3 and 4 are based on Australia's leading YTK producers, identifying just prior to the start of the project that a move from the sashimi to the 'white' fish market requires meeting the following criteria:

- A fingerling equivalent of 3.0 kg weight per fingerling within 2 years;
- A feed conversion ratio (FCR) of ≤ 1.5 and ≤ 2.2 for fish between 0.01 - 1.5 kg and 1.5 - 3.5 kg, respectively; and
- Survival of >90% from the stocking of fingerlings until harvest.

1.4. Results and key findings

The project results and key findings identified by activity were:

Activity 1: Project Initiation and Management

- The project management structure is outlined in Section 7. Communication and Extension. A project Steering Committee, Research Advisory Committee and Technical Group were formed, each with a 'Terms of Reference', to drive, manage and deliver the objectives of this project. An Executive Officer was also appointed that participated in all Committees/Groups. Membership of each Committee/Group ranged from a dominance of executives on the Steering Committee to researchers on the Technical Group. Each Committee/Group included representatives from each state research organisation and participating company involved, as well as FRDC. The Steering Committee reported to the FRDC that reported to the Department of Agriculture and Water Resources, Australian Government. The Steering Committee was required to meet at least twice yearly, but met quarterly, 3 times a year by teleconference and once a year face-to-face. The Research Advisory Committee met as required and the Technical Group typically met monthly.
- Agreements and contracts with partner organisations and service delivery agents were completed as needed and the Department of Agriculture and Water Resources informed of this through milestone reporting.
- An extension and communication strategy and monitoring and evaluation plan were created and submitted to the Department of Agriculture and Water Resources. Agtrans was contracted to provide an independent Impact Assessment of the project.

Activity 2. Nutrition: Identify economically sustainable feeds and improved diet formulations

- We were able to successfully reduce wild derived fish meal (WD FM) inclusion levels by 33.3 to 66.7% which led to reduced diet costs (up to \$150 tonne^{-1}) and improved the sustainable production of large YTK (i.e. new diets improved the fish in-fish out ratio [FIFO] by up to 35.1%) (Output 2(a); Manuscript 3.1.3.1).
- We recommend that when using soy protein concentrate (SPC), diets contain no less than 20% WD FM. When using poultry meal (PM,) we recommend that diets contain no less than 20% FM (WD or FM by-product). When using FM by-product, we recommend that diets contain a total of 30% FM, where at least 10% is derived from wild stocks, and no more than 20% is FM by-product (Output 2(a); Manuscript 3.1.3.1).
- Poultry oil is suitable for high inclusions (up to ~18%) in production diets for large YTK. This is dependent on total dietary lipid levels and season (Output 2 (a) and 2(c); Manuscript 3.1.1.2).
- Canola oil dietary inclusion in YTK production diets should be limited to ≤ 4% in a 25% total lipid diet (Output 2 (a) and 2(c); Manuscript 3.1.1.2).
- The nutrient and energy profiles as well as the apparent digestibility coefficients (ADC) of protein, amino acids, lipid and gross energy of 14 common raw materials were determined for sub-adult YTK. The raw materials examined included: two sources of fishmeal; two sources of poultry by-product meal; two sources of lupin meal; two sources of soy protein concentrate; a single source of krill meal, meat meal, blood meal, faba beans, corn gluten meal and wheat. The results indicate that sub-adult YTK are efficient at digesting nutrients and energy from marine and land animal protein sources. Plant proteins such as faba beans, and lupins appear to have relatively high protein and energy digestibility and may prove useful as secondary protein and energy sources in aquafeeds for sub-adult YTK. Digestibility of blood meal and corn gluten was poor. The ADCs derived for the raw materials examined in this study will assist in the formulation of research and commercial aquafeeds for this developing aquaculture species. (Output 2(a); Manuscript 3.1.4.1).

- The incorporation of LYSOFORTE® Liquid at a concentration of 40 mg kg lipid^{-1} in high (30%) or low (20%) lipid diets did not improve lipid utilisation or health for large YTK at cool winter water temperatures (Outputs 2(b) and 2(e); Manuscript 3.1.2.1).
- During summer, on a practical basis, we recommend that diets for large (2.0-3.5 kg) YTK at warm water temperatures contain a crude protein (CP) level of 43% (digestible protein [DP] 37%), a crude lipid (CL) level of 25% (digestible lipid [DL] 24%), a gross energy (GE) level of 20 MJ kg^{-1} (digestible energy [DE] 17 MJ kg^{-1}) with a CP:GE ratio of 21.6 g CP MJ^{-1} GE (21.8 g DP MJ^{-1} DE). Results confirm that current commercial diets are adequately formulated, in terms of protein, lipid and energy levels, for optimal growth of large YTK at warm water temperatures. However, further gains in growth performance may be achieved with advancements in our knowledge of specific essential amino acid requirements (Output 2(b); Manuscript 3.1.2.2).
- During winter, high lipid diets (up to 30%) led to improvements in weight gain and FCR (2.08 in fish fed 30% lipid diets vs 2.37 in fish fed 20% lipid diets), while health was not negatively impacted. During winter high dietary lipid (energy) level also improved whole fish yield but not dress-out yield (gutted, head on and gills in). This has implications for dietary lipid/energy selection for production, processing and market selection (Output 2(b); Manuscript 3.1.2.1).
- In summer the long chain n-3 polyunsaturated fatty acid (LC n-3 PUFA) intake rate for optimal growth and FCR of large YTK was 191 mg LC n-3 PUFA kg fish^{-1} d^{-1}(Output 2(c); Manuscript 3.1.1.1). In winter the LC n-3 PUFA intake rate for optimal growth and FCR of large YTK was more variable and ranged from 164 to 233 mg LC n-3 PUFA kg fish^{-1} d^{-1}(Output 2(c); Manuscript 3.1.1.2).
- On a practical basis, based on SGR and FCR variables this equated to estimated optimal dietary LC n-3 PUFA levels of between 2.12 to 2.26 g 100 g^{-1} (95% CI for each response variable ranged between 1.90 to 2.33 g 100 g^{-1} and 1.93 to 2.58 g 100 g^{-1} for SGR and FCR, respectively) for summer and winter water temperatures for large YTK. All estimates are model specific (Output 2(c); Manuscripts 3.1.1.1 and 3.1.1.2).
- Optimising LC n-3 PUFA levels in diets by reducing dietary fish oil inclusions lead to improved feed utilisation, diet sustainability and diet cost savings for large YTK (Output 2(c); Manuscript s 3.1.1.1 and 3.1.1.2).
- The digestible choline requirement of juvenile YTK reared at 16 °C was found to be 27.3 mg kg BW^{-1} d^{-1} when using choline deposition rate as the response variable or 26.1 mg kg BW^{-1} d^{-1} when using SGR as the response variable. The 95% CI for these estimates ranged between 20.9 to 36.1 mg kg BW^{-1} d^{-1} when based on choline deposition and 21.6 to 31.5 mg kg BW^{-1} d^{-1} when based on specific growth rate. All estimates are model specific (Output 2(d); Manuscript 3.1.5.1).
- The minimum dietary requirement for histidine in juvenile YTK was found to be < 7.45 g kg^{-1} diet. Although an absolute histidine requirement was not quantified, current industry feeds available for YTK should easily meet this specification (Output 2(c); Manuscript 3.1.5.2).
- The study on the sulphur amino acid requirements of juvenile YTK found that juvenile YTK require a digestible taurine intake of 1.71 g kg BW^{-1} d^{-1} at an average methionine intake of 3.43g kg BW^{-1} d^{-1} to optimise growth. In addition, no dietary taurine supplementation is required if enough dietary methionine is provided in the diet. Furthermore, results indicated methionine can spare taurine and cysteine can spare methionine in diets for juvenile YTK. Juvenile YTK require a methionine intake of 6.3 g kg BW^{-1} d^{-1} at an average cysteine intake of 2.3 g kg BW^{-1} d^{-1}. Exceeding a methionine intake of 7.8 g kg BW^{-1} d^{-1} at a cysteine intake of 1.6-2.7 g cysteine kg^{-1} may depress growth rate in YTK. All estimates are model specific (Output 2(c); Manuscript 3.1.5.3).
- The incorporation of LYSOFORTE® Liquid at a concentration of 40 mg kg lipid^{-1} in high (30%) or low (20%) lipid diets did not improve lipid utilisation at winter water temperatures, and as such, was un economical to use (Output 2(e); Manuscript 3.1.2.1).
- Adding small amounts of spent brewer's yeast, inulin powder, Protexin® powder or Pro(N8)ure®-IFS powder to a soy-based control diet did not improve SGR, relative feed intake or FCR in juvenile YTK. Moreover, adding these bioactives to a soy-based control diet did not alter concentrations of

cholesterol, triglycerides, total protein, glucose, lactate or aspartate aminotransferase (AST) in the plasma of juvenile YTK. Juvenile YTK reared at 20 °C can be fed optimally formulated diets containing 25% and 5% of soybean meal and SPC, respectively, without incurring any loss in short term production (Output 2(e); Manuscript 3.1.6.1).

Activity 3. Feeding strategies: Improve feeding strategies to increase profit

- Feed utilisation, oxidative stress and growth of large YTK were negatively impacted by reductions in dissolved oxygen saturation levels. More so, when fish were exposed to irregular hypoxic events similar to those experienced during dodge tides in SA waters. For example, FCRs were ≤ 1.73 and tended to increase (worsen) as dissolved oxygen levels decreased. This information has led to alterations in the criteria for site selection processes for YTK production in Australia. Results may also be used to adapt new improved feeding strategies to maximise YTK production (Output 3(a); Manuscript 3.2.1.1).
- A pond based field trial produced encouraging results on fishmeal reduction and the use of different fishmeal sources in aquafeeds for juvenile YTK reared under fluctuating abiotic conditions. The results demonstrated that the dietary level of prime fishmeal can be reduced from 55% to 15% without short term productivity being affected when fishmeal reduction is offset by inclusion of other high quality proteins. The economic (measured as reduction in raw material cost) and environmental benefits of feeding a low fishmeal diet were reflected in a 24% reduction in raw material cost and a 46% reduction in the FIFO of the low fishmeal diet, respectively (Output 3(a); Manuscript 3.2.5.1).
- A second pond trial demonstrated that 30% fishery by-product meal can be used to wholly replace an equivalent amount of prime fishmeal in diets for juvenile YTK without significantly affecting short term production outcomes. While there was little economic benefit (measured as reduction in raw material cost) in using 30% fishery by-product meal to replace an equivalent amount of prime fishmeal in diets for YTK, there was a 45% reduction in the FIFO of the fishery by-product meal diet. These results confirm there is enormous scope in not only the choice of alternative protein sources for YTK but also a high degree of formulation flexibility (Output 3(a); Manuscript 3.2.5.1).
- During winter, large YTK fed the commercial formulated diet to apparent satiation six days week^{-1} exhibited significantly higher growth rates and numerically superior FCR than fish fed the same diet at lower feed rates. By adopting the new winter feeding strategies of feeding 6 times per week, based on results from Manuscript 3.2.3.1, a saving of ~$350,000 each winter (annum) for the production of 2,000 tonnes of Yellowtail Kingfish may be achieved (personal communication, Dr C. Foster; former CEO, Clean Seas). When this practice is extrapolated and applied to the future targeted annual production levels of 34,000 tonnes of Australian YTK, a saving of $5,950,000 per annum would be achieved (Output 3(b); Manuscript 3.2.3.1)
- During summer, in order to improve growth rate and FCR large fish may be fed to apparent satiation at least twice daily at water temperatures > 20 °C, and fed to apparent satiation once daily as water temperatures drop from 20 °C to 16 °C, and potentially lower (Output 3(b); Manuscript 3.2.3.2)
- Feeding frequency trials under controlled abiotic conditions (16 °C vs 24 °C) have shown that SGR and FCR of YTK are better, respectively, in fish reared at 24 °C as opposed to 16 °C. The results also provide strong evidence that feeding sub-adult YTK a single meal to apparent satiety once per day under controlled conditions supports acceptable SGR and FCR, irrespective of water temperature. There was no evidence that dividing meals into equal sized portions during the day benefited SGR or FCR. The apparent digestibility of a commercial diet was mostly unaffected by water temperature, however lipid digestibility was slightly depressed at 16 °C. We recommend the YTK industry should continue to feed at least twice daily in farm situations to ensure all fish have an opportunity to consume enough feed to support their growth potential (Output 3(b); Manuscript 3.2.4.1).
- An experiment to examine the effect of feeding regime and diet specification on performance of sub-adult YTK indicated SGR, FCR and condition factor were better in fish fed a high-specification diet (increased protein, lipid and energy) than a lower specification diet (less protein, less lipid less energy)

by 12.2%, 22.9% and 2.8%, respectively. On average, the relative feed intake of YTK fed the high specification diet was 12.9% lower than fish fed the standard specification diet. Moreover, results indicated YTK cannot upregulate their feed intake sufficiently to compensate for lower nutrient and energy intake as a result of missed feeding days or lower diet specification. There is no performance benefit in feeding sub-adult YTK less than once daily to apparent satiation and they should be fed on a daily basis in order to maintain growth trajectory and improve feed efficiency (Output 3(b); Manuscript 3.2.4.2).

- The fecundity and diversity of offspring from YTK is higher in broodstock fed natural food sources (Australian Sardines [*Sardinops sagax*; Sardines] and Atlantic Squid [*Doryteuthis pealeii*; Squid]) as opposed to commercial feeds or specially selected proprietary broodstock preparations. The number of offspring groups identified from select spawning events (i.e. heredity testing) was also higher in broodstock fed natural Sardines and Squid compared to broodstock fed commercial pellet preparations. We also observed a reduction in fecundity after adopting 3 monthly spawning intervals in the same tanks of YTK broodstock, indicating these animals may have been placed under reproductive stress. These results will be useful in planning commercial hatchery operations for industry and guide the YTK hatchery development program at PSFI (Output 3(b); Manuscript 3.2.6.1).
- Wild and F1 broodstock failed to spawn in the second broodstock experiment following thermal-photoperiod manipulation. Reasons broodstock did not spawn are unclear, but they could relate to the sexual naivety of the wild and F1 stock, or the additional stress placed on stock at the beginning of the experiment as a result of weighing and microbiome sampling. However, gut (rectal swab) microbiome sampling found significant differences in the global community structure of the tank water and broodstock swabs, indicating that YTK broodstock are able to select, regulate and maintain their own environmentally-independent microbiome (Output 3(b); Manuscript 3.2.6.2).
- Groups of wild-caught fish fed Sardines and Squid and sampled 4 months prior to and soon after attempted spawning recorded differences in their global community structures and relative percent abundances of the top 15 operational taxonomic units (OTUs) for these groups, suggesting other factors aside from diet have an influence on the gut community structure and dynamics of these broodstock (Output 3(b); Manuscript 3.2.6.2).
- Groups of F1 broodstock held exclusively on commercial pellet (Huon 9 mm diameter) and sampled 4 months prior to and soon after attempted spawning had significant differences in their global community structures. At the bacterial phyla and taxa level, similarities were observed across tanks of broodstock before and after attempted spawning. However, clear differences were recorded at the bacterial phyla and taxa level between the pre and post spawning samples from the same tank, even though fish from different isolated tanks were fed the same commercial diet. Again this suggests other factors aside from diet have an influence on the gut community structure and dynamics of broodstock at PSFI (Output 3(b); Manuscript 3.2.6.2).
- Water temperature was shown to have a varying effect on utilisation responses in YTK, with the magnitude of the response dependent on the nutrient examined. Protein and energy utilisation efficiencies were not statistically different at different water temperatures. Maintenance requirements of all nutrients generally increased with increasing temperature. Low DO at 60% saturation negatively affected the nutrient and energy utilisation efficiencies in YTK, with this response tending to be more pronounced with increasing nutrient and energy intake. However DO did not significantly affect feed intake. This study provides insight into the effects of abiotic factors on the nutritional physiology of YTK. (Output 3(b); Manuscript 3.2.2.1).
- We have improved and updated the published bioenergetic model for YTK. This was achieved by determining the impact of changing water temperature and dissolved oxygen concentration on important model coefficients related to utilisation of nutrients (including amino acids) and energy for maintenance and growth. Models have been validated against tank and field based trials at PSFI. The new model will be extremely useful in benchmarking performance of YTK reared on-farm as well as in research trials and will be further improved by integrating reliable data from YTK farms (Output 3(b); Manuscript 3.2.2.2).

- YTK can regulate their oxygen consumption to a concentration of ~1.9 - 2.6 mg O_2 L^{-1} (equiv. ~22 - 38% saturation) at 20 °C and 15 °C respectively, after which point YTK become oxyconformers and transition to a hypoxic state. Critical oxygen concentration is strongly dependent on the acclimation water temperature. Warmer acclimation temperatures lead to less hypoxia tolerance in YTK. Oil-source (fish oil vs poultry oil) had no significant effect on the critical oxygen threshold of YTK, however YTK fed poultry-oil showed a relatively large deviation in routine metabolic rate and critical oxygen concentration (Output 3(c); Manuscript 3.2.2.3).

Activity 4. Health: Improve nutritional health to boost productivity

- A challenge test method for fish health evaluations in tank based studies was developed and validated. A reliable immune response in sub-adult YTK was generated for the challenge test by vaccination with 100 µL killed *Photobacterium damselae piscicida* 1×10^{16} cells L^{-1} culture (Output 4(a); Manuscript 3.3.2.1). During the validation of this test two experimental diets formulated to replace 66.7% of WD FM with alternative protein sources, were demonstrated to have detrimental effects on the immunity of sub-adult YTK compared to the control commercial diet. These fish were unable to maintain an antibody response to the vaccination and also had a diminished inflammatory response (Output 4(a); Manuscript 3.3.2.1).
- In general the digestive tract histology, blood haematology and biochemistry of large sub-adult YTK was not significantly impacted by dietary treatments in relation to WD FM and WD fish oil replacement, and other changing nutritional and environmental factors (Output 4(b); Across a range of Manuscripts).
- General features of gut disease (i.e. enteritis-like conditions and coccidiosis infection) in YTK on the resultant gut microbiome were established, including substantial reductions in species richness, diversity and evenness, and the occurrence of one or more dominant potentially opportunistic bacterial taxa. Within the gut, this was accompanied by a loss of barrier integrity, as marked by a reduction in the numbers of mucous-secreting cells, decrease in villi length and a thinner submucosa, muscle layer and serosa (Output 4(b); Manuscripts 3.3.1.3).
- The skin and gut microbiome of poor-performing fish can be modulated towards favourable health outcomes, with whole microbiome therapies (when delivered orally and in combination with antibiotics) resulting in increased bacterial species diversity and evenness, and a decreased abundance of potentially opportunistic pathogens in gut samples two days post administration. More relevant delivery options as well as repeated administration (and at higher concentrations) may be needed though to prolong the effects of these therapies (Output 4(c); Manuscripts 3.3.1.4).
- Variation in the gut microbiome was observed with the use of different commercial feeds, with some formulations appearing to increase microbial diversity even over more ‘natural’ diets (Output 4(c); Manuscript 3.3.1.2).
- Specific manufactured feed formulations may represent an interesting prospect for optimising gut health through the promotion of microbial diversity and reduction in the abundance of potentially opportunistic pathogens (Output 4(c); Manuscript 3.3.1.2).
- Inclusion of LC n-3 PUFA at a moderate level (2.14 g 100 g^{-1}) into the diets of sub-adult YTK was found to increase species richness, diversity and evenness, and was associated with greater representation by additional phyla and decreases in otherwise dominant, potentially opportunistic taxa. At this level of inclusion, LC n-3 PUFA is thus supported for potentially promoting improved gut health (Output 4(c); Manuscript 3.3.1.2).
- Reducing or replacing WD FM content in formulated diets is also supported, with a reduction from 20% to 10% WD FM content or replacing with 11.32% PM promoting a more diverse microbiome composition with enrichment of potentially beneficial taxa leading to the displacement of potentially opportunistic organisms in sub-adult YTK (Output 4(c); Manuscript 3.3.1.2).

- High (30%) and low (20%) lipid diets (with and without the incorporation of LYSOFORTE® Liquid) did not significantly change the gut microbiome in sub-adult YTK, though select typically environmental species constituents may be enriched at low levels from the inclusion of emulsifiers. In the absence of further information pertaining to the relevance of these organism in YTK, such effects need to be considered when using these formulations on-farm (Output 4(c); Manuscript 3.3.1.2).
- The global bacterial community composition between the environment (surrounding seawater) and YTK gut samples was markedly different, highlighting that YTK are able to regulate and maintain their own environmentally-independent bacterial communities in the gut (Output 4(d); Manuscript 3.3.1.1).
- Differences were also observed between wild and farmed fish, including between onshore (tank-based) and offshore (sea-cage) systems, as well as across YTK size/age classes. Such effects need to be considered when assessing the microbiome in health and disease (Outputs 4(b) and 4(d); Manuscript 3.3.1.1).
- Increased levels of diversity were observed in the gut microbiome of wild fish, whereas within farmed fish notably lower levels of diversity and enrichment of potentially opportunistic bacterial species were apparent, particularly in the onshore (tank-based systems. This suggests that farming practice itself has a potentially negative impact on the gut microbiome and could be associated with various factors, including the use of pelleted feeds (Output 4(d); Manuscript 3.3.1.1).
- These findings provide for the first time a detailed analysis of the active bacterial components of the gut microbiome of wild and farmed YTK, establishing baseline data of the 'normal' gut microbiome which can then be used as a critical reference point for downstream dietary and health assessments (Output 4(d); Manuscript 3.3.1.1).

Activity 5: Extending YTK capability

- As outlined in Section 7. Communication and Extension, R&D progress and outcomes were extended to project participants, both researchers and industry, through ad-hoc communications, fortnightly meetings (NSW DPI and Huon Aquaculture), monthly update reports (SARDI and Clean Seas), monthly Technical Group meetings, occasional Research Advisory Committee, and quarterly Steering Committee meetings (representatives of all participants), and four annual project Research Workshops. Project outcomes were disseminated more broadly by 21 presentations at national and at international conferences, 51 presentations at workshops, and two publications in peer-reviewed scientific journal papers in addition to three popular articles for inclusion in the FRDC FISH magazine.
- As outlined in Section 4. Student Activities, people capability was built through the project's employment of 3 postdoctoral positions (the targeted number), and 6 PhD (the targeted number) and 2 Masters/Master Intern students and 4 Honours students (the target was up to 12 Honours students), A PhD student professional development program was delivered which included an invite to participate in project Technical Group meetings attendance at three annual workshops, and national and international conferences (Section 4 Student Activities).
- The project Executive Officer worked closely with FRDC's NEAO Subprogram Leader. He attended all FRDC NEAO Subprogram meetings, contributed to grant submissions, provided project updates and comments on forwarded documentation. He also obtained advice and support from the FRDC NEAO Subprogram Leader in developing the YTK Health Training Workshop that had broader participation than just this project (e.g. included participants from the Barramundi and Cobia industries).
- The following addresses the priorities (1. Increase the profitability and productivity of primary industries) of the Rural R&D for Profit Programme as outlined at the start of this section. Agtrans, the company contracted to do the independent Impact Assessment of this project, identified (Section 5 Impact Assessment and Industry Implications) the total funding from all sources for the project was

$7.37 million (present value terms) with the Department of Agriculture and Water Resources investment totalling $3.65 million. Their analysis indicated that this investment produced an estimated total expected benefits of $27.47 million (present value terms). This gave a net present value of $20.09 million, an estimated benefit-cost ratio of 3.7 to 1, an internal rate of return of 24.9 % and a modified internal rate of return of 9.7%.

1.5. Implications for relevant stakeholders

Formulation of a nutritionally adequate diet for any species depends critically on knowledge of the animals' basic nutrient and energy requirements and the judicious use of nutrient and energy digestibility data derived from the raw materials used to formulate their diets. Only by having this information can the feed formulator limit the risk of formulating an inadequate feed that fails to promote or maximize growth rate and feed efficiency. Feed formulations based on highly digestible raw materials also have obvious benefits for the environment by reducing the waste generated from the undigested feeds. This is not just good for the environment but is good for the image of aquaculture and its social licence to operate.

The determination of digestibility coefficients for 14 common raw materials and numerous test diets used in this project have greatly increased confidence in the use of this data to accurately formulate feeds which has direct benefits for Ridley and Skretting as well as consumers of aquafeed products such as Huon Aquaculture and Clean Seas Seafood.

Prior to this project the specific requirement of YTK for choline, histidine, taurine and methionine and LC n-3 PUFA, and protein to energy ratios of sub-adult YTK, were unknown. Formulators and farm managers relied on literature values from other closely related species. Through a series of carefully designed experiments this project has now defined the requirements of these nutrients for YTK. The result of greatest interest and perhaps potential impact for industry has been research on methionine, with a higher requirement found for juvenile YTK than observed in other closely related *Seriola* species. This has the potential to improve weight gain and feed performance on-farm and also has implications for earlier nutrition studies where the level of this amino acid was thought to be sufficient.

The existing bioenergetic (factorial) model for YTK have been improved. The new iteration of the model has been refined and validated against tank and field based trials at PSFI and SARDI. The model will be extremely useful in benchmarking performance of YTK reared on-farm as well as in research trials and will be further improved by integrating reliable seasonal data from YTK farms. The goal of constructing a bioenergetic model for YTK is ongoing.

The results of several feeding studies at SARDI and PSFI have indicated there is no performance benefit in feeding sub-adult YTK less than once daily to apparent satiation under laboratory conditions. Minor deficits in the nutrient and energy content of aquafeeds (quality) for YTK, if known, might be overcome by feeding to apparent satiation at least twice per day (quantity). The growth and feed performance of sub-adult YTK is extremely sensitive to the nutrient and energy composition of aquafeeds and this has implications for raw material selection and formulation. The YTK industry should continue to feed at least twice-daily in farm situations to ensure the average fish has the opportunity to consume enough feed to support their growth potential, especially at warm water temperatures when growth rates are high.

There is enormous potential to reduce the level of wild derived fishmeal in diets for juvenile and sub-adult YTK (<1.0 kg to 4 kg body weight) using other suitably selected, high quality raw materials. Fishery by-product meal is a suitable alternative to prime quality fishmeal in carefully formulated diets for juvenile and sub-adult YTK and use of products like these, provided they are of high quality, will reduce raw material formulation costs and reduce FIFO ratio. Nonetheless very low, prime fishmeal diets such as the one tested in this report (≤ 10%) should be trialled on larger YTK under farm conditions before industry-wide changes to YTK formulations are made. Adoption of low fishmeal or low FIFO diets will improve economic outcomes as well as the environmental 'blue' footprint of Australian YTK farmers.

Our understanding of broodstock nutrition remains in its infancy. However the implications of farming progeny of poorly maintained and malnourished broodstock are profound, having negative ramifications

across the whole nursery and production cycle. In addition, the implications of quickly shifting from 'best-practice' broodstock and hatchery regimes to newer regimes without proper evidence is also profound, as the consequences of getting it wrong can be long lasting. Manipulative nutrition trials with large broodstock animals are challenging due to the scale of systems, the size of animals, the duration of experiments (especially those involving long terms spawning cycles) and often low replication. The basic nutrition research conducted on broodstock in this project has been mostly qualitative, but it has indicated that manipulating feeds and spawning cycles impacts the fecundity of wild and F1 animals. Difference in feed type (natural vs manufactured) also impacts the microbiome of broodstock in definable ways. These results demonstrate we need to pay close attention to these issues in YTK hatcheries and develop better and more rapid methods to assess the impacts of diet or abiotic shifts on the fecundity and quality of output from broodstock animals.

The importance of the microbiome in supporting health and nutrition of YTK more broadly is now becoming increasingly realised, and is a critical metric that can be used for assessing the impacts from changes in diet and practices on-farm. Microbiomes comprising diverse assemblages of bacterial species are most likely to support YTK health and nutrition in their innate ability to outcompete and displace potentially opportunistic pathogens and contribute to more diversified functions. Variations exist, however, among the commercial formulations in the extent to which they have the capacity to promote diversity in the gut, with certain diets leading to a loss of diversity in some cases. Alongside the potentially negative consequences these diminished microbiomes represent for supporting the nutrition of the animal, the emergence of potentially opportunistic species as alternate, dominant features in the gut may also bring additional challenges. This is particularly pertinent to farms where poorly understood conditions of disease may arise suddenly, and appears to be associated with previously unknown organisms that may have pathogenic potential. However, with notable changes also observed to occur in the microbiome of the outer body surfaces (skin and gills) of these animals at the very early stages of disease (where symptomatic features are not as apparent), novel non-invasive biomarkers may be developed for the early detection of changes in health on-farm and may support improved intervention or management strategies. Alongside modifying feeds to include specific ingredients (e.g. LC n-3 PUFA) at optimal levels or bioactive components, the direct replacement of potentially beneficial bacterial species (e.g. through whole microbiome therapies) represents a promising new approach in forthcoming years to support improved gut microbiome structure and robustness of YTK.

Overall, this project has been successful in generating commercially relevant information to assist the development of the Australian YTK industry. Independent information provided within this report (Section 5 Impact Assessment and Industry Implications), conservatively estimated that the research outputs from the this project, based on the current levels of SA YTK production, will result in a 5% increase in productivity and a 5% increase in profits for the YTK industry. There is also potential for the value of the R&D provided within this project to double (i.e. 10%) once YTK production is established in NSW and WA.

Information generated in this project will assist Australian YTK producers in meeting the criteria set down in the key performance indicators which were to attain a fingerling equivalent of 3.0 kg weight per fingerling within 2 years; a feed conversion ratio (FCR) of ≤ 1.5 and ≤ 2.2 for fish between 0.01 - 1.5 kg and 1.5 - 3.5 kg, respectively; and, survival rates of > 90% from the stocking of fingerlings until harvest. More specifically:

- Information for aquafeed manufacturers to:
    - Have greater flexibility to produce new improved and more cost effective and sustainable diets using increased levels of alternative ingredients that better meet the nutritional requirements of fingerling and sub-adult YTK.
- Information for Australian YTK producers to improve productivity and profits by:
    - Utilising the new improved diets, formulated to contain reduced levels of wild derived marine ingredients, for more efficient fingerling and sub-adult YTK production to improve;
    - Improve broodstock nutrition;

  - Applying the bioenergetics model to optimise feeding strategies to improve feed efficiency and reduce environmental waste;
  - Improving the FIFO ratio to enhance consumer perception and market access for Australian YTK;
  - Increasing the understanding of the microbiome of YTK to improve health management practices; and
  - Increasing knowledge and communication between them.
- Training of industry ready graduates for entrance into the Australian YTK industry, Aquafeed industries, educational institutes, government departments and other sectors of the workforce that will benefit Australia as a whole.
- The development of a stronger collaborative research, development and extension sector to aid the future development of aquaculture in Australia.

1.6. Recommendations

The current project has provided industry stakeholders with valuable and information to assist the Australian YTK industry improve productivity and profits. Overall, results from this project bode well for the future development of the YTK aquaculture industry in Australia However, dietary development work for this industry should not remain static, as important advancements in our knowledge of nutrient requirements and feeding practices will need to be ongoing to ensure the economically sustainable production of Australian YTK and a flourishing industry. Throughout the project opportunities for further research were identified and include:

Activity 2. Nutrition: Identify economically sustainable feeds and improved diet formulations;

- Nutrient requirement work must take into consideration seasonal water temperatures and fish sizes during different stages of production.
- It should be acknowledged that recommended dietary nutrient levels in commercial diets may be further reduced by optimising dietary amino acid profiles (e.g. methionine, lysine and histidine) based on new information as it comes to hand.
- Further work evaluating the dietary requirements for essential amino acids, vitamin and minerals should be undertaken for fingerling and sub-adult YTK to advance the sustainable performance.
- Currently, the recommended minimum inclusion levels of fish oil in commercial diets for sub-adult YTK is restricted by the ∑LC n-3 PUFA requirement of the fish and ranges between 5-10%, depending on the ∑LC n-3 PUFA content of the fish oil. This has implication on the FIFO ratio and sustainability. There is a pressing need to evaluate new oils rich in ∑LC n-3 PUFA with YTK as they come to hand.
- In order to be able to tailor fish for specific markets, further research is warranted to understand the kinetics associated with the uptake of ∑LC n-3 PUFA from finishing diets rich in fish oil, prior to harvest.
- LC n-3 PUFA levels and ratios in red blood cells are considered to be a good biomarker for inflammatory responses in humans and other animals. Given the importance of red blood cells in oxygen transport, fatty acid modifications in relation to saturated and unsaturated fatty acids may contribute to alterations in metabolic function. Further research is warranted to understand this aspect of YTK metabolism.
- There are opportunities to tailor the fat content of fish to specific processes and markets. In relation to dietary lipid levels and biometric measurements, the targeted processing method and markets should be taken into consideration when assessing growth performance to account for differences in fat partitioning.

- Developing functional feeds, including those that include pre- and pro-biotics and enzymes, to enhance YTK performance.
- The use of commercially available bioactives in soy-based feeds for YTK did not enhance growth or feed utilisation. However it would be prudent to explore the use of other similar products. The preliminary evidence gathered in this experiment suggests there is no major benefit in adding any of the selected bioactives into diets for juvenile YTK, at least at the levels tested. Diets for YTK that contain soybean meal and SPC and optimal levels of methionine are worthy of further investigation.
- Further investigation of the potential use of emulsifiers to improve lipid utilisation at optimal growth rates at summer water temperatures may be warranted.
- Due to the slow growth rate of large sub-adult compared to fingerling YTK at suboptimal water temperatures, it should be noted that attempting to gain an insight into the growth performance and feed utilisation of sub-adult YTK at winter water temperatures is inherently difficult. It may be beneficial to run trials with sub-adult YTK for a longer period from winter into spring to assess any dietary deficiencies or benefits that may become apparent once growth rates accelerate.
- As the aquaculture industry is tending to reduce the use of high fish meal/fish oil diets due to economic and sustainability issues, further consideration to sustainability and customer perception are needed before YTK are fed Australian Sardines under commercial conditions.

Activity 3. Feeding strategies: Improve feeding strategies to increase profit;

- The goal of constructing a bioenergetic model for YTK is ongoing and will be assisted by the provision of growth and temperature data from farms. We recommend the development of a desk-top or phone based application making it readily available and accessible to farm managers. It will also be a useful tool for feed manufacturers, allowing forecasting of feed demand from their customers.
- Fingerling and sub-adult YTK should be fed daily to ensure optimum growth and productivity.
- There did not appear to be any advantage in adopting split ration feeding to fingerling or larger sub adult YTK.
- Further research into the effectiveness of bioactive markers, such as the digestive enzyme dipeptidyl peptidase-4 (DPP4), as indicators of feed intake and digestion regulators is warranted.
- Given the reduced growth rates associated with oxygen deficiency, further research in relation to hypoxic stress is warranted for harvest sized YTK.
- Selective breeding targeted at YTK growth, efficiency of assimilation of feed nutrients and disease resistance/ health.

Activity 4. Health: Improve nutritional health to boost productivity;

- Use of the challenge model to understand the effect of WD FM and WD fish oil substitution on the health and immune system of YTK.
- Improving strategic approaches to skin and gill fluke management based on understanding which treatments are best in which circumstances.
- Approaches or management strategies which aim to enhance gut microbiome diversity in onshore systems is recommended for optimising fish robustness and may improve the natural adaptive processes of the fish to local environmental microbial communities when transferred offshore to sea-cages for grow-out.
- With changes in microbiome composition and diversity observed among major size classes associated with the commercial production cycle, there is also a need to ensure that appropriate size/age-specific controls are taken when surveying the relevance of the microbiome in changing health and nutrition in future surveys.

- Future investigations should be directed to developing dietary formulations that select for 'optimal' (diverse) gut microbiomes by conducting more detailed assessments of the underlying (gene) functions contributing to varied health and/or performance in YTK.
- Confirming the identification and determining the involvement of potentially opportunistic pathogens (namely *Mycoplasma insons*, *Brevinema andersonii*, *Photobacterium* sp.) that were found to occur at high levels in association with fish fed certain diets or that were suffering from conditions like enteritis, is recommended. With this additional information, appropriate control measures could then be implemented to improve health outcomes.
- We also recommend directing further efforts to the involvement and replenishment of organisms that may be of benefit to the host (e.g. *Bacillus* species), which were otherwise diminished in the microbiomes of diseased fish.
- Biomarkers of changing health status for coccidiosis and enteritis conditions could be established using more targeted, rapid and cost-effective tools (e.g. q-PCR), with the potential to implement non-invasive testing through the collection of swabs from the skin, which could foreseeably be implemented as part of routine health surveys for the early detection of disease.
- Strategies which promote broader microbial diversity in the gut of YTK should be investigated (e.g. probiotics, prebiotics, whole microbiome therapies), as they are most likely to improve the robustness of the fish to potentially opportunistic pathogens, ultimately improving health outcomes.
- From the findings of the microbiome manipulation trial, we believe additional experimental work is warranted. In particular, future studies should include repeat dosages of the whole microbiome inoculum (and/or at a higher concentrations) in order to sustain potential beneficial outcomes. Trialling administration of inoculum on-feed is also recommended, which would allow for easier repeated dosing and would also prove to be more applicable on-farm compared to gavage. Including more varied microbiomes or individual strains that have known therapeutic potential (or that were observed to be depleted in diseased individuals in the early work) is also suggested in a refined trial.
- Further work is also required to elucidate whether increases in diversity support improved health through the displacement of pathogens and the potential occurrence of more diversified functions. The use of more advanced omics-based techniques is recommended to investigate this further.
- As there was no significant difference in the global bacterial community structure between the three gut regions, sampling methods were refined and future work should also be directed at taking a single hindgut scraping (instead of separate fore-, mid- and hindgut), allowing for more samples to be processed at the same cost. This would increase the capacity to sample across multiple sea-cages, seasons and sites to provide a greater overview of farm-wide changes.

Activity 5: Extending YTK capability;

- Ensure that the results of the ongoing PhD projects for this project are captured and disseminated to industry as planned.
- Discuss with industry the most appropriate manner to continue YTK industry networking; this might be in the form of a new dedicated association or as part of an existing networking group (e.g. FRDC's NEAO Subprogram).
- Hold a workshop involving all YTK industry participants following completion of the project to identify and prioritise future needs to further drive the development of this industry.

1.7. Keywords

Yellowtail Kingfish, *Seriola lalandi*, nutrition, feeding strategies, health, microbiome, juvenile, sub-adult, broodstock.

2. General Introduction

2.1. Background

2.1.1. Species

Yellowtail Kingfish (YTK, *Seriola lalandi*), also known as Goldstriped Amberjack, Yellowtail Amberjack, and "hiramasa", is one of a number of *Seriola* species. Its distribution is circumglobal occurring in the Indo-Pacific and Atlantic oceans, where it frequents semi-tropical and warm temperate areas. The species lives in coastal and oceanic waters and is both pelagic and demersal. Food and Agriculture Organisation fisheries and aquaculture statistics indicate that the species, in contrast to other *Seriola* species, supports only a small wild fishery, which peaked at about 1,500 tonnes in 1998, and a small aquaculture industry. While it has been farmed in Japan for many years, the YTK aquaculture industry in Australia, Equador and New Zealand is relatively recent having only started in the late 1980s.

2.1.2. Aquaculture

In Australia, YTK aquaculture production begins with the spawning of broodstock sourced typically from selected cultured stock but sometimes from wild stock; with three to four hatchery runs typically undertaken in spring and summer. Fertilised eggs are collected from the spawning tanks and following a short incubation period of about two days, the larvae hatch and are held in larval rearing tanks where they are fed an enriched diet of live zooplankton and brine shrimp. After about 21 days the juvenile fish are transferred to nursery tanks and weaned onto a manufactured diet. At between 60 and 100 days, at a size of 25-50 grams fingerlings are transferred from the hatchery into 25 m to 60 m diameter sea-cages. Fish are then on-grown for about 16 to 18 months, during which time they are fed on commercially produced, high quality, extruded pellet diets, to a size of about 4 kg, when they are harvested and marketed. The product is sold either as premium grade sashimi or as whole fish or fillets and consumed locally as well as exported to Asia and Europe (http://www.oceanwatch.org.au/seafood/aquaculture/species/yellowtail-kingfish/).

2.1.3. Australian YTK aquaculture industry

When this project, "Growing a profitable, innovative and collaborative Australian Yellowtail Kingfish Aquaculture Industry: bringing 'white' fish to market. RnD4Profit-14-01-027", was initiated in mid-2015, the Australian YTK aquaculture industry comprised three production companies:

- Clean Seas Seafood as it is now known based on Eyre Peninsula, South Australia (SA). The Stehr Group pioneered YTK farming in Australia, conducting growout trials in 1998/99 and 1999/2000, successfully producing hatchery fingerlings in 2001, and began marketing commercial quantities of farmed product in 2014 (http://seafoodfrontier.com.au/product/kingfish/). In 2000 Clean Seas formed from the Stehr Group, in 2005 Clean Seas publically listed and in 2016 changed its name to Clean Seas Seafood. Following addressing a range of development challenges, whole weight equivalent sales volume was about 1,098 tonnes, worth $18.185 million, for the 2015 financial year and 2,353 tonnes, worth $39.7million, for the 2018 financial year. The company's 2018 financial report to shareholders indicates it is targeting sales production of 2,750-3,000 tonnes, valued at $47-50 million, for the 2019 financial year and presently has the lease area across its sites to potentially produce about 11,000 tonnes per annum of YTK (http://www.cleanseas.com.au/investors/asx-releases/).

- Indian Ocean Fresh Australia, based at Geraldton, Western Australia (WA). In 2006-2007 a publically listed company known as Western Kingfish Ltd started an aquaculture venture in

Jurian Bay, to the north of Perth, that included YTK as a species, but within 18 months had closed due to development issues. In 2008, Indian Ocean Fresh Australia started with four sea-cages in Champion Bay, Geraldton and between then and 2016 undertook three growout trials in association with the Mid West Development Commission and others; fingerlings being produced at the TAFE/Fisheries Department hatchery at Fremantle. In 2018 Indian Ocean Fresh Australia has started producing commercial quantities of YTK (https://iofa.com.au/our-story).

- Huon Aquaculture (traditionally a Tasmanian salmon aquaculture company), with its YTK operations based at Port Stephens, New South Wales (NSW). Pisces Aquaculture was granted consent for a lease to farm YTK and other species in 2001, but closed due to developmental issues in 2004. In 2013 NSW DPI was granted consent to operate a Marine Aquaculture Research Lease in Providence Bay adjacent the Pisces lease site and in 2013-14 it called for lease partners with Huon Aquaculture selected. In 2014 Huon Aquaculture bought the Pisces lease, but in 2016 NSW DPI and Huon Aquaculture sought government approval to move their two lease sites further from shore (Port Stephens Examiner Thursday 2 April 2016). At this time each lease was 62 ha in size with the capacity for 12 cages and about 1200 tonnes production. In 2018 Huon Aquaculture marketed its first YTK from two experimental cages and is about one year into a five year development plan for this site (https://www.huonaqua.com.au/about/truth/western-australia-kingfish-lease/). It has recently announced establishing leases adjacent the Abrolhos Islands, WA where the Western Australian Department of Fisheries believes some 22,000 tonnes of YTK production might be feasible within a decade (https://www.abc.net.au/news/rural/2018-10-12/huon-reveals-wa-fish-farm-plans/10366240).

2.1.4. Business Opportunity

During the preparation of FRDC's Research, Development and Extension Plan for 2015-2020, it became evident that YTK farming was likely to offer the greatest opportunity for new aquaculture development in Australia in the next few decades as defined by increases in farmed area and product, the quantity of aquafeeds that would be needed, and the growth in regional economies and employment. At the start of the project it was predicted that within 10 years, Australian YTK production could increase to 34,000 tonnes worth $440 million, using 68,000 tonnes of aquafeed worth $136 million (estimates based on the collective inputs of initial project participants). However, FRDC, in documenting its New and Emerging Aquaculture Opportunity (NEAO) Subprogram, indicated a more conservative growth of 2,500 tonnes within five years for all Australian white fish (http://www.frdc.com.au/Research/RDE-planning-and-priorities/FRDC-RDE-Plan-2015-20). At the time, the key challenge to achieving the expected growth of YTK was for the industry to diversify its focus from supplying only the relatively small volume, high price sashimi market to supplying the larger volume, lower price Australian 'white fish' market, while enhancing farm productivity and reducing operating costs to maintain profitability and improve sustainability. As such, this project sought to provide new information to assist the YTK industry to grow its position by developing more cost effective, sustainable feeds and feeding strategies to enhance YTK growth and health; the industry's highest common R&D priorities as feed and feeding costs comprised about 60% of its operating expenses.

2.1.5. Strategic Alignment

This project has focused on growing the production and profitability of the key existing Australian YTK industry participants, as well as the industry as a whole, and directly addresses FRDC's new strategic plan to build Australian sustainable aquaculture development through the activities of the new 'New and Emerging Aquaculture Opportunities' (NEAO) Subprogram. The project aligns well with the National Marine Science Plan to grow the blue economy, the national Aquaculture Statement and Strategy to grow Australian aquaculture production, and the national Research Providers Network (RPN) to better coordinate fisheries and aquaculture R&D resources. The project is also expected to build on the earlier

nutrition R&D on YTK undertaken primarily through the FRDC and the Australian Seafood Cooperative Research Centre (ASCRC).

2.1.6. Planned Networking

This project further built the relationship between two of the three Australian YTK aquaculture companies, Clean Seas Seafood, SA, which is the most advanced, and Huon Aquaculture, NSW, which only started YTK farming just prior to the start of this project. Also, between these companies and their geographically aligned fisheries and aquaculture research institutions, the South Australian Research and Development Institute (SARDI) and the New South Wales Department of Primary Industries (NSW DPI). The project also brought into this network the two key Australian aquafeed companies, Ridley and Skretting Australia, and to a lesser extent, another YTK aquaculture company, Indian Ocean Fresh Australia, Western Australia (WA). Additional to this, the project provided benefits to the broader Australian finfish aquaculture industry, including future YTK farmers and service providers, as well as the other Australian industry sectors targeting the production of 'white' fish (e.g. Barramundi, Cobia and Mulloway). Some indirect benefits have also flowed to the community through the development of more sustainable feeds, which will provide environmental benefits to the marine environment through reduced nutrient loads. Some social flow-on benefits are also expected to result from the economic growth of the Australian YTK industry, particularly in the regional areas where farming occurs.

2.2. Project Priorities, Themes, Activities and Outputs

2.2.1. Rural R&D for Profit Programme Priorities

This project was part of the Rural R&D for Profit Programme, Department of Agriculture and Water Resources, Australian Government. It aligned with the Round 1 Programmes priorities:

1. Increase the profitability and productivity of primary industries
    - Help producers increase yields and/ or reduce costs by applying innovative technologies and/ or technologies from other industries.
    - Help producers manage natural resources in an integrated way at enterprise or regional level for long-term use and profit.
2. Strengthen primary producers' ability to adapt to opportunities and threats
    - Integrate data and deliver information to help producers manage risk, benchmark performance and make production decisions for greatest profit.
3. Strengthen on-farm adoption and improve information flows
    - Consolidate knowledge of extension and adoption to better deliver practical results to primary producers, founded on what producers want from extension services.
    - Identify practical proposals to stimulate private sector extension services, particularly to fill current gaps.
    - Identify practical means to co-ordinate extension services for producers, including the development of tools and/or platforms.

2.2.2. Project Themes

To meet with these Rural R&D for Profit Programme priorities, the project addressed the Australian YTK industry's key common R&D priorities, both at conception during 2014/15, and throughout the course of the project, through three themes and their specific activities and outputs:

Theme 1: Nutrition;

Theme 2: Feeding Strategies; and

Theme 3: Nutritional Health.

2.2.3. Project Activities

The key activities of this project central to the efficient and effective delivery of its objectives and outputs were:

1. Project initiation and management;
2. Identify economically sustainable feeds and improve diet formulation;
3. Improve feeding strategies to increase profit;
4. Improve nutritional health to boost productivity; and
5. Extending YTK capability.

2.2.4. Project Outputs

Project outputs for each activity were:

Activity 1. Project initiation and management;

Output 1(a) Establish steering and research advisory committees and provide their terms of reference

Output 1(b) Execute agreements and contracts with partner organisations and service delivery agents as needed

Output 1(c) Finalise an extension and communication strategy. The strategy must include communications and extension activities including, but not limited to publications, workshops and newsletters

Output 1(d) Create a monitoring and evaluation plan for the project

Output 1(e) Undergo end of project evaluation in accordance with output 1(d) and provide a report to the department. The evaluation must report on the projects outcomes against the program objective, including quantitative information on the outcomes achieved and independent expert analysis of expected and/or demonstrated quantifiable returns on investment

Activity 2. Identify economically sustainable feeds and improved diet formulations (Nutrition theme);

Output 2(a) Evaluate alternative Australian farm protein and oil sources and identify their ideal inclusion levels in juvenile and sub-adult production diets to reduce dependence on fishmeal and fish oil

Output 2(b) Investigate protein sparing effect of using higher energy and lower protein diets

Output 2(c) Develop winter diet formulations that use ideal lipid types and levels for less than two kilogram YTK during periods of suboptimal water temperatures

Output 2(d) Determine dietary requirements of selected essential nutrients for juvenile and sub-adult YTK

- Determine the histidine requirements of juvenile YTK
- Determine the choline requirement of juvenile YTK
- Determine the taurine requirement of juvenile YTK
- Determine the methionine requirement of juvenile YTK

Output 2(e) Investigate the cost-benefit of using dietary supplements to improve the production of juvenile and sub-adult YTK

- Evaluate bioactive supplements that boost immune competence, digestive tract and skin health in YTK

Activity 3. Improve feeding strategies to increase profit (Feeding strategies theme);

Output 3(a) Evaluate optimal feeding strategies for juvenile and sub-adult YTK, including but not limited to comparing experimental nutrient-dense and commercially available feeds, floating versus sinking feeds, feed sizes and feeding strategies

- Validation trial in pond cages to asses growth and FCR on newly developed feeds and feeding strategies for juvenile and sub-adult YTK (fishmeal origin)
- Benchmark study in pond cages of a commercial diet and feeding strategies for sub-adult YTK on the NSW DPI – Huon Aquaculture Marine Aquaculture Research Lease (MARL) (fishmeal reduction)

Output 3(b) Evaluate the cost-benefit of using high versus low energy feeds for juvenile and sub-adult YTK at varying water temperatures

- Determine optimum feeding frequencies in warm water (24 °C) with sub-adult YTK grown towards market size
- Determine optimum feeding frequencies in cool water (16 °C) with sub-adult YTK
- Evaluate the effects of feeding strategy and diet specification on performance of sub-adult YTK
- Evaluate impacts of dietary shift on reproductive output and health of YTK broodstock (3 feeding experiments)

Output 3(c) Develop an improved feed ration model for on-farm YTK feed management

- Refine temperature dependant growth and bioenergetic model for YTK and develop a predictive farm-based management tool for YTK

Activity 4. Improve nutritional health to boost productivity (Health theme);

Output 4(a) Develop a challenge test method for fish health evaluations associated with tank based nutrition and feeding strategy R&D

- Further refine the challenge model by better understanding the YTK immune system

Output 4(b) Collect histopathology and blood chemistry data of diseased and healthy YTK to characterise the general health of YTK used in tank based nutrition and feeding strategy R&D

- Further refine the role of the gut microbiome in YTK gastrointestinal health by sampling additional wild fish in SA for subsequent histological and microbiomic evaluations

Output 4(c) Characterise and understand the microbiome of the digestive system of YTK in particular in relation to different diets and feeding strategies, and how this might be managed to enhance YTK health, diets or food conversion ratios

- New health theme activity - manipulation of the microbiome of diseased YTK

Output 4(d) Collect baseline data to differentiate the effects of the environment, YTK growth and farm production cycle, disease and different genetic cohorts on the microbiome

Activity 5. Extending YTK capability.

Output 5(a) Conduct workshops and provide publications to extend the outputs from the project to industry participants, and the broader aquaculture industry, scientific community and public in line with output 1(c)

Output 5(b) Student training to develop the next generation of industry R&D providers including up to three postdoctoral research fellows, up to six PhD students and up to 12 Honours students

Output 5(c) Incorporate the outcomes of the project into the new subprogram established by the FRDC or the development of new and emerging aquaculture growth opportunities to allow the direct extension and translation of outputs to potential ‘white’ fish and other new and emerging aquaculture opportunities

3. Research

3.1. Theme - Nutrition

3.1.1. Chapter - Lipid and fatty acid requirements for large Yellowtail Kingfish.

3.1.1.1. Manuscript - dietary long-chain omega-3 polyunsaturated fatty acids levels for optimum growth of large Yellowtail Kingfish (Seriola lalandi; > 2 kg) at warm water temperatures.

David A.J. Stone [a, b, c, d], Matthew S. Bansemer [a], Paul Skordas [a], Samantha Chown[e], Nicole Ruff[f], Michael Salini[g]

[a] South Australian Research and Development Institute, South Australia Aquatic Science Centre, West Beach, SA 5024, Australia.
[b] Flinders University, College of Science and Engineering, GPO Box 2100, Adelaide, SA 5001, Australia.
[c] University of Adelaide, School of Animal and Veterinary Sciences, Roseworthy, SA 5371, Australia
[d] Marine Innovation Southern Australia.
[e] The University of Adelaide, School of Agriculture, Food and Wine, Waite Campus, Urrbrae, SA 5064, Australia.
[f] Skretting Australia, Cambridge, TAS 7170, Australia.
[g] Ridley, Narangba, QLD 4504, Australia.

This manuscript may be referenced as: Stone, D.A.J., Bansemer, M.S., Skordas, P., Chown, S., Ruff, N. and Salini, M. (2019). 3.1.1.1. Manuscript - Practical dietary long-chain omega-3 polyunsaturated fatty acids levels for optimum growth of large Yellowtail Kingfish (*Seriola lalandi*; > 2 kg) at warm water temperatures (N1; Output 2c). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, December. pp.22-46.

Abstract

Understanding the level of dietary long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) for optimum growth of aquaculture species is vital to sustainably and economically utilise fish oil. The optimum dietary LC n-3 PUFA level for Yellowtail Kingfish (*Seriola lalandi*; YTK) is unknown. In this 84 day study, the growth performance, feed efficiency, hind and midgut histology and health of YTK (2.67 kg) fed graded levels of dietary fish oil, using poultry oil as the replacement, were investigated to determine the practical optimal dietary long chain omega-3 polyunsaturated fatty acid level (LC n-3 PUFA; eicosapentaenoic acid [20:5n-3, EPA], docosapentaenoic acid [22:5n-3, DPA] and docosahexaenoic acid [22:6n-3, DHA]) at warm summer water temperatures. Eight experimental diets were formulated to contain 20% fish meal and graded dietary LC n-3 PUFA levels that ranged from 0.753 to 2.950 g 100 g^{-1}. Fish were fed to apparent satiation once daily at 11:00 h. There was a moderate positive significant quadratic relationship between dietary LC n-3 PUFA and SGR (R^2 = 0.5697; P < 0.001). Based on the SGR, the optimal level of LC n-3 PUFA (turning point; y_{max}) was 2.12 g 100 g^{-1}. This equated to a LC n-3 PUFA daily intake of 191 mg kg^{-1} d^{-1}. There was no improvement in SGR by increasing LC n-3 PUFA levels above 2.39 g 100 g^{-1}. With regard to the feed conversion ratio (FCR), there was a moderate negative significant quadratic relationship between dietary LC n-3 PUFA level and FCR (R^2 = 0.5758; P < 0.001). This relationship was inversely related to the relationship between LC n-3 PUFA level and SGR. The FCR of YTK decreased (improved) as dietary LC n-3 PUFA levels

increased from 0.75 to 2.14 g 100 g^{-1}. Based on the FCR, the optimal level of LC n-3 PUFA (turning point; y_{min}) was 2.26 g 100 g^{-1}. This equated to a LC n-3 PUFA daily intake of 203 mg kg^{-1} d^{-1}. Apart from minor alterations to hindgut villus branching, changing LC n-3 PUFA level had no significant impacts on hind or midgut histology ($P > 0.05$). Based on SGR and FCR response variables it is estimated that the optimal dietary level of LC n-3 PUFA for YTK at warm temperatures is between 2.12 and 2.26 g 100 g^{-1}. The 95% CI for each response variable ranged between 1.90 to 2.33 g 100 g^{-1} and 1.93 to 2.58 g 100 g^{-1} for SGR and FCR, respectively. It is recommended further research under commercial conditions before implementing this dietary level of LC n-3 PUFA on-farm.

Introduction

In Australia, Yellowtail Kingfish (*Seriola lalandi*; YTK) are a relatively new species to aquaculture that is being developed for culture in South Australia, Western Australia and New South Wales. Currently over 90% of production stems from Clean Seas Seafood (Arno Bay, South Australia, Australia). The sustainable and economically viable production of YTK relies on cost effective diets. Over the past decades, the need to find alternative lipid sources to fish oil for aquaculture species has been highlighted due to the high price, reduced availability, and ecological issues (Glencross et al., 2007; Tacon and Metian, 2009). In order to successfully reduce dietary fish oil inclusions for aquaculture species, numerous studies have evaluated alternative dietary lipids, including poultry oil, beef tallow, and canola oil (Oliveira et al., 2008; Stone et al., 2011a; Stone et al., 2011b; Bowyer et al., 2012). These alternative lipids however, typically lack long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA; eicosapentaenoic acid [20:5n-3, EPA], docosapentaenoic acid [22:5n-3, DPA] and docosahexaenoic acid [22:6n-3, DHA]) (Higgs et al., 2006).

Long chain omega-3 polyunsaturated fatty acids are essential for carnivorous fish and required at adequate levels for numerous biological functions, including cellular membrane structure, metabolism and function to ensure optimal growth and health (Tocher, 2010). More specifically, the eicosanoids EPA, DPA and DHA are important precursors of specialized proresolving lipid mediators (SPM), the D and E series resolvins, (neuro) protectins, and maresins, that have been reported to prevent excessive inflammation, promote resolution, and expedite the return to tissue homeostasis (Serhan, 2014).

Although some alternative lipid sources do contain the precursor of EPA, DPA and DHA, α-linolenic acid (18:3n-3, ALA), unlike freshwater aquaculture species and terrestrial livestock species, marine fish species lack enzymes, elongase 2 and Δ-6 desaturase, at appreciable levels to chain elongate and de-saturate ALA to EPA, DPA and DHA. As a result, fish oil is currently the best option to supply LC n-3 PUFA for carnivorous marine aquaculture species (Tocher, 2010; Bowyer et al., 2012).

Understanding the LC n-3 PUFA requirements of aquaculture species is vital to sustainably and economically utilise fish oil. The LC n-3 PUFA requirement for a number of aquaculture species is known, including the Gilthead Sea Bream (*Sparus aurata*) and Japanese Yellowtail (*Seriola quinqueradiata*) (Deshimaru et al., 1982; Kalogeropoulos et al., 1992). However, the LC n-3 PUFA requirement for marine aquaculture species, is species-specific, and also depends on life stage, water temperature and EPA:DHA ratio (Yone, 1978; Masumoto, 2002; Sargent et al., 2002; Oliva-Teles, 2012). Information pertaining to nutritional requirements of YTK, including the optimal dietary LC n-3 PUFA level to promote optimum growth, is lacking in the literature. In Australia currently, commercial diet formulations are based on the limited available nutritional information for YTK. When YTK-specific nutritional information is unknown information from surrogate fish species, including Japanese Yellowtail, salmonoid species and Barramundi is used (Stone and Bellgrove, 2013). Recent research has suggested that the growth and feed utilisation of juvenile YTK (95 g) fed a diet (45% crude protein and 25% crude lipid) is not compromised when replacing fish oil with poultry oil (100% replacement; ∑LC n-3 PUFA = 0.87%; Bowyer et al., 2012). In the study of Bowyer et al. (2012), the fish meal component of the diet provided levels of LC n-3 PUFA close to the reported requirement for Japanese Yellowtail (Deshimaru et al., 1982). It should also be noted that the study by Bowyer et al. (2012) was short term, and in a longer term study, YTK (1.67 kg) fed a diet that contained 1.41% ∑LC n-3 PUFA exhibited inferior growth performance to fish fed a diet that contained > 2% ∑LC n-3 PUFA (Stone et al., 2016). In order to efficiently utilise fish oil replacements in diets for YTK, further research to understand the

specific LC n-3 PUFA requirements for optimal growth of this species is needed. This research will ultimately improve the sustainable and economically viable production of YTK through cost-effective diet formulations with low, but optimal, marine ingredient use.

Tocher (2010) suggested that the quantitative estimation of essential fatty requirements (EFA) may be described on three levels:

1) The physiological level required to prevent classical nutritional pathology (EFA deficiency signs);
2) EFA requirement to support optimum growth and health, although this is currently not well defined for any species and would likely vary dependent upon other dietary factors and fish metabolism (Tocher, 2003).
3) The level that maintains nutritional quality for human consumption, based on n-3 LC-PUFA content of the flesh (Simopoulos, 2000; Tocher, 2009).

The first two levels address the actual EFA requirements of the fish, while the later does not. It addresses EFAs in terms of producing a nutritionally healthy product for human consumption, and is useful for marketing purposes. For the current experiment we chose to adopt selected responses from levels one and two for the purpose of estimating the optimum levels of dietary n-3 LC-PUFA for large YTK at warm water temperatures. A PhD project conducted by Samantha Chown (Appendix 4) is underway and is addressing issues associated with level 3.

Aim

The aim of this study was to determine the practical optimum levels of dietary LC n-3 PUFA on the growth performance, feed utilisation and health of large YTK (> 1.5 kg) at warm summer water temperatures.

Methods

Experimental design and diets

In this study, the pellet kernel (9 mm diameter), fish oil and poultry oil were supplied by Skretting Australia (Cambridge, Tasmania, Australia). Diets were formulated based on a Skretting YTK diet (20% fish meal; ~43% crude protein [CP], ~27% crude lipid [CL] and a gross energy [GE] level of ~21 MJ kg^{-1}). The pellet kernel utilised in the current study contained ~10% crude lipid, which was top coated with an additionally 20% lipid (fish oil and poultry oil; total crude lipid level 27%) at Aquafeeds Australia (Mount Barker, South Australia, Australia).

Eight experimental diets were designed in the current study to be deficient, meet or exceed the requirements of LC n-3 PUFA by YTK, which was manipulated by changing the proportion of fish oil and poultry oil (Table 3.1.1.1.1). Poultry oil was selected as the fish oil replacement due its lack of LC n-3 PUFA, and also due to the promising results previously reported in two separate studies that utilised fish oil replacement diets (Bowyer et al., 2012; Stone et al., 2016). For Diet 1 for example, the pellet kernel was top-coated with an additional 8% fish oil and 12% poultry oil in addition to the inherent pellet kernel lipids (2.95 g 100 g^{-1} ∑LC n-3 PUFA), which was hypothesised to exceed the LC n-3 PUFA requirements of YTK, based on the reported requirements of the closely related Japanese Yellowtail (*Seriola quinqueradiata*; 45-85 g;) of 2.00 g 100 g^{-1} ∑LC n-3 PUFA (Deshimaru et al., 1982). Diet 8 was formulated to contain no additional fish oil, but the inherent LC n-3 PUFA from the dietary inclusions of fish meal was supplied. Diet 8 was formulated based on preliminary research with YTK (1.67 kg; Stone et al., 2016) to be LC n-3 PUFA deficient (Diet 8: 0.753 g 100 g^{-1} ∑LC n-3 PUFA). The LC n-3 PUFA and fish oil replacement levels were selected in the current study to cover the range required observe a dose-dependent effect of graded LC n-3 PUFA levels (i.e. to exceed the LC n-3 PUFA requirements in Diet 1, be deficient in LC n-3 PUFA in Diet 8, and meet the requirements between these two diets), which was required to estimate the practical LC n-3 PUFA requirements of large YTK. The biochemical composition of the eight experimental diets are displayed in Table 3.1.1.1.2 and 3.1.1.1.3. Fish were fed to apparent satiation at 11:00 h daily. Apparent satiation was achieved by providing feed

to the tank and monitoring feed intake of fish over a period of four min tank^{-1}. Care was taken to minimise waste by dispersing feed evenly and slowly across each tank. Once small quantities of uneaten feed were observed on the tank bottom, fish were judged to have reached apparent satiation. Tanks were cleaned every second day. As required, mortalities were removed, weighed, measured and recorded required and replaced with tagged fish (T-tags) of a similar weight. Tagged fish were included in biomass calculations for FCR (see Performance indice section), but excluded from all other analyses. This study ran for a total of 84 days.

Experimental fish

Experimental work was conducted in the pool-farm facility at the South Australian Research and Development Institute, South Australia Aquatic Science Centre (SARDI SAASC; West Beach, South Australia, Australia). YTK (n = 480; 2.67 ± 0.02 kg; 556 ± 3 mm (fork length; mean ± standard deviation) were obtained from Clean Seas Seafood (Port Lincoln, South Australia, Australia). Upon arrival at the SARDI SAASC facility, YTK were transferred to 5000 L tanks supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated, aerated sea water at ambient temperature and held for ~3.5 months and fed a standard Ridley Pelagica diet (crude protein 46%; crude lipid 24%; gross energy 19.30 MJ kg^{-1}).

Skin and gill fluke treatment

Upon arrival at SARDI SAASC, YTK were inspected, and were observed to have a low burden of skin flukes (*Benedenia seriola*) and gill flukes (*Zeuxapta seriola*). Treatment was deemed necessary, and was prescribed by Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd., Ballina, New South Wales, Australia). Prior to the commencement of the trial, fish were exposed to two treatments (16/11/15 and 30/11/15) of formalin (250 ppm for 30 min) at 19-22 °C.

Experimental Stocking and Intermediate weight checks

At the commencement of the current study (February 2016), YTK were anaesthetised in 5000 L tanks (total water volume 2500 L) using AQUI-S® (AQUI-S® New Zealand Ltd., Lower Hutt, New Zealand) at a concentration of 14 mg L^{-1} of seawater. Twenty fish were removed from their tank, measured, weighed and stocked into one of the three replicate 5000 L tanks treatment combination^{-1} (n = 8 treatments; n = 24 tanks).

Tanks were supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated sea water at ambient temperature. All tanks were supplied with aeration and oxygenation throughout the study.

At day 28 and 56, post-stocking, all fish were anaesthetised using AQUI-S® at a concentration of 14 mg L^{-1} of seawater. YTK were measured, weighed and visually inspected for skin and gill flukes, before fish were returned to their respective tanks.

Water quality analyses

Water quality parameters were measured daily at 14:30 h and maintained at appropriate levels for acceptable growth of YTK throughout the study (Table 3.1.1.1.4). Water temperature was measured using a thermometer. Dissolved oxygen (mg L^{-1} and % saturation) was measured using a dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark). The pH was measured daily using a meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, Illinois, United States of America). Salinity (g L^{-1}) was measured weekly using a portable salinity refractometer (model RF20, Extech Instruments, Nashua, New Hampshire, United States of America).

Biochemical and histological analyses

The proximate composition analyses of diets and whole body tissue were conducted according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardization (DIN) (2000). A one kg sample of each diet was collected, ground and analysed for proximate composition (moisture, protein, fat, ash, carbohydrate and energy), amino acid profile, taurine level, mineral composition, fatty acids profile and cholesterol level and rancidity (p-anisidine and peroxide value). In addition, a total of twelve fish (n = 12 fish) at the start of the experiment, and four fish from each tank (n = 4 fish tank^{-1}; n = 24 tanks; n = 96 fish) at the conclusions of the experiment were collected and stored frozen at -20 °C. Whole fish samples were partially thawed, homogenised and analysed for proximate composition (moisture, protein, fat, ash, carbohydrate and energy), amino acid profile, mineral composition, fatty acids profile.

Blood samples from three fish per tank (n = 3 fish tank^{-1}; n = 24 tanks; n = 72 fish) were collected using a 19 G needle with a 5 mL syringe in two separate Vacuette® tubes (lithium heparin and EDTA). Blood samples were analysed for blood haematocrit at SARDI SAASC, and haematology and biochemistry analyses conducted by IDEXX (Unley, South Australia, Australia). These blood sampled fish were then dissected and the visceral and liver was weighed in order to calculate visceral index (VSI; %) and hepatosomatic index (HSI; %), respectively. From blood sampled fish, a 1 cm^3 section of liver, and a 1cm^2 longitudinally opened midgut and hindgut section were collected for histology. In brief, samples were fixed in 10% seawater formalin for > 48 h, processed and embedded in paraffin wax. Tissue sections were cut using a microtome and floated onto Starfrost® glass slides and dried for > 24 h at room temperature before being stained. Liver sections were stained with hematoxylin and eosin, and were subjectively scored for fatty change, inflammation, melanomacrophage centres, proliferation of bile ducts and haemorrhage by Dr Fran Stephens (Aquatilia Healthcare, Western Australia, Australia). Subjective scores ranged from 0: not observed, 1: Rare, 2: Mild, 3: Moderate, 4: Severe. Hindgut and midgut sections were stained with both hematoxylin and eosin and high iron diamine/alcian blue pH 2.5 (HID/AB pH 2.5). Villus height, width, perimeter, area and branching, total goblet cell number and composition were measured in the hindgut and midgut.

Performance indices

All data reported for each treatment for animal performance were based on the mean of the replicate tanks. All calculations using fish weight and diets were based on wet or as fed values, respectively:

- Weight gain = final weight - initial weight
- Biomass gain (kg tank^{-1}) = (final weight + ∑mortality weight) - (initial weight + ∑replacement weight)
- Specific growth rate (SGR; % d^{-1}) = ([ln final weight - ln initial weight] / d) × 100
- Length growth rate (mm d^{-1}) = (final fish length - initial fish length) / d
- Condition factor = (fish weight [g] / fish length [cm]^3) × 100
- Apparent feed conversion ratio (FCR) = feed consumed / fish weight gain
- Apparent protein deposition = ([final whole protein - initial whole protein] / protein intake) × 100
- Apparent energy deposition = ([final whole energy - initial whole energy] / energy intake) × 100
- Haematocrit count = red blood cell (mm) / total blood (red blood cell and plasma [mm]) × 100
- Visceral index (VSI; %) = wet visceral wt × 100 / final wet fish wt
- Hepatosomatic index (HSI; %) = wet liver wt × 100 / final wet fish wt

Statistical analyses

IBM SPSS (version 24 for Windows; IBM SPSS Inc., USA) was used for all statistical analyses. Homogeneity of variances and normality among mean values were assessed using Levene's test for equality of variance errors and Shapiro-Wilk test, respectively. Data were compared across all treatments using a one-factor ANOVA. When significant effects were observed, post-hoc tests were used to detect significant differences between all treatments (Student-Newman-Keuls test). Quadratic polynomial regression analyses were also applied to determine the relationship between dietary LC n-3 PUFA levels and SGR, FCR and energy deposition. A significance level of $P < 0.05$ was used for all statistical tests. All values are presented as means ± standard error (SE) of the mean unless otherwise stated.

Results

General observations

There were no significant differences in the initial weight or fork length of YTK between treatments at the commencement of the current study ($P > 0.05$; Table 3.1.1.1.5). The average initial weight and fork length were 2.67 ± 0.02 kg and 556 ± 3 mm (fork length; mean ± standard deviation), respectively. YTK fed actively during the experiment, with no apparent differences observed between dietary treatments. The overall mortality for fish in the study was low (1.46%), and no apparent signs of disease were observed. Moreover, apart from the initial presence of gill and skin flukes, there were negligible gill and skin fluke burdens observed throughout the study.

Growth performance

Final weight, biomass gain, SGR, final fork length and length growth rate were significantly influenced by dietary LC n-3 PUFA level ($P < 0.05$; one-factor ANOVA; Table 3.1.1.1.5). Generally, the growth performance of fish fed the diet containing 0.75 g 100 g^{-1} LC n-3 PUFA was significantly lower than fed dietary LC n-3 PUFA above 1.83 g 100 g^{-1}. Final condition factor was not significantly influenced by dietary LC n-3 PUFA level ($P = 0.146$; one-factor ANOVA; Table 3.1.1.1.5). There was a moderate positive significant quadratic relationship between dietary LC n-3 PUFA level and SGR ($R^2 = 0.5697$; $P < 0.001$; Figure 3.1.1.1.1). Based on the SGR, the estimated optimal dietary level of LC n-3 PUFA (turning point; ymax) was 2.12 g 100 g^{-1}. The 95% CI for the SGR response variable ranged between 1.90 to 2.33 g 100 g^{-1}. There appeared to be no improvement to growth by increasing LC n-3 PUFA levels above 2.39 g 100 g^{-1}.

Feed utilisation

Apparent feed consumption (kg $tank^{-1}$) and feed intake (% BW d^{-1}) was not significantly influenced by dietary LC n-3 PUFA level ($P > 0.05$; one-factor ANOVA; Table 3.1.1.1.5). Apparent FCR was significantly influenced by dietary LC n-3 PUFA level ($P = 0.008$; one-factor ANOVA; Table 3.1.1.1.5). The apparent FCR of fish fed the diet containing 0.75 g 100 g^{-1} LC n-3 PUFA was significantly higher than those fed all diets containing higher LC n-3 PUFA levels. In addition, there was a moderate negative significant quadratic relationship between dietary LC n-3 PUFA level and FCR ($R^2 = 0.5758$; $r = 0.7588$; $P < 0.001$; Figure 3.1.1.1.2). This relationship was inversely related to the relationship between LC n-3 PUFA level and SGR. The FCR of YTK decreased (improved) as dietary LC n-3 PUFA levels increase from 0.75 to 2.14 g 100 g^{-1}. Based on the FCR, the estimated optimal dietary level of LC n-3 PUFA (turning point; y_{min}) was 2.26 g 100 g^{-1}. The 95% CI for the FCR response variable ranged between and 1.93 to 2.58 g 100 g^{-1}.

Whole fish proximate and energy composition

The tissue moisture (58.7-60.0%), protein (19.58-20.76% wet), lipid (17.6-19.4% wet) ash (2.0-2.7% wet), carbohydrate (< 1.5% wet) and energy contents (9.94-10.57 MJ kg^{-1} wet) of fish were not significantly influenced by dietary LC n-3 PUFA level ($P > 0.05$; one-factor ANOVA; Table 3.1.1.1.5).

Nutrient utilisation

Dietary LC n-3 PUFA level did not significantly affect apparent protein deposition (18.51-22.95%) and apparent energy deposition (23.20-32.05%; $P > 0.05$; one-factor ANOVA; Table 3.1.1.1.5). There was a tendency for the apparent protein deposition and apparent energy deposition of fish to be lower when fed the diet containing 0.75 g 100 g^{-1} LC n-3 PUFA diet compared to those fed diets containing higher dietary LC n-3 PUFA levels.

There was a moderate positive significant quadratic relationship between dietary LC n-3 PUFA level and energy deposition ($R^2 = 0.3225$; $P = 0.017$; Figure 3.1.1.1.3). This relationship was inversely related to the relationship between LC n-3 PUFA level and energy deposition. Based on the energy deposition, the estimated optimal dietary level of LC n-3 PUFA (turning point; y_{min}) was 2.51 g 100 g^{-1}.

Whole fish fatty acid, amino acid and mineral composition

There were numerous significant differences of the whole fish fatty acid levels between dietary treatments ($P < 0.05$; one-factor ANOVA; Table 3.1.1.1.6). Typically, the fatty acid profile of whole fish mirrored the fatty profile of the diets. Most noteworthy, the EPA, DPA, DHA and ∑LC n-3 PUFA of fish significantly increased with increasing provision of dietary fish oil and dietary EPA, DPA, DHA and ∑LC n-3 PUFA ($P < 0.05$; one-factor ANOVA; Table 3.1.1.1.6).

Whole fish amino acid levels (essential [arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine] and non-essential [alanine, aspartic acid, glutamic acid, glycine, proline, hydroxyl proline, serine and tyrosine]) were not significantly affected by dietary LC n-3 PUFA level ($P > 0.05$; one-factor ANOVA; Table 3.1.1.1.7).

Whole fish mineral levels (calcium, copper, iodine, iron, magnesium, manganese, potassium, phosphorus, selenium, zinc) were not significantly influenced by dietary LC n-3 PUFA levels ($P > 0.05$; one-factor ANOVA; Table 3.1.1.1.8).

Blood biochemistry and haematology

Serum protein level was significantly affected by dietary LC n-3 PUFA level ($P = 0.042$; one-factor ANOVA; Table 3.1.1.1.9). Serum protein level of fish fed the diet containing 2.13 g 100 g^{-1} LC n-3 PUFA was significantly lower than those fed the diets containing either 1.83 g 100 g^{-1} LC n-3 PUFA or 1.29 g 100 g^{-1} LC n-3 PUFA ($P < 0.05$). Serum protein level of fish was not significantly different between other dietary treatments ($P > 0.05$). All other measured blood biochemistry and haematology parameters were not significantly influenced by dietary LC n-3 PUFA level ($P > 0.05$; one-factor ANOVA; Table 3.1.1.1.9).

Visceral somatic parameters, liver and gastrointestinal tract morphology

Dietary LC n-3 PUFA level did not significantly affect viscerosomatic index (5.58-6.77%) and hepatosomatic index (1.16-1.36%; $P > 0.05$; one-factor ANOVA; Table 3.1.1.1.10).

Liver morphology scores for fatty change, inflammation, melanomacrophage centres, proliferation of bile ducts and haemorrhages were not significantly affected by dietary LC n-3 PUFA level ($P > 0.05$; one-factor ANOVA; Table 3.1.1.1.10). While inflammation, melanomacrophage centres, proliferation of bile ducts and haemorrhages scores were low (0-1), fatty change was high, and scored 3 for all dietary treatments.

Hindgut and midgut morphology was variable. Dietary LC n-3 PUFA level significantly influenced villus branching in the hindgut (P = 0.028; one-factor ANOVA; Table 3.1.1.1.10). Fish fed the diet containing 2.13 g 100 g^{-1} LC n-3 PUFA had significantly higher hindgut villus branching than those fed diets containing either 1.29 g 100 g^{-1} LC n-3 PUFA or 0.75 g 100 g^{-1} LC n-3 PUFA ($P < 0.05$), while villus branching of fish fed other dietary LC n-3 PUFA levels were statistically similar ($P > 0.05$). Villus branching in the midgut was not significantly affected by dietary LC n-3 PUFA level ($P > 0.05$). Additionally, hindgut and midgut villus height, width, perimeter, area, and total goblet cell number and composition were not significantly influenced by dietary LC n-3 PUFA level ($P > 0.05$; one-factor ANOVA; Table 3.1.1.1.10).

Discussion

Our aim in the current study was to improve current diet formulations and economic viability for YTK production during summer by reducing/optimising dietary fish oil levels by understanding the practical dietary LC n-3 PUFA requirements for optimum growth of large YTK (> 2 kg) at warm summer water temperatures. In order to achieve this, YTK were fed graded dietary LC n-3 PUFA levels (fish oil). Optimum SGR and FCR were obtained when YTK consumed 191 and 203 mg LC n-3 PUFA kg $fish^{-1}$ d^{-1}, respectively. Based on the combined results for SGR and FCR, the optimal dietary level of LC n-3 PUFA for large YTK at warm summer water temperatures was estimated to be between 2.12 and 2.26 g 100 g^{-1}. The 95% CI for each response variable ranged between 1.90 to 2.33 g 100 g^{-1} and 1.93 to 2.58 g 100 g^{-1} for SGR and FCR, respectively. It is important to recognise the aforementioned estimates were made by fitting quadratic polynomial regression models to the data and that other dose-response models may provide different values. In terms of digestive tract health, apart from minor alterations to hindgut villus branching, changing dietary LC n-3 PUFA level had no significant impacts on hind or midgut histology of large YTK during this study. Further research under commercial conditions are needed before implementing the recommended dietary level of LC n-3 PUFA on-farm.

These recommended levels compare to, albeit slightly higher than, the reported requirement of 2 g LC n-3 PUFA 100 g^{-1} for juveniles of the closely related Japanese Yellowtail (Deshimaru et al., 1982). The differences in recommendations may, in part be explained by species and size differences. Differences may also be explained by differences in diet specifications and potential growth rates between studies in relation to lipid and energy levels. The study of Deshimaru et al. (1982) was run using low energy diets containing ~15% total lipid, whereas the current study used higher energy diets containing ~26% lipid. More recently, the NRC (2011) suggested that EFA requirements should perhaps be reassessed for fast growing fish species fed high energy/lipid diets. As such contrasting results from current studies with previous older research using lower energy diets, may be misleading. Bearing this in mind, and given the interest of the Australian YTK industry to use higher energy diets, the LC n-3 PUFA recommendation for large YTK in the current study appears to be valid.

Stubhaug et al. (2007) suggested in general, fish do not preferentially retain LC n-3 PUFA. However, Brodtkord et al. (1997) suggested that DHA appears to be an exception, when dietary levels of DHA are deficient, DHA is preferentially retained in the tissue for most fish species. Results from an Honours project linked to the experiment in the current study, conducted by Samantha Chown, indicated that DHA deposition in white muscle tissue of large YTK was significantly altered by dietary LC n-3 PUFA level (Figure 3.1.1.1.4). Below dietary threshold levels of LC n-3 PUFA, of between 2.13 and 2.39 g 100 g^{-1}, tissue levels of DHA appeared to be significantly reduced (Figure 3.1.1.1.4). Additionally, when dietary levels of LC n-3 PUFA were below 2.13 g 100 g^{-1}, DHA levels in white muscle tissue appeared to be conserved when dietary levels of LC n-3 PUFA were between 0.75 and 1.83 g 100 g^{-1} (Figure 3.1.1.1.4). This could be in part due to the Δ4 double bond in DHA being relatively resistant to mitochondrial β-oxidation (Madsen et al., 1998). Therefore, it appears that when dietary levels of LC n-3 PUFA are below 2.13 g 100 g^{-1}, Large YTK may not have reserve DHA levels required for other essential metabolic processes related to growth and health. This condition may become exacerbated when fish are challenged with routine or unexpected culture stresses. Nevertheless, this result further supports the estimated optimal dietary level of LC n-3 PUFA (between 2.12 and 2.26 g 100 g^{-1}), for

growth and feed utilisation of large YTK at warm summer water temperatures, put forward in the current study.

Several important issues need to be taken into consideration when formulating production diets to economically deliver the targeted level of LC n-3 PUFA:

- There is not set recommended level of fish oil inclusion, as such, to deliver a set dose rate of LC n-3 PUFA to meet the recommended daily requirement. The actual quality of the fish oil, in terms of LC n-3 PUFA content, will determine the amount required, and must be ascertained prior to formulation and manufacture. For example, if the fish oil contains high levels of LC n-3 PUFA (high quality) lower inclusion levels will be required, and vice versa if the fish oil is of lower quality a higher amount of oil will be required.
- The delivery of the recommended dose rate of LC n-3 PUFA may also be impacted if the total lipid content of the diet is altered. Research investigating optimum protein and lipid levels with large YTK at warm water temperatures (Manuscript 3.1.2.2) indicated that increasing dietary lipid level from 25 to 30% resulted in significant reductions in feed intake of 9 and 14% at 40 and 44% crude protein levels, respectively. This significant reduction in feed intake means that to achieve the target dose rate of 191 to 203 mg LC n-3 PUFA kg^{-1} d^{-1}, the amount of fish oil added to the diet will need to be increased.
- Currently fish oil is the practical, although expensive, source of LC n-3 PUFA (Glencross et al., 2007; Tacon and Metian, 2009). Limited supplies of fishery by-product oils are also available. Alternatives ingredients rich in LC n-3 PUFA, such as algal oil and genetically modified varieties of plant oils (canola and soy oil) are on the horizon. Although being touted as economically viable, the genetically modified plant oils tend to be perceived as socially un-acceptable. Nevertheless, once LC n-3 PUFA rich alternate oils become commercially available further reductions in the use of marine fish oil will be attainable.

Collectively, all of the aforementioned issues will impact the sustainable production of YTK production diets

In addition to LC n-3 PUFA, fish oil is also a rich source of cholesterol (870 mg 100 g^{-1}), while alternative lipid sources such as poultry oils contain lower levels (270 mg 100 g^{-1}). The cholesterol levels of the diets progressively declined from 295 to 243 mg 100 g^{-1} as fish oil was substituted with poultry oil (Table 3.1.1.1.1). Cholesterol has many important biological functions including disease resistance and taurine metabolism (Hernández et al., 2004; Moschetta et al., 2005; Maita et al., 2006; NRC, 2011). Cholerstrol can be synthesised by most vertebrates from sterol precursors; however, the abaility of YTK to do so is unknown. In addition to understanding the LC n-3 PUFA requirements of YTK, dietary cholesterol levels need to be considered when fish oil is replaced with alternative lipid sources, including poultry oil. Previous studies have recommended that the supplementation of dietary cholesterol is necessary when substituting high levels of fish oil with oil ingredients low in cholesterol, to prevent hypocholesterolemia and associated health issues (Stone and Bellgrove, 2013). Currently however, the dietary cholesterol requirement, and cholesterol *de novo* synthesise rate is unknown for large YTK, particularly when utilising low dietary inclusions of fish oil. Further research in this area is needed.

Conclusions and Recommendations

Based on results for SGR and FCR from the current study, it is estimated that the conservative and practical dietary ∑LC n-3 PUFA level for optimal growth of YTK (2.67-3.84 kg) at warm water temperatures is between 2.12 and 2.26 g 100 g^{-1}. The 95% CI for each response variable ranged between 1.90 to 2.33 g 100 g^{-1} and 1.93 to 2.58 g 100 g^{-1} for SGR and FCR, respectively. This level compares to the reported requirement for LC n-3 PUFA of the closely related Japanese yellowtail (*Seriola quinqueradiata*; 45-80 g) of 2.00 g 100 g^{-1} (Deshimaru et al., 1982). It is important to recognise the aforementioned estimates were made by fitting quadratic polynomial regression models to the data and that other dose-response models may provide different values. Pilot scale commercial research trials are

recommended to validate these requirements under commercial conditions before implementing this level of LC n-3 PUFA in commercial diets.

Findings

- Optimising LC n-3 PUFA levels in YTK diets by reducing dietary fish oil inclusions lead to improved diet sustainability and diet cost savings, compared to current commercial diets.
- We recommend that these results are followed up with further pilot scale commercial trials before the implementing this LC n-3 PUFA level on-farm. Levels of DHA in white muscle tissue of YTK were conserved when dietary LC n-3 PUFA levels were limiting growth.
- All FCRs in the current Manuscript ranged from 2.43 down to 2.03. Apparent feed conversion ratio (FCR) was significantly influenced by diet, and tended to be improved in fish fed diets containing > 1.83 g 100 g^{-1} LC n-3 PUFA (2.03 -2.11) compared to other diets.
- An improvement in FCR based on the information provided within this Manuscript, will assist feed manufacturers in formulating commercial diets that achieve one of the overarching goals of the K4P project, which was to provide information to assist producers to achieve FCRs of < 2.2 for large YTK between 1.5-3.5 kg.

Publications

No publications have resulted from this R&D to date.

Acknowledgements

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The authors would also like to acknowledge the South Australian Research and Development Institute (SARDI) and Marine Innovation Southern Australia for their financial support of Assoc. Prof. David Stone and the provision of the SARDI SAASC experimental facilities at West Beach, South Australia. We would also like to thank Dr Richard Smullen and Dr Simon Tabrett of Ridley for their input into experimental design, and Dr Matthew Bransden of Skretting Australia for their input into experimental design, experimental diet formulation and manufacture. Thanks to Dr Amber Tomas (Educational Statistical Consultancy) for her assistance with modelling and statistical analyses. Thanks to Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd.) for the diagnosis and prescription of skin and gill fluke treatments. We also thank Leigh Kuerschner, Krishna-Lee Currie, Jessica Buss, Nicole Thompson, Filipa Isabel, Samantha Chown and Aaron Teoh for their technical assistance during the experiment.

References

Bowyer, J.N., Qin, J.G., Smullen, R.P., Stone, D.A.J., 2012. Replacement of fish oil by poultry oil and canola oil in Yellowtail Kingfish (*Seriola lalandi*) at optimal and suboptimal temperatures. Aquaculture 356-357, 211-222.

Brodtkord, T., Rosenlund, G., Lie, O., 1997. Effects of dietary levels of 20:5n-3 and 22:6n-3 on tissue lipid composition in juvenile Atlantic salmon, *Salmo salar*, with emphasis on brain and eye. Aquaculture Nutrition 3, 175-187.

Deshimaru, O., Kuroki, K., Yone, Y., 1982. Suitable levels of lipids and Ursodeoxycholic acid in diet of yellowtail. Bulletin of Japanese Society of Scientific Fisheries 48, 1265-1270.

Glencross, B.D., Booth, M., Allan, G.L., 2007. A feed is only as good as its ingredients - a review of ingredient evaluation strategies for aquaculture feeds. Aquaculture Nutrition 13, 17-34.

Hernández, P.V., Olvera-Novoa, M.A., Rouse, D.B., 2004. Effect of dietary cholesterol on growth and survival of juvenile redclaw crayfish *Cherax quadricarinatus* under laboratory conditions. Aquaculture 236, 405-411.

Higgs, D.A., Balfry, S.K., Oakes, J.D., Rowshandeli, M., Skura, B.J., Deacon, G., 2006. Efficacy of an equal blend of canola oil and poultry fat as an alternative dietary lipid sources for Atlantic Salmon (*Salmo salar* L.) in sea water. I: effects on growth performance, and whole body and fillet proximate and lipid composition. Aquaculture Research 37, 180-191.

Kalogeropoulos, N., Alexis, M.N., Henderson, R.J., 1992. Effects of dietary soybean and cod-liver oil levels on growth and body composition of gilthead bream (*Sparus aurata*). Aquaculture 104, 293-308.

Maita, M., Maekawam, J., Satoh, K., Sutami, K., Satoh, S., 2006. Disease resistance and hypocholesterolemia in yellowtail, *Seriola quinqueradiata*, fed non-fishmeal diet. Fisheries Science 72, 513-519.

Madsen, L., Frøyland, L., Dyrøy, E., Helland, K., Berge, R.K., 1998. Docosahexaenoic and eicosapentaenoic acids are differently metabolized in rat liver during mitochondria and peroxisome proliferation. Journal of Lipid Research 39, 583-593.

Masumoto, T., 2002. Yellowtail, *Seriola quinqueradiata*. In: Webster, C.D., Lim, C.E. (Eds), Nutrient Requirements and Feeding of Finfish for Aquaculture. CABI Publishing, Oxon, UK, pp. 131-146.

Moschetta, A., Xu, F., Hagey, L. R., van Berge-Henegouwen, G.P., van Erpecum, K.H., Browers, J.F., Cohen, J.C., Bierman, M., Hobbs, H.H., Steinbach, J.H., Hofmann, A.F., 2005. A phyolgenetic survey of bilary lipids in vertebrates. Journal of Lipid Research 46, 2221-2232.

National Research Council (NRC), 2011. Nutrient Requirements of Fish and Shrimp, National Academy Press, Washington, DC, pp. 1-376.

Oliva-Teles, A., 2012. Nutrition and health of aquaculture fish: review article. Journal of Fish Disease 35, 83-108.

Oliveira, A.C.M., Stone, D.A.J., Plante, S., Smiley, S., Bechtel, P.J., Hardy, R.W., 2008. Fish oils from Alaskan seafood processing by-products: an un-exploited sustainable resource for aquaculture. World Aquaculture 39, 50-69.

Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The Lipids. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition. Academic Press, San Diego, USA, pp. 181-257.

Serhan, C.N., 2014. Novel pro-resolving lipid mediators in inflammation are leads for resolution physiology. Nature 510, 92-101.

Simopoulos, A.P., 2000. Human requirement for n-3 polyunsaturated fatty acids. Poultry Science 79, 961-70

Stone, D.A.J., Oliviera, A.C.M., Plante, S., Smiley, S., Bechtel, P., Hardy, R.W., 2011a. Enhancing highly unsaturated ω-3 fatty acids in phase-fed rainbow trout (*Oncorhynchus mykiss*) using Alaskan fish oils. Aquaculture Nutrition 17, 501-510.

Stone, D.A.J, Oliviera, A.C.M., Ross, C., Plante, S., Smiley, S., Bechtel, P., Hardy, R.W., 2011b. The effects of phase-feeding rainbow trout (*Oncorhynchus mykiss*) with canola oil and Alaskan pollock fish oil on fillet fatty acid composition and sensory attributes. Aquaculture Nutrition 17, 521-529.

Stone D.A.J., Bellgrove, E., 2013. A literature review: the current status of knowledge of the nutritional requirements of yellowtail kingfish. In: Stone, D.A.J. and Bowyer, J.N. (Eds). Final Report. Sustainable Feeds and Feed Management for Yellowtail Kingfish (*Seriola lalandi*). South Australian Research and

Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2013/000200-1. SARDI Research Report Series No. 751. pp. 92-121.

Stone, D.A.J., D'Antignana, T.D., Bansemer, M.S., 2016. Final Report. Refining Yellowtail Kingfish feeds and feed management. Prepared by the South Australian Research and Development Institute (Aquatic Sciences), Adelaide. AS-CRC Project No. 2013/730. 144 pp.

Stubhaug, I., Lie, Ø., Torstensen, B.E., 2007. Fatty acid productive value and β-oxidation capacity in Atlantic salmon tissues (*Salmo salar* L.) fed on different lipid sources along the whole growth period. Aquaculture Nutrition 13, 145-155.

Tacon, A.G., Metian, M., 2009. Fishing for feed or fishing for food: increasing global competition for small pelagic forage fish. Ambio 38, 294-302.

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Reviews in Fisheries Science 11, 107-184.

Tocher, D.R., 2009. Issues surrounding fish as a source of omega-3 long-chain polyunsaturated fatty acids. Lipid Technology 21, 13-16.

Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquaculture Research 41, 717-732.

Yone, Y., 1978. Essential fatty acids and lipid requirement of marine fish. In: Yone, Y. (Ed.), Dietary Lipids in Aquaculture. Koseisya-Koseikaku, Tokyo, (in Japanese). pp. 43-59.

Table 3.1.1.1.1. Experimental design of dietary treatments to investigate the practical conditional dietary requirements for long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA; EPA, DPA and DHA) in large Yellowtail Kingfish (*Seriola lalandi*) at warm water temperatures.

Item	Pellet kernel (%)[1,2]	Fish oil (%)[1,2]	Poultry oil (%)[1,2]	∑LC n-3 PUFA level (g 100 g^{-1})
D2.95	80	8	12	2.950
D2.39	80	6	14	2.390
D2.13	80	5	15	2.140
D1.83	80	4	16	1.830
D1.61	80	3	17	1.610
D1.29	80	2	18	1.293
D1.01	80	1	19	1.012
D0.75	80	0	20	0.753

[1] Pellet kernel, fish oil and poultry oil supplied by Skretting Australia (Cambridge, Tasmania, Australia).
[2] Pellet kernel, fish oil and poultry oil were analysed for total crude lipid and fatty acid profile prior to top-coating pellet kernel (lipid level and ∑LC n-3 PUFA level for the pellet kernel, fish oil and poultry oil was 9.55, 100.00 and 100.00%, and 1.03, 34.10 and 0.40 g 100 g^{-1}, respectively).

Table 3.1.1.1.2. Analysed proximate, mineral and amino acid composition of the eight test diets used in the current trial.

Diet[1]	D2.95	D2.39	D2.13	D1.83	D1.61	D1.29	D1.01	D0.75
Item (as fed)								
Proximate composition (g 100 g^{-1})								
Moisture	7.3	7.3	7.2	7.2	7.3	7.2	7.3	7.3
Crude protein	43.19	43.31	42.94	43.00	42.58	43.00	43.06	42.94
Crude lipid	26.2	26.0	26.9	26.6	27.1	26.9	26.8	27.1
Ash	9.7	9.8	9.6	9.9	9.6	9.8	9.7	9.5
Carbohydrate[2]	13.6	13.6	13.4	13.3	13.4	13.1	13.1	13.2
Gross energy (MJ kg^{-1})	19.35	19.29	19.52	19.41	19.55	19.49	19.47	19.56
Rancidity test								
p-Anisidine Value	20.7	19.0	19.3	17.5	15.6	15.1	15.3	14.5
Peroxide Value	4.1	4.0	2.9	<0.1	<0.1	<0.1	<0.1	<0.1
Analysed minerals (mg kg^{-1})								
Calcium	28000	28000	26000	26000	26000	27000	27000	26000
Copper	13	12	12	19	12	12	12	12
Iodine	4.5	1.9	3.0	2.9	2.8	3.4	2.5	3.1
Iron	750	690	720	700	670	740	750	690
Magnesium	1500	1500	1400	1500	1500	1500	1500	1500
Manganese	35	41	39	36	35	38	40	41
Phosphorus	19000	18000	18000	18000	18000	18000	18000	18000
Potassium	6000	6000	5900	6000	5900	5900	5900	5900
Selenium	1.8	1.8	1.8	1.9	1.7	1.7	1.9	1.8
Zinc	200	190	190	200	190	190	190	190
Analysed amino acids (g 100 g^{-1})								
Alanine	2.650	2.624	2.626	2.663	2.625	2.598	2.633	2.647
Arginine	2.497	2.499	2.511	2.580	2.560	2.467	2.534	2.576
Aspartic acid	4.176	3.913	2.675	3.149	4.067	3.199	3.129	3.980
Glutamic acid	4.823	5.418	6.542	5.983	5.009	5.773	5.716	5.103
Glycine	3.013	2.998	2.956	3.174	3.055	2.958	3.062	3.054
Histidine	1.212	1.128	1.139	1.178	1.192	1.125	1.177	1.153
Isoleucine	1.439	1.436	1.445	1.428	1.439	1.415	1.459	1.435
Leucine	3.337	3.307	3.357	3.353	3.316	3.293	3.349	3.332
Lysine	2.764	2.705	2.856	3.017	2.912	2.750	2.959	2.781
Methionine	1.064	1.032	1.046	1.087	1.041	1.056	1.074	1.035
Phenylalanine	1.967	2.072	2.150	2.019	2.016	2.072	1.991	2.030
Proline	2.345	2.393	2.429	2.511	2.460	2.409	2.412	2.344
Hyroxy Proline	0.775	0.767	0.757	0.796	0.782	0.782	0.768	0.789
Serine	1.647	1.586	1.586	1.687	1.636	1.627	1.655	1.604
Threonine	1.609	1.598	1.628	1.644	1.638	1.599	1.628	1.613
Tyrosine	1.187	1.155	1.191	1.175	1.168	1.169	1.192	1.167
Valine	2.365	2.328	2.368	2.397	2.353	2.317	2.384	2.349
Total amino acid	38.87	38.96	39.26	39.84	39.27	38.61	39.12	38.99
Other (mg 100 g^{-1})								
Taurine	686	720	745	820	715	753	708	704
Choline (Hydroxide)	358.18	353.96	350.96	353.18	353.84	345.76	342.28	342.41
Cholesterol	295	285	286	276	268	251	250	243

[1] Pellet kernel, fish oil and poultry oil to manufacture diets were supplied by Skretting Australia (Cambridge, Tasmania, Australia).
[2] Carbohydrate = 100 - (moisture + lipid + protein + ash).

Table 3.1.1.1.3. Analysed fatty acid composition of the eight test diets used in the current trial.

Diet[1]	D2.95	D2.39	D2.13	D1.83	D1.61	D1.29	D1.01	D0.75
Analysed fatty acids (mg 100 g^{-1})								
Saturated Fatty Acids								
C4:0 Butyric	<10	<10	<10	<10	<10	<10	<10	<10
C6:0 Caproic	<10	<10	<10	<10	<10	<10	<10	<10
C8:0 Caprylic	<10	<10	<10	<10	<10	<10	<10	<10
C10:0 Capric	<10	<10	<10	<10	<10	<10	<10	<10
C12:0 Lauric	<10	<10	<10	<10	<10	<10	<10	<10
C13:0 Trisdecanoic	<10	<10	<10	<10	<10	<10	<10	<10
C14:0 Myristic	900	760	730	660	620	540	480	420
C15:0 Pentadecanoic	89	77	77	74	69	66	59	53
C16:0 Palmitic	5550	5570	5760	5780	5860	5890	5880	5930
C17:0 Margaric	110	100	100	98	95	93	90	89
C18:0 Stearic	1670	1690	1770	1790	1810	1840	1860	1870
C20:0 Arachidic	53	49	51	47	44	48	44	36
C22:0 Docosanoic	31	31	30	28	26	24	23	25
C24:0 Tetracosanoic	21	<10	24	<10	<10	<10	<10	<10
Mono-unsaturated Fatty Acids								
C10:1 Decenoic	<10	<10	<10	<10	<10	<10	<10	<10
C14:1 Myristoleic	78	79	78	77	79	75	77	76
C15:1 Pentadecenoic	<10	<10	<10	<10	<10	<10	<10	<10
C16:1 Palmitoleic	1610	1570	1560	1530	1540	1490	1440	1450
C17:1 Heptadecenoic	50	46	51	50	53	52	50	53
C18:1n-6 Octadecenoic	<10	<10	<10	<10	<10	<10	<10	<10
C18:1n-7 Octadecenoic	670	650	660	650	660	650	640	640
C18:1n-9 Oleic	8020	8700	9290	9530	10080	10310	10580	11050
C20:1n-9 Eicosenoic	160	156	150	150	140	150	140	130
C20:1n-11,13 Eicosenoic	44	38	34	36	36	31	34	31
C20:1 Eicosenoic (total)	200	190	180	190	180	180	170	160
C22:1n-9 Docosenoic	47	<10	18	<10	<10	<10	<10	<10
C22:1n-11,13 Docosenoic	64	46	44	36	31	23	<10	<10
C24:1 Tetracosenoic	48	37	41	35	32	24	24	20
Poly-unsaturated Fatty Acids								
C18:2n-6 Linoleic	2300	2500	2650	2730	2900	2960	3040	3150
C18:2 Conjugated 9c 11t Octadecadienoic	33	37	38	39	42	40	45	46
C18:3n-6 Gamma Linolenic	43	38	41	37	36	35	30	31
C20:2n-6 Eicosadienoic	<10	<10	<10	<10	<10	<10	<10	<10
C20:3n-6 Dihomo-gamma-linoleic	33	33	35	28	28	25	27	24
C20:4n-6 Arachidonic	170	160	50	140	140	120	110	110
C22:4n-6 Docosatetraenoic	30	26	19	21	<10	<10	<10	<10
C22:5n-6 Docosapentaenoic	52	44	34	28	22	22	<10	<10
C18:3n-4 Octadectrenoic	<10	<10	<10	<10	<10	<10	<10	<10
C18:3n-3 Alpha Linolenic	430	460	490	490	520	530	540	550
C18:4n-3 Steridonic	270	220	190	160	130	110	75	51
C20:3n-3 Eicosatrienoic	<10	<10	<10	<10	<10	<10	<10	<10
C20:4n-3 Eicosatetraenoic	170	44	45	41	35	27	38	21
C20:5n-3 Eicosapentanaeoic	1350	1060	930	790	680	530	400	270
C21:5n-3 Heneicosapentaenoic	<10	<10	<10	<10	<10	<10	<10	<10
C22:5n-3 Docosapentaenoic	160	140	130	110	100	83	72	63
C22:6n-3 Docosahexaenoic	1440	1190	1080	930	830	680	540	420
∑LC n-3 PUFA	2950	2390	2140	1830	1610	1293	1012	753
n-3 FA:n-6 FA	1.45	1.11	1.01	0.84	0.73	0.62	0.52	0.41

[1] Pellet kernel, fish oil and poultry oil to manufacture diets were supplied by Skretting Australia (Cambridge, Tasmania, Australia)

Table 3.1.1.1.4. Summary of water quality parameters.

Item[1]	Temperature (°C)	Dissolved oxygen (mg L^{-1})	Dissolved oxygen (% saturation)	pH	Salinity (mg L^{-1})	Ammonia (ppm)	CO_2 (mg L^{-1})
Mean	19.7 ± 2.2	7.3 ± 0.6	97.7 ± 6.7	7.90 ± 0.12	36 ± 0	0.35 ± 0.25	2 ± 1
Range	15.5 - 24.5	4.8 - 9.9	70.0 - 119.0	7.58 - 8.16	36 - 36	0.00 - 2.00	1 - 5

[1] Values means ± standard deviation.

Table 3.1.1.1.5. Growth performance, feed utilisation, proximate composition and nutrient retention of Yellowtail Kingfish fed graded dietary long chain omega-3 polyunsaturated fatty acid levels for 84 days at warm summer water temperatures.

Diet[1]	D2.95	D2.39	D2.13	D1.83	D1.61	D1.29	D1.01	D0.75	ANOVA[2]
Growth performance									
Initial weight (kg)	2.67±0.02	2.67±0.02	2.66±0.01	2.67±0.01	2.67±0.01	2.66±0.02	2.66±0.02	2.67±0.02	$P = 0.994$
Final weight (kg)	3.77±0.04^{ab}	3.84±0.06^{a}	3.79±0.01^{ab}	3.84±0.04^{a}	3.81±0.05^{ab}	3.75±0.02^{ab}	3.71±0.04^{ab}	3.61±0.07^{b}	$P = 0.036$
Biomass gain (kg tank^{-1})	21.88±0.85^{ab}	23.28±0.80^{a}	22.59±0.11^{a}	23.44±0.78^{a}	22.84±0.93^{a}	21.90±0.46^{ab}	20.92±1.09^{ab}	18.70±1.09^{b}	$P = 0.017$
SGR (% d^{-1})	0.41±0.02^{a}	0.43±0.01^{a}	0.42±0.00^{a}	0.43±0.01^{a}	0.42±0.01^{a}	0.41±0.01^{a}	0.39±0.02^{ab}	0.35±0.02^{b}	$P = 0.016$
Initial fork length (mm)	559.2±1.3	556.3±3.0	554.1±0.6	555.3±1.7	558.0±1.4	556.7±0.3	555.0±0.7	556.2±1.2	$P = 0.370$
Final fork length (mm)	610.6±0.9^{ab}	612.6±2.1^{a}	607.2±0.8^{ab}	608.8±1.8^{ab}	610.0±1.4^{ab}	608.3±2.5^{ab}	605.1±1.6^{ab}	602.0±2.9^{b}	$P = 0.028$
Length growth rate (mm d^{-1})	0.61±0.02	0.67±0.01	0.63±0.00	0.63±0.02	0.61±0.01	0.61±0.03	0.59±0.02	0.54±0.02	$P = 0.014$
Final Condition factor	1.65±0.01	1.67±0.01	1.69±0.00	1.70±0.01	1.68±0.01	1.67±0.02	1.67±0.02	1.65±0.01	$P = 0.146$
Feed utilisation (as fed)									
Apparent feed consumption (kg tank$^{-1)}$	45.99±0.49	47.23±0.70	47.58±0.78	48.34±0.76	48.45±1.99	47.45±0.49	45.98±1.21	45.26±1.45	$P = 0.394$
Apparent feed intake (% BW d^{-1})	0.88±0.01	0.89±0.01	0.90±0.01	0.91±0.02	0.92±0.03	0.91±0.01	0.89±0.02	0.88±0.01	$P = 0.629$
Apparent FCR	2.11±0.09^{b}	2.03±0.05^{b}	2.11±0.04^{b}	2.07±0.05^{b}	2.12±0.06^{b}	2.17±0.03^{b}	2.20±0.07^{b}	2.43±0.07^{a}	$P = 0.008$
Proximate composition (wet basis)									
Moisture (%)	59.1±1.1	58.8±0.4	59.2±0.4	58.8±0.4	59.6±0.8	58.7±0.4	59.0±0.3	60.0±0.2	$P = 0.751$
Protein (%)	20.06±0.25	19.58±0.14	20.35±0.16	19.88±0.13	20.19±0.43	20.69±0.33	20.76±0.16	20.11±0.35	$P = 0.093$
Lipid (%)	19.1±1.0	18.7±0.5	19.0±0.7	19.4±0.2	18.2±0.9	17.9±0.7	18.8±0.4	17.6±0.3	$P = 0.500$
Ash (%)	2.0±0.1	2.6±0.4	2.3±0.4	2.7±0.2	2.4±0.4	2.3±0.2	2.2±0.2	2.2±0.2	$P = 0.708$
Carbohydrate (%)	<1.5	<1.5	<1.5	<1.5	<1.5	<1.5	<1.5	<1.5	$P = 1.000$
Energy (MJ kg^{-1})	10.48±0.31	10.28±0.19	10.50±0.23	10.57±0.09	10.16±0.25	10.17±0.23	10.47±0.13	9.94±0.14	$P = 0.401$
Nutrient retention (%)[3]									
Apparent PD	21.14±0.56	20.12±0.51	22.34±0.41	21.02±0.58	21.72±1.54	22.95±1.16	22.86±1.22	18.51±1.05	$P = 0.067$
Apparent ED	31.30±3.30	30.39±2.12	30.93±2.35	32.05±0.19	27.90±1.99	27.52±2.00	29.77±0.63	23.20±0.61	$P = 0.088$

[1] Values are mean ± SE; $n = 3$. Initial fish proximate composition (wet basis): Moisture 61.8%, protein 20.41%, lipid 16.5%, ash 2.2%, carbohydrate (by difference) 1.5%, energy 9.57 MJ kg^{-1}.
[2] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).
3 ED = energy deposition; PD = protein deposition.

Table 3.1.1.1.6. Cholesterol and fatty acid composition (mg 100 g^{-1}) of Yellowtail Kingfish fed graded dietary long chain omega-3 polyunsaturated fatty acid levels for 84 days at warm water temperatures.

Diet[1,2]	Initial	D2.95	D2.39	D2.13	D1.83	D1.61	D1.29	D1.01	D0.75	ANOVA[3]
Cholesterol (mg 100 g^{-1} wet)	-	100±2	96±4	102±4	101±4	98±6	98±4	100±2	97±1	$P = 0.933$
Saturated Fatty Acids										
C14:0 Myristic	500	550±26^{a}	510±21^{a}	490±23^{ab}	483±9^{ab}	433±27^{bc}	407±15^{c}	427±3^{bc}	390±6^{c}	$P < 0.001$
C15:0 Pentadecanoic	59	62±3^{a}	60±2^{ab}	56±3^{abc}	57±1^{abc}	53±3^{abc}	50±1^{bc}	53±1^{abc}	48±0^{c}	$P = 0.002$
C16:0 Palmitic	3010	3293±141	3267±131	3223±145	3297±42	3117±169	3040±121	3207±43	2970±60	$P = 0.411$
C17:0 Margaric	63	67±2	64±3	63±4	65±0	59±4	56±2	60±1	55±1	$P = 0.063$
C18:0 Stearic	910	1000±35	997±41	1010±40	1007±3	970±55	947±38	1007±15	920±12	$P = 0.492$
C20:0 Arachidic	34	32±1	32±1	31±2	34±1	30±2	31±0	31±1	27±1	$P = 0.070$
C22:0 Docosanoic	16	17±1	15±1	16±2	16±1	14±0	13±0	15±1	14±0	$P = 0.096$
Saturated Fat (g 100 g^{-1})	4.6	5.0±0.2	4.9±0.2	4.9±0.2	5.0±0.1	4.7±0.3	4.6±0.2	4.8±0.1	4.4±0.1	$P = 0.252$
Mono-unsaturated Fatty Acids										
C14:1 Myristoleic	30	39±2^{a}	39±2^{a}	40±1^{a}	39±1^{a}	36±2^{ab}	35±2^{ab}	36±2^{ab}	32±1^{b}	$P = 0.026$
C16:1 Palmitoleic	1050	1260±64	1220±53	1230±46	1243±15	1153±67	1130±44	1173±22	1100±20	$P = 0.197$
C17:1 Heptadecenoic	45	55±3	55±1	55±2	56±1	52±2	51±2	55±3	51±1	$P = 0.501$
C18:1n-7 Octadecenoic	500	583±26	567±23	567±15	573±9	540±30	523±20	543±12	517±9	$P = 0.218$
C18:1n-9 Oleic	5490	6693±303	6783±298	7010±188	7290±74	6963±393	6933±315	7253±233	6940±167	$P = 0.741$
C20:1 Eicosenoic (total)	240	217±3	217±3	213±9	217±7	207±15	200±10	210±0	200±0	$P = 0.535$
C20:1n-9 Eicosenoic	210	190±6	190±0	190±6	190±6	177±9	177±7	187±3	173±3	$P = 0.170$
C20:1n-11,13 Eicosenoic	30	28±1	28±0	27±2	27±1	29±3	27±1	26±1	25±0	$P = 0.742$
C22:1n-9 Docosenoic	22	17±1	17±1	16±1	18±0	16±1	15±1	17±1	16±1	$P = 0.315$
C24:1 Tetracosenoic	48	44±2	41±1	41±2	41±0	37±2	36±1	39±1	34±2	$P = 0.001$
Mono Unsaturated Fat (g 100 g^{-1})	7.5	9.0±0.4	9.0±0.4	9.3±0.3	9.6±0.1	9.1±0.5	9.0±0.4	9.4±0.3	9.0±0.2	$P = 0.883$

[1] Values are mean ± SE; $n = 3$.

[2] Samples below the detectable range and were assigned the value of 0. Values for the following fatty acids < 10 mg 100 g-1 and were excluded from the table: C4:0 Butyric, C6:0 Caproic, C8:0 Caprylic, C10:0 Capric, C12:0 Lauric, C13:0 Trisdecanoic, C24:0 Tetracosanoic, C10:1 Decenoic C15:1 Pentadecenoic C18:1n-6 Octadecenoic, C22:1n-11, 13 Docosenoic, C18:3n-6 Gamma Linolenic C20:3n-3 Eicosatrienoic2 C21:5n-3 Heneicosapentaenoic.

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values within each row without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

Table 3.1.1.1.6. Continued: Cholesterol and fatty acid composition (mg 100 g^{-1}) of Yellowtail Kingfish fed graded dietary long chain omega-3 polyunsaturated fatty acid levels for 84 days at warm water temperatures.

Diet[1,2]	Initial	D2.95	D2.39	D2.13	D1.83	D1.61	D1.29	D1.01	D0.75	ANOVA[3]
C18:2n-6 Linoleic	1670	1883±104	1910±44	1980±78	2067±17	1963±90	1960±86	2060±50	1980±44	$P = 0.571$
C18:3n-3 Alpha Linolenic	220	287±20	283±3	300±10	313±3	290±6	297±12	303±12	283±9	$P = 0.480$
C18:3n-4 Octadecatrienoic	17	16±1	17±2	17±1	16±1	12±1	12±0	13±1	12±1	$P = 0.092$
C18:4n-3 Steridonic	93	$130±12^{a}$	$110±0^{b}$	$103±3^{bc}$	$102±4^{bc}$	$86±3^{cd}$	$79±3^{d}$	$76±2^{d}$	$67±5^{d}$	$P < 0.001$
C20:2n-6 Eicosadienoic	27	31±1	31±1	32±2	31±1	31±2	29±1	34±1	31±1	$P = 0.476$
C20:3n-6 Dihomo-gamma-linoleic	22	24±2	25±1	24±1	25±1	22±1	23±1	23±1	21±1	$P = 0.392$
C20:4n-3 Eicosatetracenoic	120	$109±6^{a}$	$100±0^{ab}$	$98±7^{ab}$	$97±2^{ab}$	$88±4^{bc}$	$83±3^{bc}$	$88±1^{bc}$	$79±4^{c}$	$P = 0.001$
C20:4n-6 Arachidonic	120	133±12	117±3	120±6	120±0	110±6	107±3	113±3	105±5	$P = 0.063$
C20:5n-3 Eicosapentaenoic	630	$743±73^{a}$	$623±18^{b}$	$603±24^{b}$	$597±13^{b}$	$510±29^{bc}$	$480±21^{bc}$	$483±9^{bc}$	$443±32^{c}$	$P < 0.001$
C22:4n-6 Docosatetraenoic	34	$38±3^{a}$	$33±1^{ab}$	$32±1^{ab}$	$33±1^{ab}$	$28±2^{b}$	$28±0^{b}$	$29±2^{b}$	$28±2^{b}$	$P = 0.007$
C22:5n-3 Docosapentaenoic	180	$217±20^{a}$	$190±6^{ab}$	$187±7^{ab}$	$187±3^{ab}$	$163±9^{b}$	$157±3^{b}$	$160±0^{b}$	$150±10^{b}$	$P = 0.002$
C22:5n-6 Docosapentaenoic	35	$38±4^{a}$	$34±1^{ab}$	$31±2^{abc}$	$30±2^{abc}$	$26±1^{bc}$	$24±2^{bc}$	$24±1^{bc}$	$22±3^{c}$	$P = 0.001$
C22:6n-3 Docosahexaenoic	790	$993±93^{a}$	$840±38^{ab}$	$827±27^{abc}$	$827±19^{abc}$	$723±33^{bc}$	$687±34^{bc}$	$707±19^{bc}$	$627±49^{c}$	$P < 0.001$
∑LC n3 PUFA	1600	$1953±186^{a}$	$1653±59^{ab}$	$1617±57^{ab}$	$1610±35^{ab}$	$1397±70^{bc}$	$1323±58^{bc}$	$1350±25^{bc}$	$1220±90^{c}$	$P < 0.001$
Poly Unsaturated Fat (g 100 g^{-1})	4.1	$4.8±0.3^{a}$	$4.5±0.0^{ab}$	$4.5±0.2^{ab}$	$4.6±0.1^{ab}$	$4.2±0.2^{ab}$	$4.1±0.2^{ab}$	$4.2±0.1^{ab}$	$4.0±0.1^{b}$	$P = 0.030$
Trans Fat content (g 100 g^{-1})	0.3	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.3±0.0	0.2±0.0	$P = 0.786$
Total Omega 3	2050	$2487±221^{a}$	$2167±62^{ab}$	$2130±75^{ab}$	$2137±38^{ab}$	$1877±84^{bc}$	$1787±73^{bc}$	$1830±23^{bc}$	$1667±104^{c}$	$P < 0.001$
Total Omega 6	1920	2153±130	2157±38	2227±90	2317±18	2203±97	2177±88	2297±48	2197±48	$P = 0.734$
Total Omega 9	5770	6943±309	7030±301	7260±199	7540±79	7197±404	7157±324	7490±236	7163±164	$P = 0.760$

[1] Values are mean ± SE; $n = 3$.

[2] Samples below the detectable range and were assigned the value of 0. Values for the following fatty acids < 10 mg 100 g-1 and were excluded from the table: C4:0 Butyric, C6:0 Caproic, C8:0 Caprylic, C10:0 Capric, C12:0 Lauric, C13:0 Trisdecanoic, C24:0 Tetracosanoic, C10:1 Decenoic C15:1 Pentadecenoic C18:1n-6 Octadecenoic, C22:1n-11, 13 Docosenoic, C18:3n-6 Gamma Linolenic C20:3n-3 Eicosatrienoic2 C21:5n-3 Heneicosapentaenoic.

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values within each row without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

Table 3.1.1.1.7. Essential and non-essential amino acid composition (g 100 g^{-1}) of Yellowtail Kingfish fed graded dietary long chain omega-3 polyunsaturated fatty acid levels for 84 days at warm summer water temperatures.

Diet[1]	Initial	D2.95	D2.39	D2.13	D1.83	D1.61	D1.29	D1.01	D0.75	ANOVA[2]
Essential										
Arginine	1.531	1.233±0.036	1.235±0.018	1.178±0.069	1.195±0.026	1.220±0.057	1.230±0.026	1.143±0.043	1.173±0.060	P = 0.780
Histidine	0.979	1.107±0.031	1.087±0.031	1.132±0.072	1.052±0.007	1.078±0.035	1.038±0.018	1.021±0.063	1.082±0.055	P = 0.692
Isoleucine	0.802	0.941±0.037	0.910±0.046	0.927±0.038	0.900±0.034	0.940±0.032	0.887±0.048	0.862±0.032	0.957±0.014	P = 0.635
Leucine	1.335	1.428±0.029	1.403±0.036	1.424±0.016	1.393±0.029	1.425±0.012	1.351±0.035	1.325±0.046	1.424±0.021	P = 0.182
Lysine	1.655	1.107±0.344	1.589±0.097	1.618±0.061	1.602±0.154	1.830±0.113	1.549±0.046	1.657±0.042	1.631±0.058	P = 0.129
Methionine	0.467	0.420±0.031	0.432±0.055	0.470±0.041	0.427±0.021	0.413±0.050	0.427±0.018	0.439±0.043	0.401±0.017	P = 0.944
Phenylalanine	0.777	0.785±0.016	0.766±0.022	0.774±0.018	0.768±0.013	0.784±0.008	0.753±0.012	0.745±0.030	0.787±0.014	P = 0.636
Threonine	0.838	0.815±0.022	0.849±0.029	0.835±0.021	0.815±0.003	0.853±0.016	0.838±0.018	0.785±0.037	0.821±0.015	P = 0.465
Valine	1.006	1.029±0.020	1.012±0.010	1.022±0.022	1.015±0.017	1.016±0.004	0.980±0.011	0.958±0.030	1.029±0.027	P = 0.180
Non-essential										
Alanine	1.721	1.220±0.062	1.267±0.049	1.218±0.036	1.280±0.025	1.328±0.038	1.295±0.059	1.169±0.043	1.228±0.054	P = 0.367
Aspartic acid	2.013	1.908±0.038	1.926±0.044	1.895±0.027	1.867±0.037	1.953±0.050	1.655±0.218	1.831±0.049	1.980±0.061	P = 0.299
Glutamic acid	2.744	2.607±0.025	2.713±0.126	2.666±0.104	2.559±0.118	2.848±0.050	2.765±0.187	2.434±0.028	2.829±0.139	P = 0.202
Glycine	2.699	1.186±0.157	1.366±0.162	1.235±0.064	1.451±0.113	1.516±0.113	1.548±0.205	1.254±0.065	1.265±0.111	P = 0.409
Proline	1.793	0.878±0.095	0.978±0.077	0.910±0.052	1.029±0.060	1.060±0.071	1.072±0.115	0.900±0.046	0.924±0.065	P = 0.452
Hydroxy proline	0.606	0.234±0.031	0.282±0.043	0.239±0.030	0.299±0.015	0.303±0.029	0.348±0.059	0.245±0.012	0.249±0.027	P = 0.265
Serine	0.879	0.729±0.040	0.726±0.015	0.740±0.049	0.730±0.011	0.746±0.014	0.739±0.013	0.688±0.033	0.726±0.017	P = 0.879
Tyrosine	0.567	0.645±0.013	0.642±0.024	0.658±0.010	0.627±0.016	0.637±0.005	0.609±0.012	0.587±0.027	0.640±0.014	P = 0.138
Total amino acids	22.41	18.27±0.48	19.18±0.24	18.94±0.39	19.01±0.35	19.95±0.42	19.09±0.34	18.04±0.58	19.15±0.32	P = 0.099

[1] Values are mean ± SE; n = 3;

[2] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

Table 3.1.1.1.8. Mineral composition (mg kg^{-1}) of Yellowtail Kingfish fed graded dietary long chain omega-3 polyunsaturated fatty acid levels for 84 days at warm summer water temperatures.

Diet[1]	Initial	D2.95	D2.39	D2.13	D1.83	D1.61	D1.29	D1.01	D0.75	ANOVA[2]
Calcium	4800	4633±578	7033±1559	4067±433	6167±884	4967±1244	7600±872	6567±578	4433±186	$P = 0.092$
Copper	0.92	0.63±0.02	0.65±0.01	0.68±0.05	0.72±0.01	0.70±0.02	0.68±0.02	0.74±0.10	0.79±0.06	$P = 0.306$
Iodine	0.33	0.33±0.03	0.37±0.01	0.41±0.04	0.38±0.07	0.33±0.03	0.38±0.05	0.40±0.06	0.35±0.08	$P = 0.914$
Iron	23	19±2	18±1	18±1	20±2	17±1	19±1	18±1	23±5	$P = 0.573$
Magnesium	340	313±12	350±23	297±7	330±15	320±15	353±15	337±7	303±3	$P = 0.072$
Manganese	1.30	0.45±0.04	0.62±0.11	0.42±0.02	0.57±0.07	0.50±0.11	0.63±0.07	0.61±0.06	0.50±0.04	$P = 0.317$
Potassium	3200	3200±58	3233±33	3200±0	3200±58	3300±100	3167±33	3200±58	3200±58	$P = 0.828$
Phosphorus	4400	4367±318	5633±801	4000±231	5100±503	4500±608	5800±404	5233±240	4200±115	$P = 0.087$
Selenium	0.83	0.64±0.01	0.70±0.01	0.67±0.01	0.66±0.02	0.67±0.03	0.69±0.02	0.65±0.02	0.67±0.01	$P = 0.444$
Zinc	15	12±1	14±1	12±0	13±0	12±1	14±1	14±1	12±1	$P = 0.125$

[1] Values are mean ± SE; $n = 3$.
[2] A significance level of $P < 0.05$ was used for all statistical tests.

Table 3.1.1.1.9. Blood haematology and biochemistry of Yellowtail Kingfish fed graded dietary long chain omega-3 polyunsaturated fatty acid levels for 84 days at warm summer water temperatures.

Diet[1]	D2.95	D2.39	D2.13	D1.83	D1.61	D1.29	D1.01	D0.75	ANOVA[2]
Biochemistry[3]									
Sodium (mmol L^{-1})	189.56±0.80	191.00±7.84	191.56±2.15	191.56±5.53	190.22±1.06	181.44±8.22	184.78±3.82	198.33±7.02	$P = 0.548$
Potassium (mmol L^{-1})	10.03±0.25	12.42±0.57	7.88±0.75	8.78±0.90	9.86±2.54	14.74±4.24	12.32±1.71	9.90±2.18	$P = 0.360$
Urea (mmol L^{-1})	1.34±0.26	1.70±0.30	1.52±0.04	1.61±0.21	1.97±0.21	1.56±0.27	1.76±0.11	2.14±0.28	$P = 0.325$
Creatinine (mmol L^{-1})	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.05±0.04	0.02±0.00	$P = 0.412$
Calcium (mmol L^{-1})	3.11±0.07	3.17±0.05	2.94±0.05	3.13±0.06	3.08±0.07	3.02±0.11	3.05±0.12	3.14±0.05	$P = 0.497$
Protein (g L^{-1})	40.78±0.59^{ab}	42.22±0.11^{ab}	37.78±0.87^{b}	43.33±0.84^{a}	41.22±0.56^{ab}	43.00±2.22^{a}	41.44±0.87^{ab}	41.56±0.78^{ab}	$P = 0.042$
Albumin (g L^{-1})	22.00±1.35	20.78±0.11	19.44±0.97	21.00±0.51	20.89±0.29	23.44±3.02	21.11±1.56	22.00±0.51	$P = 0.647$
Globulin (g L^{-1})	18.78±1.28	21.44±0.11	18.33±1.50	22.33±1.26	20.33±0.38	21.78±1.46	20.33±1.17	19.56±1.06	$P = 0.208$
Total Bilirubin (mmol L^{-1})	0.56±0.22	0.67±0.00	0.44±0.22	0.78±0.11	0.33±0.19	0.56±0.40	0.67±0.33	0.56±0.11	$P = 0.914$
ALT (IU L^{-1})	14.44±1.87	9.67±0.88	16.33±4.30	13.56±1.57	14.56±1.44	12.11±2.76	14.11±0.11	11.89±1.47	$P = 0.526$
ALP (IU L^{-1})	11.89±1.06	13.33±1.35	11.22±1.37	12.78±2.82	10.89±1.16	11.44±1.49	11.33±0.67	10.44±2.26	$P = 0.923$
Magnesium (mmol L^{-1})	1.68±0.13	1.52±0.13	1.63±0.08	1.69±0.14	1.83±0.23	1.72±0.13	1.54±0.12	1.66±0.09	$P = 0.812$
Cholesterol (mmol L^{-1})	5.94±0.23	6.12±0.13	5.21±0.03	5.72±0.06	5.39±0.51	6.44±1.53	5.02±1.11	5.03±0.37	$P = 0.779$
Triglyceride (mmol L^{-1})	1.20±0.15	1.71±0.19	1.52±0.20	1.32±0.10	1.35±0.22	1.26±0.46	1.40±0.18	1.88±0.11	$P = 0.428$
Bile Acids (mmol L^{-1})	5.14±0.93	4.58±0.74	16.62±8.69	7.08±2.84	6.86±2.66	2.99±0.68	11.26±3.57	3.49±0.08	$P = 0.205$
Haematology[4]									
RBC ($\times 10^{12}$)	3.39±0.04	3.55±0.15	3.07±0.01	3.44±0.18	3.23±0.16	3.59±0.03	3.38±0.09	3.50±0.04	$P = 0.065$
HGB (g L^{-1})	123±2	127±1	113±4	124±3	124±4	124±4	117±6	124±5	$P = 0.212$
PCV (L L^{-1})	0.51±0.00	0.51±0.00	0.49±0.01	0.49±0.01	0.48±0.00	0.49±0.03	0.50±0.01	0.48±0.03	$P = 0.846$
MCV (fl)	170.5±3.4	160.7±7.1	177.5±6.2	164.8±11.2	173.5±6.9	165.3±4.1	167.8±4.6	164.8±6.0	$P = 0.683$
MCH (pg)	36.3±0.6	35.2±1.0	36.8±1.2	35.1±1.3	38.2±1.0	35.6±0.6	38.1±4.3	35.5±1.5	$P = 0.851$
MCHC (g L^{-1})	213±1	222±2	209±0	217±8	217±3	212±3	208±11	217±2	$P = 0.545$
WBC ($\times 10^{9}$)	5.7±0.3	5.3±0.1	5.8±0.1	5.8±0.2	5.7±0.3	5.6±0.2	5.5±0.0	5.8±0.2	$P = 0.591$
Granulocytes (%)	9±0	8±1	9±0	10±0	8±1	9±1	8±0	9±0	$P = 0.176$
Lymph (%)	90±1	91±1	91±0	89±0	91±1	91±1	91±1	90±0	$P = 0.201$
Mono (%)	1±0	1±0	1±0	1±0	1±0	1±0	1±0	1±0	$P = 0.993$
Eosin (%)	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	$P = 1.000$
Baso (%)	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	$P = 1.000$
Platelets ($\times 10^{9}$)	20±1	9±1	21±5	13±5	18±3	12±1	19±2	14±2	$P = 0.151$

[1] Values are mean ± SE; $n = 3$. SE less than 0.01 are reported as "0.00.

[2] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

[3] ALT = alanine aminotransferase; ALP = alkaline phosphatase.

[4] Smear content: red and white cell normal; Baso = basophil; Eosin = eosinophil; HGB = haemoglobin; Lymph = lymphocytes; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; MCV = mean corpuscular volume; Mono = monocytes; PCV = packed cell volume; RBC = red blood cell count; WBC = white blood cell count.

Table 3.1.1.1.10. Visceral somatic parameters and gastrointestinal morphology of Yellowtail Kingfish fed graded dietary long chain omega-3 polyunsaturated fatty acid levels for 84 days at warm summer water temperatures.

Diet[1]	D2.95	D2.39	D2.13	D1.83	D1.61	D1.29	D1.01	D0.75	ANOVA[2]
Visceral somatic parameters									
Viscerosomatic index (VSI; %)	6.23±0.39	6.04±0.12	6.27±0.23	6.74±0.27	6.12±0.34	5.58±0.14	6.77±0.30	5.94±0.12	$P = 0.071$
Hepatosomatic index (HSI; %)	1.19±0.05	1.24±0.02	1.30±0.05	1.36±0.01	1.25±0.05	1.16±0.01	1.22±0.11	1.18±0.00	$P = 0.166$
Hind gut morphology									
Villus height (µm)	672±61	916±56	857±56	891±107	810±42	661±149	833±6	573±58	$P = 0.057$
Villus width (µm)	293±44	357±22	456±72	397±21	369±56	296±70	400±14	254±44	$P = 0.121$
Villus perimeter (µm)	2626±801	4105±734	3653±17	3448±441	3070±110	2227±456	2697±241	2038±633	$P = 0.118$
Villus area (µm^2)	137216±49908	227201±31168	228191±10298	203767±26273	169075±34874	118500±46408	173318±4517	106592±42207	$P = 0.139$
Villus branching	2±1^{ab}	2±0^{ab}	3±0^{a}	2±0^{ab}	3±0^{ab}	2±0^{b}	2±0^{ab}	2±0^{b}	$P = 0.028$
Total goblet cell number[3]	346±136	281±64	252±81	305±84	152±6	165±31	162±19	215±79	$P = 0.483$
Sialylated goblet cell number[4]	231±69	217±42	194±43	212±39	135±4	141±27	138±14	169±42	$P = 0.493$
Sulphated goblet cell number[5]	116±68	64±22	57±38	93±65	16±8	24±6	24±6	47±36	$P = 0.586$
Mid gut morphology									
Villus height (µm)	793±133	742±111	821±117	709±156	606±53	609±23	607±38	609±109	$P = 0.614$
Villus width (µm)	318±96	256±50	318±53	298±117	224±18	215±9	235±30	246±56	$P = 0.869$
Villus perimeter (µm)	2520±765	2431±436	2952±736	2490±629	2009±195	2177±101	1893±504	2146±659	$P = 0.902$
Villus area (µm^2)	130929±56103	111514±27904	142259±43831	134485±49900	91848±9588	91744±4610	102070±31914	103725±37952	$P = 0.949$
Villus branching	2±1	2±0	2±1	2±1	1±0	2±0	1±0	2±1	$P = 0.879$
Total goblet cell number[3]	289±46	310±51	289±78	300±133	210±22	366±110	276±109	300±117	$P = 0.974$
Sialylated goblet cell number[4]	288±46	309±50	289±78	297±130	210±22	365±110	273±106	300±117	$P = 0.973$
Sulphated goblet cell number[5]	1±1	1±1	0±0	3±3	0±0	0±0	2±2	0±0	$P = 0.764$
Liver morphology[6]									
Fatty change	3±0	3±0	3±0	3±0	3±0	3±0	3±0	3±0	NA
Inflammation	0±0	1±0	0±0	1±0	0±0	0±0	1±0	0±0	$P = 0.511$
Melanomacrophage centres	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	$P = 0.466$
Proliferation of bile ducts	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	NA
Haemorrhage	0±0	0±0	0±0	0±0	1±0	0±0	0±0	1±0	$P = 0.094$

[1] Values are mean ± SE; $n = 3$.
[2] A significance level of $P < 0.05$ was used for all statistical tests.
[3] Expressed as the sum of goblet cells observed in samples stained with PAS/AB pH 2.5 and HID/AB pH 2.5 per millimetre villus height.
[4] Expressed as total number of sialylated goblet cells per millimetre height.
[5] Expressed as total number of sulphated goblet cells per millimetre villus height.
[6] Subjective scoring by Dr Fran Stephens (Aquatilia Healthcare, WA). Scoring is based on 0 = less to 4 = most.

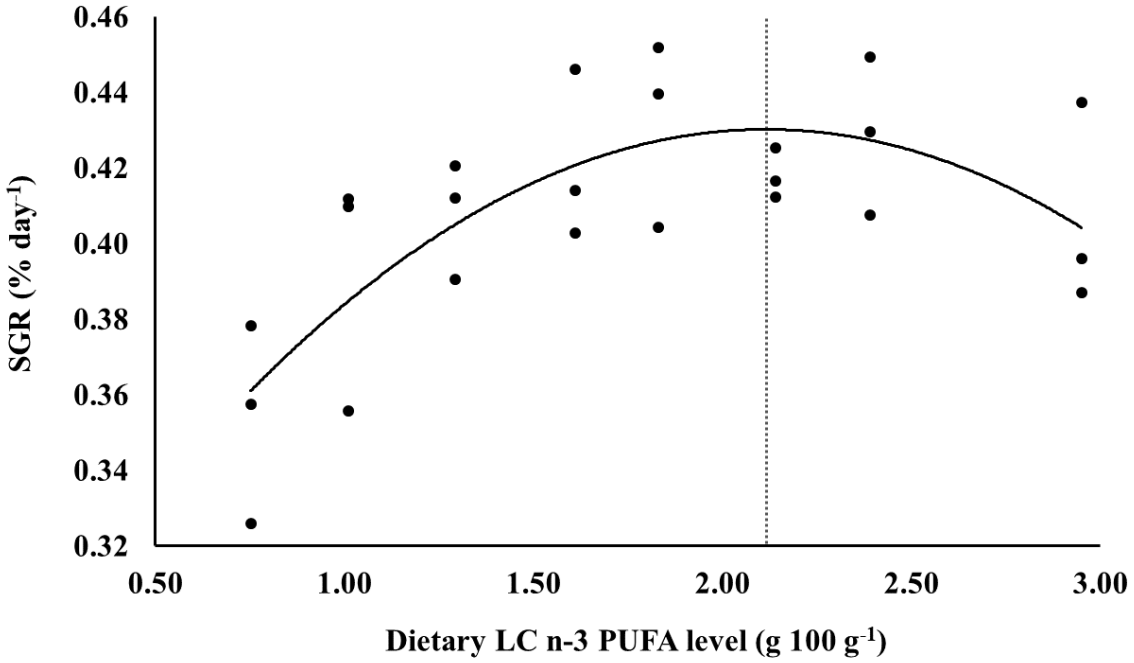


Figure 3.1.1.1.1. The relationship between dietary long chain omega-3 polyunsaturated fatty acid (LC n-3 PUFA; ∑EPA, DPA, DHA) and specific growth rate (SGR; % d^{-1}) for Yellowtail Kingfish fed graded dietary LC n-3 PUFA levels for 84 days at warm summer water temperatures. Quadratic polynomial relationship: $y = -0.0372x^2 + 0.1575x + 0.2636$; $R^2 = 0.5697$; $r = 0.7548$; $P < 0.001$; (turning point; y_{max}) = 2.12 g 100 g^{-1}. The 95% CI for the SGR response variable ranged between and 1.90 to 2.33 g 100 g^{-1}.

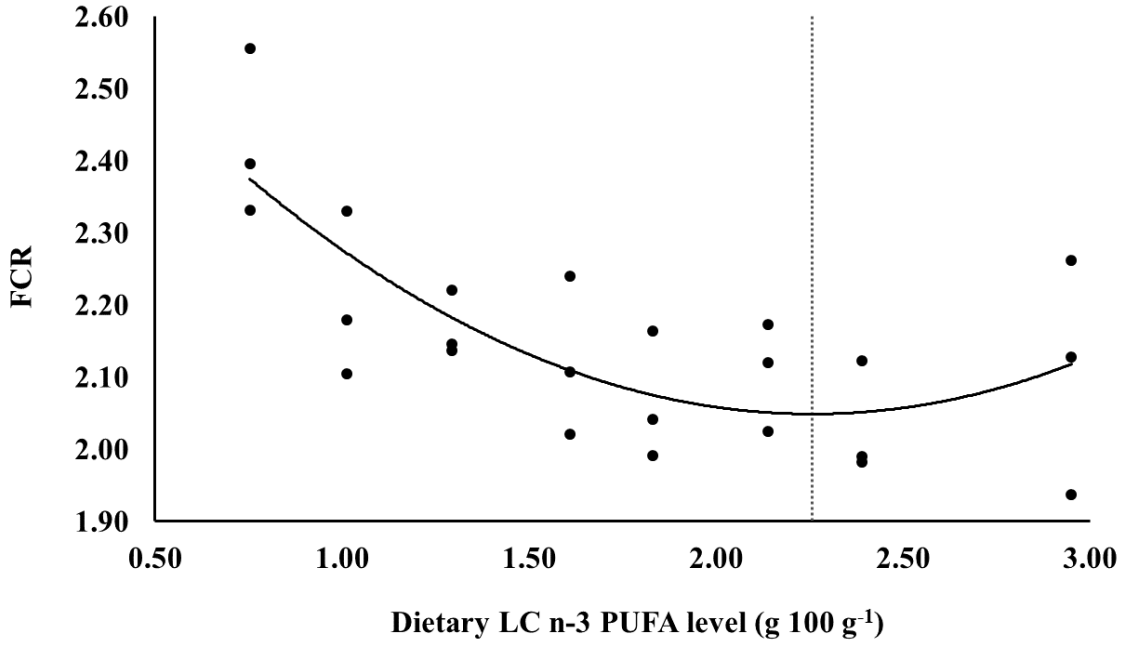


Figure 3.1.1.1.2. The relationship between dietary long chain omega-3 polyunsaturated fatty acid (LC n-3 PUFA; ∑EPA, DPA, DHA) and apparent feed conversion ratio (FCR) for Yellowtail Kingfish fed graded dietary LC n-3 PUFA levels for 84 days at warm summer water temperatures. Quadratic polynomial relationship: $y = 0.1441x^2 - 0.6505x + 2.7832$; $R^2 = 0.5758$; $r = 0.7588$; $P < 0.001$; (turning point; y_{min}) = 2.26 g 100 g^{-1}. The 95% CI for the FCR response variable ranged between and 1.93 to 2.58 g 100 g^{-1}.

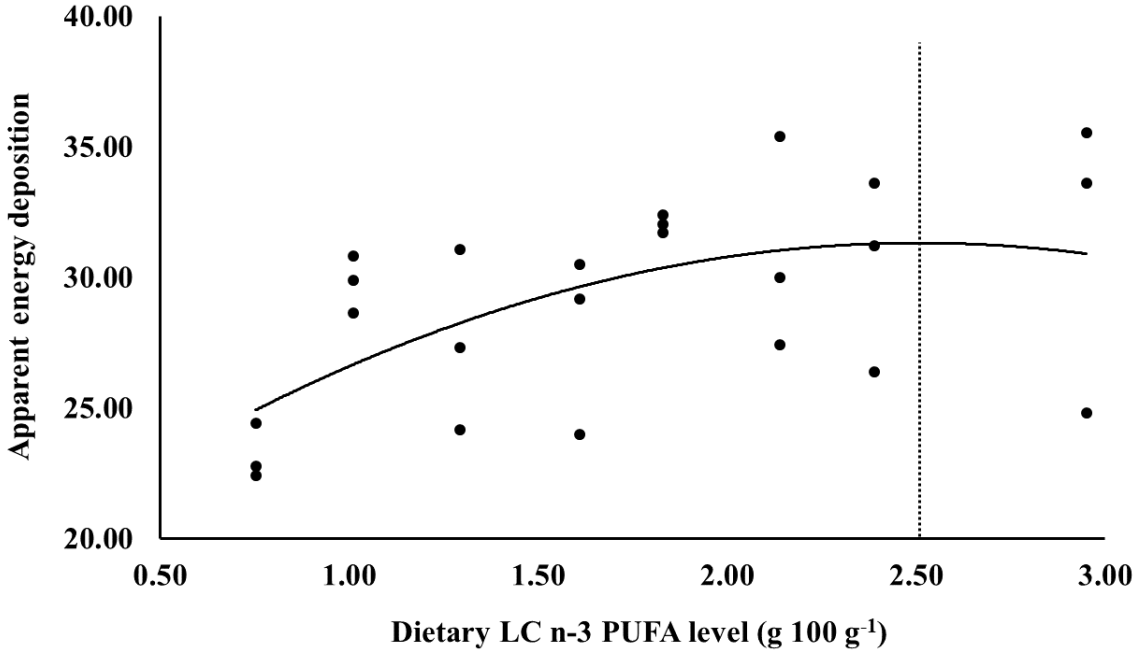


Figure 3.1.1.1.3. The relationship between dietary long chain omega-3 polyunsaturated fatty acid (LC n-3 PUFA; ∑EPA, DPA, DHA) and energy deposition for Yellowtail Kingfish fed graded dietary LC n-3 PUFA levels for 84 days at warm summer water temperatures. Quadratic polynomial relationship: $y = -2.0793x^2 + 10.425x + 18.247$; $R^2 = 0.3225$; $r = 0.5679$; $P = 0.017$; (turning point; y_{max}) = 2.51 g 100 g^{-1}.

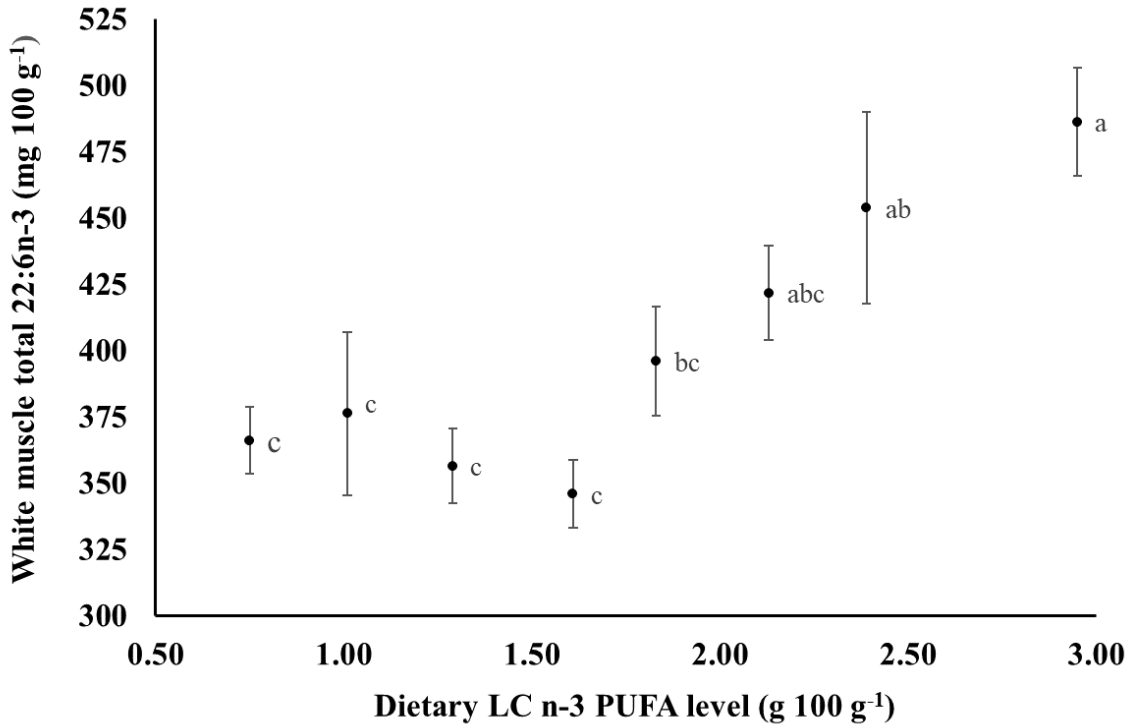


Figure 3.1.1.1.4. DHA deposition in the white muscle tissue of Yellowtail Kingfish fed graded dietary LC n-3 PUFA levels for 84 days at warm summer water temperatures.

Data from Samantha Chown, PhD project (Appendix 4). Values are mean ± SE; $n = 3$. A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

3.1.1.2. Manuscript - Alternative oils for Yellowtail Kingfish (Seriola lalandi) at cool water temperatures.

David A.J. Stone [a, b, c, d], Matthew S. Bansemer [a], Paul Skordas [a], Michael Salini[e]

[a] South Australian Research and Development Institute, South Australian Aquatic Sciences Centre, West Beach, SA 5024, Australia.
[b] Flinders University, School of Science and Engineering, GPO Box 2100, Adelaide, SA 5001, Australia.
[c] University of Adelaide, School of Animal and Veterinary Sciences, Roseworthy, SA 5371, Australia
[d] Marine Innovation Southern Australia.
[e] Ridley, Narangba, QLD 4504, Australia.

This manuscript may be referenced as: Stone, D.A.J., Bansemer, M.S., Skordas, P. and Salini, M. (2019). 3.1.1.2. Manuscript - Alternative oils for Yellowtail Kingfish (*Seriola lalandi*) at cool water temperatures (N6; Output 2c). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.47-66.

Abstract

The dietary LC n-3 PUFA requirement for Yellowtail Kingfish (*Seriola lalandi*; YTK) and the effect of replacing poultry oil (PO) with canola oil (CO) at sub-optimal cool water temperatures are unknown. There were two major aims in this 84 day study. The first aim was to determine the practical dietary long chain omega-3 polyunsaturated fatty acid level for optimal growth (LC n-3 PUFA; eicosapentaenoic acid [20:5n-3, EPA], docosapentaenoic acid [22:5n-3, DPA] and docosahexaenoic acid [22:6n-3, DHA]) at cool water temperatures, in a series of three diets that contained 1.42, 2.34 and 3.33 g 100 g^{-1} ∑LC n-3 PUFA. The second aim was to investigate the effect of replacing poultry oil with canola oil in a series of four diets. In this diet series, fish oil was added to satisfy the estimated optimum dietary levels of LC n-3 PUFA reported at warm water temperatures (Stone et al., Manuscript 3.1.1.1; > 2.12 g 100 g^{-1}), and PO and CO were used to satisfy the remaining lipid/energy requirements at different ratio (100.00 + 0.00%, 66.67 + 33.33%, 33.33 + 66.67% and 0.00 + 100.00%, PO + CO, respectively). Fish were fed to apparent satiation once daily at 09:00 h. In terms of growth, feed utilisation, nutrient digestibility, hindgut histology or blood haematology and biochemistry indices measured there was no significant difference between diets. However, there were trends to suggest that fish performance and feed utilisation declined once the LC n-3 PUFA levels were below 2.34 g 100 g^{-1} diet. Based on previous research at warm water temperatures (Stone et al., Manuscript 3.1.1.1) and current results, it was conservatively estimated that diets for large YTK at cool water temperatures should be formulated to contain 2.12 g 100 g^{-1} LC n-3 PUFA (95% CI ranged between 1.90 to 2.33 g 100 g^{-1}). With regard to canola oil, fish performance and feed utilisation declined, and we recommend that diets for large YTK contain up to ~4% dietary inclusion (24.13% of the added oil in a 25% total lipid diet). In contrast, fish performance and feed utilisation improved as poultry oil replaced canola oil. Poultry oil appeared to be a suitable lipid source for high inclusion (73.5% of total added lipid) in diets for large YTK at cool water temperatures. We recommend further pilot scale research under commercial conditions before implementing these suggestions on-farm.

Introduction

Understanding the dietary long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) requirements and increasing the inclusion of non-marine raw materials (lipid sources) to satisfy the energy requirements of aquaculture species is vital to improve diet sustainably and formulation flexibility, and decrease diet costs, while optimising production. Over the past decades, the need to find alternative lipid sources to fish oil for aquaculture species has been highlighted due to the high price, reduced availability, and ecological issues (Glencross et al., 2007; Tacon and Metian, 2009; Stone et al., 2011a; Stone et al., 2011b). In order to successfully reduce dietary fish oil inclusions for aquaculture species, numerous

studies have evaluated alternative dietary lipids, including poultry oil, canola oil and beef tallow (Oliveira et al., 2008; Stone et al., 2011a; Stone et al., 2011b; Bowyer et al., 2012). These alternative lipids however, typically lack long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA; eicosapentaenoic acid [20:5n-3, EPA], docosapentaenoic acid [22:5n-3, DPA] and docosahexaenoic acid [22:6n-3, DHA]) (Higgs et al., 2006).

Long chain omega-3 polyunsaturated fatty acids are essential for carnivorous fish are required for numerous biological functions, including cellular membrane structure, metabolism and function to ensure optimal growth and health (Tocher, 2010). Although some alternative lipid sources do contain the precursor of EPA, DPA and DHA, α-linolenic acid (18:3n-3, ALA), unlike freshwater aquaculture species and terrestrial livestock species, marine fish species lack enzymes, elongase 2 and Δ-6 desaturase, at appreciable levels to chain elongate and de-saturate ALA to EPA, DPA and DHA. As a result, fish oil is currently the best option to supply LC n-3 PUFA for carnivorous marine aquaculture species (Tocher, 2010; Bowyer et al., 2012).

Understanding the LC n-3 PUFA requirements of aquaculture species is vital to sustainably and economically utilise fish oil. The LC n-3 PUFA requirement for a number of aquaculture species is known, including the Gilthead Sea Bream (*Sparus aurata*) and Japanese Yellowtail (*Seriola quinqueradiata*) (Deshimaru et al., 1982; Kalogeropulos et al., 1992), and more recently Yellowtail Kingfish (*Seriola lalandi*; YTK) at warm water temperatures (Stone et al., Manuscript 3.1.1.1). However, the LC n-3 PUFA requirement for carnivorous marine aquaculture fish, is species-specific, and also is water temperature-dependent (Yone, 1978; Masumoto, 2002; Sargent et al., 2002; Oliva-Teles, 2012). In Australia, the dietary LC n-3 PUFA level in commercial diet formulations for large YTK are currently based on the LC n-3 PUFA requirements determined at warm water temperatures (Stone et al., Manuscript 3.1.1.1). This research suggested that optimal dietary LC n-3 PUFA level for the growth and feed utilisation of large YTK (2.66 kg) is 2.12 g 100 g^{-1} of LC n-3 PUFA, when using poultry oil as the alternative lipid source (Stone et al., Manuscript 3.1.1.1). Information pertaining to the optimal dietary LC n-3 PUFA level for large YTK at cool water temperatures is lacking in the literature.

Currently, poultry oil is the primary alterative lipid source utilised to replace fish oil in diets for YTK. However, other alterative oils, including canola oil, are available in commercial quantities that may be used to reduce the reliance on a single alterative lipid source. The price of canola oil and poultry oil are relatively similar and are both cheaper than fish oil (Bowyer et al., 2012). Previous studies have explored the effect of replacing fish oil with either poultry or canola oil for juvenile YTK (Bowyer et al., 2012). Results suggested that poultry oil was preferable to canola oil for juvenile YTK (Bowyer et al., 2012). However, for large YTK at cool water temperatures, the effect of replacing poultry oil (industry standard alternative oil) with canola oil is unknown. This research will ultimately improve the sustainable and economically viable production of YTK through cost-effective diet formulations by optimising marine ingredient use. It will also provide information to improve formulation flexibility for feed manufacturers.

Aim

In the current study we investigated the growth performance, feed utilisation and health of large YTK (> 1.5 kg) at cool water temperatures in two separate diet series to determine:

(i) The practical optimum levels of dietary LC n-3 PUFA; and

(ii) The effects of graded dietary poultry and canola oil blends.

Methods

Experimental design and diets

To address the two aims of the study simultaneously in one large two part experiment, six experimental diets were prepared by top coating 9 mm diameter diet pellet kernels with either fish oil, poultry oil or canola oil, or a blend of these oils. Ridley (Narangba, Queensland, Australia supplied the pellet kernels, fish oil, poultry oil and canola oil. Diets were formulated based on a YTK commercial diet (30% fish

meal; ~48% crude protein [CP], ~25% crude lipid [CL] and a gross energy [GE] level of ~19.80 MJ kg^{-1}). The pellet kernel utilised in the current study contained ~10% crude lipid, which was top coated at normal atmospheric pressure with an additional 17.3% lipid (fish oil, poultry oil and/or canola oil; total crude lipid level 25%) at Aquafeeds Australia (Mount Barker, South Australia, Australia).

In the first part of the experiment, the estimation of the optimal practical level of LC n-3 PUFA at cool water temperatures, a series of three diets (Diet 1, Diet 2 and Diet 3) were formulated. The diets were formulated to be deficient, meet or exceed the warm water requirements of LC n-3 PUFA by YTK, based on previous research that investigated the LC n-3 PUFA requirements of YTK (Stone et al., Manuscript 3.1.1.1). Poultry oil was used as the fish oil replacement in this three diet series due its low LC n-3 PUFA level, and also due to the promising results previously reported in a number of separate studies that utilised fish oil replacement diets (Bowyer et al., 2012; Stone et al., 2016; Stone et al., Manuscript 3.1.1.1). For example, the pellet kernel for Diet 1 was formulated to contain no additional fish oil, but the inherent LC n-3 PUFA from the dietary inclusions of fish meal was supplied (Diet 1: 1.42 g 100 g^{-1} ∑LC n-3 PUFA). Diet 1 was hypothesised to be deficient in LC n-3 PUFA. Further, in addition to the inherent pellet kernel lipids, Diet 2 and 3 were top-coated with an additional 4.61 and 8.69% fish oil and 12.69 and 8.64% poultry oil, respectively (Table 3.1.1.2.1 and 3.1.1.2.2; 2.34 and 3.33 g 100 g^{-1} ∑LC n-3 PUFA, respectively). Dietary LC n-3 PUFA in Diet 2 and 3 were hypothesised to meet and exceed the LC n-3 PUFA requirements of YTK, based on the requirements at warm water temperatures (2.12 g 100 g^{-1} ∑LC n-3 PUFA; Stone et al., Manuscript 3.1.1.1).

In the second part of the experiment, the effects of graded dietary poultry and canola oil blends on growth performance, a separate diet series comprised of four diets were formulated (Diet 2, Diet 4, Diet 5 and Diet 6) so that poultry oil (commercially used alternative lipid source) was replaced with canola oil at 0%, 33.3%, 66.7% and 100% for Diet 2, 4, 5 and 6, respectively (Table 3.1.1.2.1 and 3.1.1.2.2). Please note, Diet 2 from the three diet LC n-3 PUFA diet series was also used as the control for the poultry and canola oil blend series.

Fish were fed to apparent satiation at 09:00 h daily, which involved feeding fish for four min $tank^{-1}$ or until a feed refusal response was observed. Tanks were cleaned every second day. This study ran for a total of 84 days.

Experimental fish

Experimental work was conducted in the pool-farm facility at the South Australian Research and Development Institute, South Australian Aquatic Science Centre (SARDI SAASC; West Beach, South Australia, Australia). YTK (n = 342; 1.45 ± 0.12 kg; 459 ± 14 mm (fork length; mean ± standard deviation) were obtained from Clean Seas Seafood (Port Lincoln, South Australia, Australia). Upon arrival at the SARDI SAASC facility, YTK were transferred to 5000 L tanks supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated, aerated sea water at ambient temperature and held for ~4 weeks and fed a standard Ridley Pelagica diet (crude protein 46%; crude lipid 24%; gross energy 19.30 MJ kg^{-1}).

Skin and gill fluke treatment

Upon arrival at SARDI SAASC, YTK were inspected, and were observed to have a low burden of gill flukes (*Zeuxapta seriola*). Treatment was deemed necessary and was prescribed by Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd., East Ballina, New South Wales, Australia).

Experimental stocking and intermediate weight checks

At the commencement of the current study (August 2017), YTK were anaesthetised in 5000 L tanks (total water volume 2500 L) using AQUI-S® (AQUI-S® New Zealand Ltd., Lower Hutt, New Zealand) at a concentration of 14 mg L^{-1} of seawater. Nineteen fish were removed from their tank, measured,

weighed and stocked into one of the three replicate 5000 L tanks per treatment combination (n = 6 treatments; n = 18 tanks).

Tanks were supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated sea water at ambient temperature. All tanks were supplied with aeration and oxygenation throughout the study.

At 28 and 56 days, post-stocking, all fish were anaesthetised using AQUI-S® at a concentration of 14 mg L^{-1} of seawater. YTK were weighed and measured then returned back to their respective tanks. .

As required, mortalities were removed form the tanks, weighed, measured and recorded and replaced with tagged fish (T-tags) of a similar weight. Tagged fish were included in biomass calculations for FCR (see Performance indice section), but excluded from all other analyses.

Water quality analyses

Water quality parameters were measured daily at 12:30 h and maintained at appropriate levels for acceptable growth of YTK throughout the study (Table 3.1.1.2.3). Water temperature was measured using a thermometer (Figure 3.1.1.2.1). Dissolved oxygen (mg L^{-1} and % saturation) was measured using a dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark). Oakton pHtestr 20; Oakton Instruments, Vernon Hills, Illinois, United States of America). Salinity (g L^{-1}) was measured weekly using a portable salinity refractometer (model RF20, Extech Instruments, Nashua, New Hampshire, United States of America).

Final harvest sampling

At day 84, all fish were anaesthetised using AQUI-S® at a concentration of 14 mg L^{-1} of seawater and weighed and measured. Three fish from each tank (n = 3 fish $tank^{-1}$; n = 18 tanks; n = 54 fish) were whole collected and stored frozen at -20 °C for biochemical analysis. Blood from three separate fish per tank (n = 3 fish $tank^{-1}$; n = 18 tanks; n = 54 fish) were collected using a 19 G needle with a 5 mL syringe at the conclusion of the experiment. Blood samples were transferred to three separate Vacuette® or BD vacutainer® tubes (Z serum clot activator or EDTA tubes). A sub-sample of blood collected in EDTA Vacuette® tubes were analysed for blood haematocrit at SARDI SAASC. Serum was analysed for blood biochemistry and whole blood was analysed for blood haematology conducted by IDEXX (Unley, South Australia, Australia).

These blood sampled fish were then dissected and the visceral, liver and visceral fat was weighed in order to calculate visceral index (VSI; %), hepatosomatic index (HSI; %) and intraperitoneal fat (%), respectively. The stomach from these fish were opened longitudinally, and were subjectively scored for gastric dilation (Chown, 2015). In addition, $1cm^2$ longitudinally opened hindgut sections were collected from blood sampled fish for histology. In brief, hindgut samples were fixed in 10% seawater formalin for > 48 h, processed and embedded in paraffin wax. Tissue sections were cut using a microtome and floated onto Starfrost® glass slides and dried for > 24 h at room temperature before being stained with hematoxylin and eosin (H and E) and periodic acid-schiff alcian blue (PAS/AB pH 2.5). Gastrointestinal morphological parameters in the hindgut including muscle and serosa thickness, villi length, lamina propria thickness, total goblet cell number, eosinophilic droplets in epithelial cells and melanomacrophage centres were measured.

Apparent digestibility coefficients and nutrient digestion

At the conclusion of the 84 day growth experiment, a digestibility experiment was undertaken. After fish (n = 13 $tank^{-1}$) were weighed and measured they were returned to their tank and fed daily to apparent satiation for six days. After six days, fish were anaesthetised using AQUI-S® at a concentration of 20 mg L^{-1} of seawater (to enable handling and faecal matter collection), manually stripped and the faecal matter was collected. In brief, manual stripping involved placing the forefinger and thumb on either side of the fish abdomen at the pelvic fin. Moderate pressure was applied by the forefinger and thumb, and

at the same time moved towards the anus, this process was repeated six times. Uncontaminated faecal samples (free from blood, urine and mucus) were collected in a 250 mL container. Fish were then revived in their respective tank and fed daily to apparent satiation for a further six days. Fish were manually stripped again to ensure adequate samples were collected. Faecal material from all fish from a tank from both stripping events were pooled for analysis.

Biochemical and histological analyses

The proximate composition analyses of diets and whole body tissue were conducted according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardization (DIN) (2000). A 1 kg sample of each diet was collected, ground and analysed for proximate composition (moisture, protein, fat, ash, total carbohydrate and energy) and fatty acids profile. In addition, a total of twelve fish (n = 12 fish) at the start of the experiment, and three fish from each tank (n = 4 fish tank^{-1}; n = 24 tanks; n = 96 fish) at the conclusions of the experiment were collected and stored frozen at -20 °C. Whole fish samples were partially thawed, homogenised and analysed for proximate composition (moisture, protein, fat, ash, carbohydrate and energy) and fatty acid profile.

Performance indices

All data reported for each treatment for animal performance were based on the mean of the replicate tanks. All calculations using fish weight and diets were based on wet or as fed values, respectively:

- Weight gain = final weight - initial weight
- Biomass gain (kg tank^{-1}) = (final weight + ∑mortality weight) - (initial weight + ∑replacement weight)
- Specific growth rate (SGR; % d^{-1}) = ([ln final weight - ln initial weight] / d) × 100
- Length growth rate (mm d^{-1}) = (final fish length - initial fish length) / d
- Condition factor = (fish weight [g] / fish length [cm]^3) × 100
- Apparent feed conversion ratio (FCR) = feed consumed / fish weight gain
- Apparent protein deposition = ([final whole protein - initial whole protein] / protein intake) × 100
- Apparent energy deposition = ([final whole energy - initial whole energy] / energy intake) × 100
- Visceral index (VSI; %) = wet visceral wt × 100 / final wet fish wt
- Hepatosomatic index (HSI; %) = wet liver wt × 100 / final wet fish wt

Statistical analyses

IBM SPSS (version 24 for Windows; IBM SPSS Inc., USA) was used for all statistical analyses. Homogeneity of variances and normality among mean values were assessed using Levene's test for equality of variance errors and Shapiro-Wilk test, respectively. Data were compared across all treatments using a one-factor ANOVA. When significant effects were observed, post-hoc tests were used to detect significant differences between all treatments (Student-Newman-Keuls test). A significance level of $P < 0.05$ was used for all statistical tests. All values are presented as means ± standard error (SE) of the mean unless otherwise stated.

Results

General observations

There were no significant differences in the initial weight and fork length of YTK between treatments in the current study ($P > 0.05$; one-factor ANOVA; Table 3.1.1.2.4). The average initial weight and fork length were 1.45 ± 0.12 kg; 459 ± 14 mm (fork length; mean ± standard deviation; n = 342). YTK fed actively during the experiment, with no apparent differences observed between dietary treatments. The overall mortality for fish in the study was low (0.87%), and there were no apparent signs of disease observed.

Growth performance

Final weight (P = 0.313), biomass gain (P = 0.171), specific growth rate (SGR; P = 0.161), final fork length (P = 0.463), length growth rate (P = 0.211) and final condition factor (P = 0.579) of YTK was not significantly influenced by diet (one-factor ANOVA; Table 3.1.1.2.4).

Feed utilisation

Feed intake (% BW d^{-1}; P = 0.447) and feed consumption (kg $tank^{-1}$; P = 0.335) were not significantly affected by diet (one-factor ANOVA; Table 3.1.1.2.4). Feed conversion ratio (FCR) of YTK was also not significantly influenced by diet (P = 0.442; one-factor ANOVA; Table 3.1.1.2.4).

Whole fish proximate and energy composition

Diet did not significantly influence moisture (64.9-65.6%), protein (19.8-20.3% wet), lipid (13.1-14.2% wet), ash (1.9-2.8% wet), carbohydrate (< 1% wet; by difference), energy (8.07-8.80 MJ kg^{-1} wet) (P > 0.05; one-factor ANOVA; Table 3.1.1.2.4).

Nutrient utilisation

Diet did not significantly influence apparent protein deposition (21.49-24.52%) and apparent energy deposition (27.44-36.54%) (P > 0.05; one-factor ANOVA; Table 3.1.1.2.4).

Whole fish fatty acid composition

Diet significantly affected a number of fatty acid levels in whole fish (P < 0.05; one-factor ANOVA; Table 3.1.1.2.5). For example, arachidic (C20:0), eicosenic (C20:1), cetoleic (C22:1), docosenoic (C22:1), nervonic (C24:1) and alpha-linolenic acids (C18:3n3) levels were significantly influenced by diet. For these fatty acids, the whole fish mirrored what was in the diet (Table 3.1.1.2.2 and 3.1.1.2.5).

Blood biochemistry and haematology

All measured blood haematology and biochemistry parameters were not significantly affected by diet (P > 0.05; one-factor ANOVA; Table 3.1.1.2.6).

Fatty acid composition of plasma and red blood cell

Diet significantly affected the level of a number of fatty acids in blood plasma (P < 0.05; one-factor ANOVA; Table 3.1.1.2.7). For example, arachidic (C20:0), eicosenic (C20:1), docosenoic (C22:1), nervonic (C24:1), alpha-linolenic acids (C18:3n3), eicosatrienoic (C20:3n6) and docosahexaenoic (C22:6n3) were significantly affected by diet (P < 0.05). Fatty acid level in the whole red blood cells were also significantly affected by diet (P < 0.05; one-factor ANOVA; Table 3.1.1.2.8). For example, margaric (C17:0), arachidic (C20:0), Oleic (C18:1n9), eicosenic (C20:1n9), erucic (C22:1n9), nervonic (C24:1), linoleic (C18:2n6), alpha-Linolenic (C18:3n3), eicosatrienoic (C20:3n6) were significantly affected by diet (P < 0.05). These fatty acids in both the blood plasma and red blood cells typically mirrored what was in the diet (Table 3.1.1.2.2; Table 3.1.1.2.7; Table 3.1.1.2.8).

Gastrointestinal tract morphology

Intraperitoneal fat (1.14-1.90%), visceral index (6.50-6.84%) and hepatosomatic index (0.99-1.07%) of YTK was not significantly influenced by diet (P > 0.05; one-factor ANOVA; Table 3.1.1.2.9).

Diet did not significantly influence the gastric dilation score ($P > 0.05$; one-factor ANOVA; Table 3.1.1.2.9). All except for two fish fed Diet 6 (Stage 1), were determined to be Stage 0 (healthy/no gastric dilation; Table 3.1.1.2.9). Stage 0 is defined as having pronounced/well defined folds throughout the pylorus, anterior and distal stomach, while Stage 1 is defined as having minimal or absent folds throughout the pylorus and anterior stomach, but has pronounced/well defined folds in the distal stomach.

Muscularis and submucosa thickness, villus length and thickness, lamina propria thickness, total goblet cell number, eosinophilic droplets in epithelial cells and melanomacrophage centres in the hindgut were not significantly influenced by diet ($P > 0.05$; one-factor ANVOA; Table 3.1.1.2.9).

Apparent digestibility coefficients

Apparent digestibility coefficient for dietary dry matter (43.3-56.4%), crude protein (72.4-77.2%) and gross energy (65.0-70.6%) was not significantly influenced by diet ($P > 0.05$; one-factor ANOVA; Table 3.1.1.2.9).

Discussion

Our aim in the current study was to improve the sustainable and economically viable production of YTK through cost-effective diet formulations by optimising marine ingredient use (FO) and also increasing the type of raw materials (lipid sources) available to use to satisfy the energy requirements of the animal. This overarching aim was addressed in two parts: (i) to determine the practical optimum levels of dietary LC n-3 PUFA on the growth performance, feed utilisation and health of large YTK (> 1.5 kg) at cool water temperatures; and (ii) to determine the effects of graded dietary poultry and canola oil blends for YTK at cool water temperatures.

In the current study, YTK readily accepted and consumed all experimental diets. In terms of growth, feed utilisation, diet digestibility, hindgut histology indices or blood haematology and biochemistry measured there was no significant difference between diets. There was however, a tendency for fish fed Diet 2 (26.71% fish oil + 73.52% poultry oil [added oil]; 2.34 g 100 g^{-1} LC n-3 PUFA) and Diet 3 (50.35% fish oil + 50.06% poultry oil [added oil]; 3.33 g 100 g^{-1} LC n-3 PUFA) to perform better in terms of growth and feed utilisation than those fed Diet 1 (0.00% fish oil + 100% poultry oil [added oil]; 1.42 g 100 g^{-1} LC n-3 PUFA). The SGRs of fish fed all diets in the LC n-3 PUFA requirement component of the study declined from 0.39 to 0.37 % d^{-1} as dietary LC n-3 PUFA levels decreased. While the corresponding FCRs also worsened (ranged from 1.73 to 1.87) at the dietary LC n-3 PUFA level of 1.42 g 100 g^{-1}. While these differences were not significant, results in the current study for LC n-3 PUFA intake rates ranged from 164-233 mg LC n-3 PUFA kg $fish^{-1}$ d^{-1} and were similar to daily intake rates of LC n-3 PUFA in the N1 study (Stone et al., Manuscript 3.1.1.1; 191 mg LC n-3 PUFA kg $fish^{-1}$ d^{-1}). These results suggest that the estimated optimal dietary level of LC n-3 PUFA for YTK at cool water temperatures may be similar to those at warm water temperatures (~2.12 g 100 g^{-1} LC n-3 PUFA [95% CI ranged between 1.90 to 2.33 g 100 g^{-1}]) (Stone et al., Manuscript 3.1.1.1).

With regard to the poultry and canola oil diet series, there was a tendency for fish fed Diet 2 (26.71% fish oil + 73.52% poultry oil + 0% canola oil [added oil]) and Diet 4 (27.81% fish oil + 48.26% poultry oil + 24.13% canola oil [added oil]) to perform better than fish fed diets containing higher canola oil levels (Diet 5 [8% total canola oil] and Diet 6 [12% total canola oil]). In practical terms, Diet 4 contained a total of 4% canola oil. FCR tended to increase (worsen) as dietary inclusion of canola increased above 4%. While not significant, results for maximum canola oil inclusion are consistent with previous research by Bowyer et al. (2012). The authors reported that a 50% replacement of fish oil with canola oil (~10% total canola oil) tended to reduce growth, while 100% substitution of fish oil with canola oil (~20% total canola oil) resulted in significantly reduced growth for juvenile YTK (Bowyer et al., 2012). In contrast, FCR tended to decrease (improve) as dietary inclusion of poultry oil replaced canola oil and results suggest poultry oil is a suitable lipid source for high inclusion (73.5% of total added lipid) in diets for large YTK at cool water temperatures. This is consistent with results fish oil substitution with

poultry oil (50-100% substitution with 10 or 20% total poultry oil) reported for juvenile YTK by Bowyer et al. (2012).

As previously reported for a range of fish species, including YTK (Tocher, 2010; Stone et al., 2011a; Stone et al., 2011b; Bowyer et al., 2012), the fatty acid profiles of whole fish mirrored that of the diets (Table 3.1.1.2.2 and 3.1.1.2.5). Fish fed high levels of canola and poultry oil had reduced LC n-3 PUFA contents which may not be as desirable for consumers. This may have marketing implications for YTK producers. Further research is warranted to understand the kinetics associated with the uptake of LC n-3 PUFA from finishing diets rich in fish oil, prior to harvest (Stone et al., 2011a; Stone et al., 2011b).

A similar response in terms of fish oil substitution was observed for the fatty acid profiles of the blood plasma and red blood cells (Table 3.1.1.2.7). LC n-3 PUFA levels and ratios in red blood cells are considered to be a good biomarker for inflammatory responses in humans and other animals (Fontes et al., 2015). Interestingly, the changes in red blood cell fatty acids were observed predominantly in the saturated and monounsaturated fatty acid classes, with little change in the LC n-3 PUFA values. This suggests that is unlikely the alterations of fish oil, poultry oil and canola oils levels in the current study had a negative effect on red blood cell function in relation to inflammatory response. Nevertheless, given the importance of red blood cells in oxygen transport, the fatty acid modifications in relation to saturated and unsaturated fatty acids may contribute to alterations in metabolic function. Further research is warranted to understand this aspect of YTK metabolism.

It should be noted that attempting to gain an insight into the growth performance and feed utilisation of large YTK at winter water temperatures is inherently difficult. This is due to the slow growth rate of large compared to small fish at suboptimal water temperatures (Bowyer et al., 2012), combined with the short growth period (84 d, ~3 months). However, large YTK commercially cultured in South Australian waters are exposed to a water temperature profile that is similar to the one tested in the current study. Therefore, given the study was well controlled and well replicated, trends in results for SGR and FCR in response to dietary LC n-3 PUFA levels, and fish oil substitution with canola and poultry oil, provide new and valuable information. This new information will aid in the development of improved commercial diets for the production of YTK at cool suboptimal water temperatures.

Conclusions and Recommendations

Based on results for growth performance and feed intake rates from the current study and the optimal LC n-3 PUFA level for YTK at warm water temperatures (Stone et al., Manuscript 3.1.1.1), it is conservatively estimated that diets for large sub-adult YTK at cool water temperatures should be formulated to contain 2.12 g 100 g^{-1} LC n-3 PUFA (95% CI ranged between 1.90 to 2.33 g 100 g^{-1}). With regard to canola oil, we may recommend that diets for large YTK contain up to ~4% dietary inclusion (24.13% of the added oil in a 25% total lipid diet). FCR tended to increase (worsen) as dietary inclusion of canola increased above 4%. Poultry oil appears to be a suitable lipid source for high inclusion (73.5% of total added lipid) in diets for large YTK at cool water temperatures. FCR tended to decrease (improve) as dietary inclusion of poultry oil replaced canola oil.

Findings

- Based on intake results from this study, it is conservatively estimated that diets for large YTK at cool water temperatures should be formulated to contain similar LC n-3 PUFA levels to those at warm water temperatures (~2.12 g 100 g^{-1} LC n-3 PUFA [95% CI ranged between 1.90 to 2.33 g 100 g^{-1}]) (Stone et al., Manuscript 3.1.1.1).
- Canola oil dietary inclusion in YTK production diets should be limited (≤ 4%).
- All FCRs in the current winter study were ≤ 1.87.
- FCR tended to increase (worsen) as dietary inclusion of canola increased above 4%.
- Poultry oil is suitable for high inclusions in production diets for large YTK. FCR tended to decrease (improve) as dietary inclusions of poultry oil replaced canola oil.

- An overarching goal of the K4P project was to provide information to assist feed companies to formulate and develop commercial diets for large YTK that would result in FCRs of < 2.2 for fish between 1.5-3.5 kg.

The results from this study will provide feed manufactures with information to formulate commercial diets to improve FCRs for large YTK to meet the project goal. This information may improve flexibility in diet formulations for feed manufactures to select raw materials that most economically meet the nutrient criteria. These conservative recommendations are based on growth, feed utilisation and blood hematology and biochemistry parameters, and hindgut histology data. This is a commercial decision for the YTK industry. Further research in pilot scale commercial trials are needed before implementing these diets on-farm.

Publications

No publications have resulted from this R&D to date.

Acknowledgements

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The authors would also like to acknowledge the South Australian Research and Development Institute (SARDI) Marine Innovation Southern Australia for their financial support of Assoc. Prof. David Stone and the provision of the SARDI SAASC experimental facilities at West Beach, South Australia. We would also like to thank Dr Trent D'Antignana of Nutrisea for his input into experimental design. Dr Richard Smullen of Ridley for his input into experimental design, experimental diet formulation and manufacture, and Dr Leo Nankervis of Skretting Australia for his input into experimental design. Thanks to Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd.) and Dr Marty Deveney for the diagnosis and prescription of gill fluke treatments. We also thank Leigh Kuerschner, Krishna-Lee Currie, Mark Purvis, Jessica Buss, Filipa Isabel, Jackson Doherty, Nicole Thompson and Samantha Chown for their technical assistance during the experiment.

References

Bowyer, J.N., Qin, J.G., Smullen, R.P., Stone, D.A.J., 2012. Replacement of fish oil by poultry oil and canola oil in Yellowtail Kingfish (*Seriola lalandi*) at optimal and suboptimal temperatures. Aquaculture 356-357, 211-222.

Chown, S., 2015. Effects of diet retention of yellowtail kingfish (*Seriola lalandi*) physiology. Honours thesis. Flinders University, South Australia, Australia.

Deshimaru, O., Kuroki, K., Yone, Y., 1982. Suitable levels of lipids and Ursodeoxycholic acid in diet of yellowtail. Bulletin of Japanese Society of Scientific Fisheries 48, 1265-1270.

Fontes, J.D., Rahman, F., Lacey, S., Larson, M.G., Vasan, R.S., Benjamin, E.J., Harris, W.S., Robins, S.J., 2015. Red blood cell fatty acids and biomarkers of inflammation: a cross-sectional study in a community-based cohort. Atherosclerosis 240, 431-6.

Glencross, B.D., Booth, M., Allan, G.L., 2007. A feed is only as good as its ingredients - a review of ingredient evaluation strategies for aquaculture feeds. Aquaculture Nutrition 13, 17-34.

Higgs, D.A., Balfry, S.K., Oakes, J.D., Rowshandeli, M., Skura, B.J., Deacon, G., 2006. Efficacy of an equal blend of canola oil and poultry fat as an alternative dietary lipid sources for Atlantic Salmon (*Salmo salar* L.) in sea water. I: effects on growth performance, and whole body and fillet proximate and lipid composition. Aquaculture Research 37, 180-191.

Kalogeropoulos, N., Alexis, M.N., Henderson, R.J., 1992. Effects of dietary soybean and cod-liver oil levels on growth and body composition of gilthead bream (*Sparus aurata*). Aquaculture 104, 293-308.

Masumoto, T., 2002. Yellowtail, *Seriola quinqueradiata*. In: Webster, C.D., Lim, C.E. (Eds), Nutrient Requirements and Feeding of Finfish for Aquaculture. CABI Publishing, Oxon, UK, pp. 131-146.

Oliva-Teles, A., 2012. Nutrition and health of aquaculture fish: review article. Journal of Fish Disease 35, 83-108.

Oliveira, A.C.M., Stone, D.A.J., Plante, S., Smiley, S., Bechtel, P.J., Hardy, R.W., 2008. Fish oils from Alaskan seafood processing by-products: an un-exploited sustainable resource for aquaculture. World Aquaculture 39, 50-69.

Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The Lipids. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition. Academic Press, San Diego, USA, pp. 181-257.

Stone, D.A.J., Oliviera, A.C.M., Plante, S., Smiley, S., Bechtel, P., Hardy, R.W., 2011a. Enhancing highly unsaturated ω-3 fatty acids in phase-fed rainbow trout (*Oncorhynchus mykiss*) using Alaskan fish oils. Aquaculture Nutrition 17, 501-510.

Stone, D.A.J, Oliviera, A.C.M., Ross, C., Plante, S., Smiley, S., Bechtel, P., Hardy, R.W., 2011b. The effects of phase-feeding rainbow trout (*Oncorhynchus mykiss*) with canola oil and Alaskan pollock fish oil on fillet fatty acid composition and sensory attributes. Aquaculture Nutrition 17, 521-529.

Stone, D.A.J., D'Antignana, T., Bansemer, M.S., 2016. Final Report. Refining Yellowtail Kingfish feeds and feed management. Prepared by the South Australian Research and Development Institute (Aquatic Sciences), Adelaide. AS-CRC Project No. 2013/730. 144pp.

Tacon, A.G., Metian, M., 2009. Fishing for feed or fishing for food: increasing global competition for small pelagic forage fish. Ambio 38, 294-302.

Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquaculture Research 41, 717-732.

Yone, Y., 1978. Essential fatty acids and lipid requirement of marine fish. In: Yone, Y. (Ed.), Dietary Lipids in Aquaculture. Koseisya-Koseikaku, Tokyo, (in Japanese). pp. 43-59.

Table 3.1.1.2.1. Dietary oil addition levels to the experimental diets for the graded fish oil levels diet series (Diet 1, 2 and 3) and graded poultry and canola oil blend diet series (Diet 2, 4, 5 and 6).

Diet	Fish oil (% added)	Poultry oil (% added)	Canola oil (% added)	Target ∑LC n-3 PUFA (g 100 g^{-1} diet)	Analysed ∑LC n-3 PUFA (g 100 g^{-1} diet)
1	0.00	100.00	0.00	1.45	1.42
2	26.58	73.42	0.00	2.12	2.34
3	50.14	49.86	0.00	2.88	3.33
4	27.75	48.19	24.06	2.12	2.39
5	28.61	23.78	47.61	2.12	2.43
6	29.25	0.00	70.75	2.12	2.46

Table 3.1.1.2.2. The proximate and fatty acid composition of the six experimental diets used in the current experiment.

Item (as fed)[1]	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Proximate composition (g 100 g^{-1})						
Moisture	4.8	4.9	4.8	4.6	4.6	5.0
Crude protein	47.8	47.9	48.4	48.1	48.1	48.1
Crude lipid	24.9	24.6	25.3	24.6	25.1	25.1
Ash	8.2	8.1	8.2	8.1	8.2	8.3
Carbohydrate[2]	14.3	14.5	13.3	14.6	14	13.5
Gross energy (MJ kg^{-1})	19.8	19.7	19.9	19.8	19.8	19.8
Analysed fatty acids (g 100 g^{-1} diet)						
Saturated Fatty Acids						
C13:0	0.01	0.01	0.01	0.01	0.01	0.01
C14:0 Myristic	0.41	0.53	0.67	0.50	0.48	0.45
C15:0 Pentadecanoic	0.07	0.09	0.11	0.08	0.08	0.08
C16:0 Palmitic	6.21	5.84	5.70	4.97	4.18	3.36
C17:0 Margaric	0.12	0.12	0.12	0.11	0.10	0.09
C18:0 Stearic	1.88	1.67	1.55	1.44	1.23	1.02
C20:0 Arachidic	0.05	0.06	0.07	0.09	0.11	0.13
C22:0 Docosanoic	0.02	0.03	0.03	0.04	0.05	0.06
C24:0 Tetracosanoic[2]	0.05	0.06	0.08	0.07	0.07	0.08
Total Saturated Fat	8.85	8.45	8.41	7.34	6.37	5.33
Mono-unsaturated Fatty Acids						
C16:1n-7 Palmitoleic	1.44	1.40	1.42	1.16	0.93	0.70
C18:1n-9 Oleic	7.71	6.91	6.41	7.39	8.07	8.48
C18:1n-7 Vaccenic	0.64	0.70	0.77	0.73	0.77	0.83
C20:1n-9 Eicosenoic	0.22	0.60	1.00	0.65	0.70	0.74
C22:1n-9 Erucic acid	0.02	0.09	0.16	0.09	0.09	0.10
C24:1 Tetracosenoic	0.03	0.09	0.16	0.11	0.12	0.13
Total Monos	10.05	9.80	9.91	10.12	10.68	10.97
Poly-unsaturated Fatty Acids						
C18:2n-6 Eicosadienoic	3.55	3.00	2.56	3.37	3.79	4.19
C18:3n-3 Alpha Linolenic	0.61	0.52	0.46	0.94	1.37	1.79
C18:3n-6 Gamma Linolenic	0.05	0.05	0.07	0.05	0.04	0.04
C20:2n-6 Eicosadienoic	0.04	0.06	0.07	0.05	0.06	0.05
C20:3n-6 Dihomo-gamma-linoleic	0.03	0.04	0.05	0.03	0.03	0.03
C20:4n-6 Arachidonic	0.14	0.17	0.20	0.16	0.15	0.15
C20:5n-3 Eicosapentanaeoic	0.39	0.66	0.96	0.68	0.69	0.70
C22:5n-3 Docosapentaenoic	0.11	0.20	0.31	0.20	0.20	0.20
C22:6n-3 Docosahexaenoic	0.92	1.47	2.07	1.51	1.53	1.56
∑C22:4n-6 + 22:3n-3	0.02	0.03	0.04	0.03	0.03	0.02
∑LC n-3 PUFA	1.42	2.33	3.34	2.39	2.42	2.46
Total Omega 3	2.02	2.86	3.79	3.33	3.80	4.25
Total Omega 6	3.84	3.34	2.99	3.69	4.10	4.48
Total Omega 7	2.07	2.11	2.19	1.89	1.70	1.53
Total Omega 9	7.95	7.60	7.56	8.12	8.87	9.32
Trans Fatty Acids						
t18:1n-7	0.11	0.11	0.10	0.08	0.07	0.05
t18:2	0.05	0.06	0.07	0.05	0.05	0.05
Total Trans Fatty Acids	0.16	0.16	0.17	0.14	0.12	0.09

[1] Diet 1 - 0% fish oil + 100% poultry oil [added oil]; 1.42 g 100 g^{-1} LC n-3 PUFA, Diet 2 - 26.71% fish oil + 73.52% poultry oil [added oil]; 2.34 g 100 g^{-1} LC n-3 PUFA, Diet 3 - 50.35% fish oil + 50.06% poultry oil [added oil]; 3.33 g 100 g^{-1} LC n-3 PUFA, Diet 4 - 27.81% fish oil + 48.26% poultry oil + 24.13% canola oil [added oil], Diet 5 - 28.68% fish oil + 23.84% poultry oil + 47.68% canola oil [added oil], Diet 6 - 29.37% fish oil + 0% poultry oil + 70.86% canola oil [added oil].

2 Carbohydrate = 100 - (moisture + lipid + protein + ash).

Table 3.1.1.2.3. Summary of water quality parameters.

Item[1]	Temperature (°C)	Dissolved oxygen (mg L^{-1})	Dissolved oxygen (% saturation)	pH	Salinity (mg L^{-1})	Ammonia (ppm)	CO_2 (mg L^{-1})
Mean	16.2 ± 2.1	8.2 ± 0.6	104.1 ± 4.6	7.78 ± 0.18	38 ± 0	0.00 ± 0.02	1 ± 0
Range	13.0 - 20.0	6.6 - 11.2	89.0 - 119.0	7.40 - 8.17	38 - 38	0.00 - 0.25	0 - 2

[1] Values means ± standard deviation.

Table 3.1.1.2.4. Growth performance and feed utilisation of Yellowtail Kingfish fed different fish oil, poultry oil and canola oil blend diets for 84 days.

Diet[1,2]	1	2	3	4	5	6	ANOVA[3]
Growth performance							
Initial weight (kg)	1.45±0.01	1.45±0.01	1.45±0.01	1.45±0.01	1.45±0.01	1.45±0.01	$P = 0.996$
Final weight (kg)	1.95±0.03	1.99±0.02	2.00±0.01	1.99±0.02	1.94±0.04	1.94±0.03	$P = 0.313$
Biomass gain (kg tank^{-1})	9.46±0.57	10.13±0.22	10.44±0.23	10.11±0.10	9.30±0.45	9.21±0.45	$P = 0.171$
SGR (% d^{-1})	0.35±0.02	0.37±0.01	0.39±0.01	0.37±0.00	0.35±0.01	0.35±0.01	$P = 0.161$
Initial fork length (mm)	458±2	458±3	459±1	460±0	460±0	461±2	$P = 0.676$
Final fork length (mm)	491±1	493±2	496±0	494±1	493±2	493±3	$P = 0.463$
Length growth rate (mm d^{-1})	0.40±0.02	0.42±0.00	0.44±0.01	0.41±0.01	0.39±0.02	0.38±0.01	$P = 0.211$
Final Condition factor	1.65±0.02	1.65±0.03	1.64±0.01	1.65±0.02	1.62±0.01	1.61±0.01	$P = 0.579$
Feed utilisation (as fed)							
Apparent feed consumption (kg tank$^{-1)}$	17.61±0.62	17.52±0.25	18.19±0.29	17.54±0.56	16.87±0.29	17.02±0.31	$P = 0.335$
Apparent feed intake (% BW d^{-1})	0.68±0.02	0.67±0.01	0.70±0.01	0.68±0.02	0.66±0.01	0.67±0.02	$P = 0.447$
Apparent FCR	1.87±0.08	1.73±0.04	1.74±0.01	1.74±0.05	1.82±0.07	1.86±0.08	$P = 0.442$
Proximate composition (wet basis)							
Moisture (%)	64.8±0.2	65.2±0.4	65.2±0.3	64.9±0.4	65.6±0.3	64.9±0.3	$P = 0.526$
Protein (%)	20.0±0.3	19.8±0.3	20.2±0.3	20.3±0.4	19.8±0.2	19.8±0.1	$P = 0.670$
Lipid (%)	13.6±1.1	14.2±0.6	13.2±0.6	13.6±0.4	13.1±0.3	13.8±0.6	$P = 0.814$
Ash (%)	2.8±0.3	2.3±0.2	2.1±0.0	2.3±0.2	1.9±0.1	2.0±0.1	$P = 0.085$
Carbohydrate (%)	<1	<1	<1	<1	<1	<1	NA
Energy (MJ kg^{-1})	8.43±0.43	8.80±0.00	8.30±0.17	8.47±0.18	8.07±0.03	8.53±0.18	$P = 0.326$
Nutrient retention[4]							
Apparent PD	22.73±1.42	22.85±1.28	24.05±1.53	24.52±1.88	21.49±0.75	21.63±0.75	$P = 0.539$
Apparent ED	31.14±4.99	36.54±0.72	30.22±1.54	32.28±2.20	27.44±0.77	32.40±2.72	$P = 0.317$

[1] Values are mean ± SE; $n = 3$.

[2] Diet 1 - 0% fish oil + 100% poultry oil [added oil]; 1.42 g 100 g^{-1} LC n-3 PUFA, Diet 2 - 26.71% fish oil + 73.52% poultry oil [added oil]; 2.34 g 100 g^{-1} LC n-3 PUFA, Diet 3 - 50.35% fish oil + 50.06% poultry oil [added oil]; 3.33 g 100 g^{-1} LC n-3 PUFA, Diet 4 - 27.81% fish oil + 48.26% poultry oil + 24.13% canola oil [added oil], Diet 5 - 28.68% fish oil + 23.84% poultry oil + 47.68% canola oil [added oil], Diet 6 - 29.37% fish oil + 0% poultry oil + 70.86% canola oil [added oil].

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$; * indicates Student-Newman-Keuls test was unable to detect significant differences between treatments).

[4] ED = energy deposition; PD = protein deposition.

Table 3.1.1.2.5. Fatty acid composition (mg 100 g^{-1}) of whole Yellowtail Kingfish fed different fish oil, poultry oil and canola oil blend diets for 84 days.

Diet[1,2,3]	1	2	3	4	5	6	ANOVA[4]
Saturated Fatty Acids							
C10:0 Capric	19±6	15±9	18±5	<10	13±8	14±8	$P = 0.727$
C14:0 Myristic	250±23	276±23	293±11	277±22	266±11	271±5	$P = 0.642$
C15:0 Pentadecanoic	41±3	43±2	44±3	41±1	39±1	42±2	$P = 0.780$
C16:0 Palmitic	2209±213	2334±160	2241±110	2216±145	2043±67	2038±38	$P = 0.602$
C17:0 Margaric	41±3	43±2	48±5	41±1	44±4	42±2	$P = 0.598$
C18:0 Stearic	754±72	750±42	680±40	691±48	655±24	654±16	$P = 0.441$
C20:0 Arachidic	14±1[b]	14±1[b]	13±1[b]	14±0[b]	26±1[a]	28±1[a]	$P < 0.001$
C22:0 Behenic	13±8	19±5	13±1	14±0	22±5	14±1	$P = 0.621$
C24:0 Lignoceric	24±11	24±10	31±9	32±9	35±5	41±6	$P = 0.736$
Total Saturated	3376±324	3547±247	3395±178	3356±222	3147±111	3165±65	$P = 0.756$
Mono-unsaturated Fatty Acids							
C16:1 Palmitoleic	746±75	780±55	746±42	713±39	650±22	639±5	$P = 0.249$
C18:1 Oleic	5192±488	5223±290	4827±261	5214±260	5119±148	5471±125	$P = 0.754$
C18:1 Vaccenic	399±34	432±25	421±25	427±23	415±14	437±9	$P = 0.886$
C20:1 Eicosenic	221±13[b]	285±16[a]	333±13[a]	290±12[a]	288±2[a]	304±6[a]	$P < 0.001$
C22:1 Cetoleic	158±10[b]	194±8[a]	224±10[a]	199±1[a]	201±5[a]	203±13[a]	$P = 0.006$
C22:1 Docosenoic	49±4[b]	66±4[a]	74±2[a]	68±2[a]	66±2[a]	69±3[a]	$P < 0.001$
C24:1 Nervonic	31±2[b]	43±2[ab]	48±5[a]	41±1[ab]	44±4[ab]	46±3[a]	$P = 0.041$
Total Mono-unsaturated	6806±619	7033±394	6690±352	6957±341	6795±181	7183±160	$P = 0.939$
Poly-unsaturated Fatty Acids							
C18:2n6 Linoleic	1767±131	1735±56	1449±85	1665±21	1626±82	1806±116	$P = 0.142$
C18:3n3 Alpha-Linolenic	217±17[b]	218±8[b]	176±16[b]	253±15[b]	262±23[b]	348±38[a]	$P = 0.002$
C20:2n6 Eicosadienoic	27±2	33±6	30±3	32±5	35±8	32±3	$P = 0.932$
C20:4n6 Arachidonic	81±10	90±8	71±13	72±11	61±12	65±12	$P = 0.490$
C20:5n3 Eicosapentaenoic	255±39	306±45	260±53	243±47	219±42	234±55	$P = 0.838$
C22:5n3 Docosapentaenoic	130±15	155±18	141±25	131±22	118±23	122±29	$P = 0.862$
C22:6n3 Docosahexaenoic	558±111	676±116	551±139	508±124	464±114	493±135	$P = 0.861$
Omega 3 Fatty Acids	1348±196	1573±205	1317±257	1319±231	1243±232	1383±291	$P = 0.945$
Omega 6 Fatty Acids	1925±140	1910±64	1603±94	1819±35	1766±94	1955±130	$P = 0.197$
Total Poly-unsaturated	3312±259	3540±229	2977±335	3187±268	3057±313	3388±421	$P = 0.797$
Trans Fatty Acids							
Total Mono Trans Fatty Acids	46±8	43±2	40±2	32±5	35±5	32±3	$P = 0.214$
Total Poly Trans Fatty Acids	59±7	71±3	61±2	68±2	66±2	69±3	$P = 0.234$

[1] Values are mean ± SE; $n = 3$.

[2] Diet 1 - 0% fish oil + 100% poultry oil [added oil]; 1.42 g 100 g^{-1} LC n-3 PUFA, Diet 2 - 26.71% fish oil + 73.52% poultry oil [added oil]; 2.34 g 100 g^{-1} LC n-3 PUFA, Diet 3 - 50.35% fish oil + 50.06% poultry oil [added oil]; 3.33 g 100 g^{-1} LC n-3 PUFA, Diet 4 - 27.81% fish oil + 48.26% poultry oil + 24.13% canola oil [added oil], Diet 5 - 28.68% fish oil + 23.84% poultry oil + 47.68% canola oil [added oil], Diet 6 - 29.37% fish oil + 0% poultry oil + 70.86% canola oil [added oil].

[3] Values for the following fatty acids < 10 mg 100 g^{-1} and were excluded from the table: C4:0 Butyric, C6:0 Caproic, C8:0 Caprylic, C12:0 Lauric, C14:1 Myristoleic, C17:1 Heptadecenoic, C18:3n6 gamma-Linolenic, C20:3n6 Eicosatrienoic, C20:3n3 Eicosatrienoic, C22:2n6 Docosadienoic, C22:4n6 Docosatetraenoic.

[4] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$.

Table 3.1.1.2.6. Blood biochemistry of serum and blood haematology on whole blood of Yellowtail Kingfish fed different fish oil, poultry oil and canola oil blend diets for 84 days.

Diet[1,2]	1	2	3	4	5	6	ANOVA[3]
Biochemistry[4]							
Sodium (mmol L^{-1})	196.49±1.62	195.75±0.89	194.28±1.08	197.20±1.99	197.34±1.66	197.82±0.97	$P = 0.554$
Potassium (mmol L^{-1})	2.71±0.67	3.38±0.18	3.51±0.56	3.86±0.61	2.86±0.53	3.76±0.95	$P = 0.725$
Urea (mmol L^{-1})	3.7±0.4	3.8±0.5	3.9±0.5	3.7±0.4	3.6±0.3	4.1±0.3	$P = 0.952$
Creatinine (mmol L^{-1})	0.017±0.001	0.016±0.001	0.016±0.000	0.016±0.001	0.017±0.001	0.017±0.000	$P = 0.944$
Calcium (mmol L^{-1})	2.97±0.03	2.94±0.02	2.97±0.08	2.96±0.08	3.03±0.03	3.05±0.03	$P = 0.651$
Protein (g L^{-1})	38±1	37±1	37±1	36±0	37±0	36±2	$P = 0.760$
Albumin (g L^{-1})	11±0	11±0	11±0	11±0	11±0	10±0	$P = 0.334$
Globulin (g L^{-1})	27±1	26±1	26±0	25±0	26±0	26±1	$P = 0.777$
Total Bilirubin (mmol L^{-1})	3±1	2±1	1±0	1±0	1±0	1±0	$P = 0.587$
ALT (IU L^{-1})	15±3	11±1	10±1	9±1	9±1	10±3	$P = 0.366$
ALP (IU L^{-1})	22±3	27±2	31±3	24±4	30±3	31±3	$P = 0.303$
Magnesium (mmol L^{-1})	1.33±0.06	1.21±0.02	1.25±0.07	1.26±0.03	1.28±0.09	1.26±0.05	$P = 0.813$
Cholesterol (mmol L^{-1})	3.9±0.2	4.0±0.3	4.5±0.2	4.3±0.2	4.2±0.4	4.1±0.1	$P = 0.561$
Triglyceride (mmol L^{-1})	2.00±0.47	2.32±0.34	1.68±0.04	1.79±0.45	1.94±0.14	2.26±0.12	$P = 0.658$
Bile Acids (mmol L^{-1})	2.9±0.1	5.4±1.9	5.2±1.1	11.1±8.4	6.2±3.3	5.5±0.6	$P = 0.761$
Haematology[5]							
RBC ($\times 10^{12}$)	2.86±0.09	2.29±0.20	2.45±0.24	2.47±0.19	2.66±0.06	2.72±0.17	$P = 0.257$
HGB (g L^{-1})	115±3	103±4	106±2	104±3	102±2	111±4	$P = 0.060$
PCV (L L^{-1})	0.59±0.01	0.57±0.01	0.57±0.01	0.57±0.02	0.59±0.01	0.60±0.00	$P = 0.208$
MCV (fl)	181.88±1.84	177.90±6.93	179.51±1.35	181.11±3.07	181.69±1.55	179.46±1.71	$P = 0.950$
MCH (pg)	40.5±1.1	48.4±5.5	45.5±4.1	44.0±5.4	38.4±0.3	42.1±4.5	$P = 0.570$
MCHC (g L^{-1})	223±4	273±31	254±22	244±29	211±0	235±24	$P = 0.443$
WBC ($\times 10^{9}$)	6.9±0.2	6.9±0.2	6.8±0.1	7.0±0.3	6.8±0.2	7.0±0.2	$P = 0.990$
Granulocytes (%)	8±0	7±1	6±1	8±2	7±0	7±2	$P = 0.692$
Lymph (%)	92±0	93±1	94±1	91±2	93±0	93±2	$P = 0.783$
Mono (%)	0±0	0±0	0±0	0±0	0±0	0±0	NA
Eosin (%)	0±0	0±0	0±0	0±0	0±0	0±0	NA
Baso (%)	0±0	0±0	0±0	0±0	0±0	0±0	NA
Platelets ($\times 10^{9}$)	21±9	30±13	26±7	24±4	17±3	27±4	$P = 0.863$

[1] Values are mean ± SE; $n = 3$.

[2] Diet 1 - 0% fish oil + 100% poultry oil [added oil]; 1.42 g 100 g^{-1} LC n-3 PUFA, Diet 2 - 26.71% fish oil + 73.52% poultry oil [added oil]; 2.34 g 100 g^{-1} LC n-3 PUFA, Diet 3 - 50.35% fish oil + 50.06% poultry oil [added oil]; 3.33 g 100 g^{-1} LC n-3 PUFA, Diet 4 - 27.81% fish oil + 48.26% poultry oil + 24.13% canola oil [added oil], Diet 5 - 28.68% fish oil + 23.84% poultry oil + 47.68% canola oil [added oil], Diet 6 - 29.37% fish oil + 0% poultry oil + 70.86% canola oil [added oil].

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

[4] ALT = alanine aminotransferase; ALP = alkaline phosphatase

[5] Smear content: red and white cell normal; Baso = basophil; Eosin = eosinophil; HGB = haemoglobin; Lymph = lymphocytes; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; MCV = mean corpuscular volume; Mono = monocytes; PCV = packed cell volume; RBC = red blood cell count; WBC = white blood cell count.

Table 3.1.1.2.7. Fatty acid composition of plasma of Yellowtail Kingfish fed different fish oil, poultry oil and canola oil blend diets for 84 days.

Diet[1,2]	1	2	3	4	5	6	ANOVA[3]
Saturated Fatty Acids							
C14:0 Myristic	2.6±0.5	3.7±0.4	4.5±0.3	3.5±1.0	3.2±0.1	4.3±0.4	$P = 0.161$
C15:0 Pentadecanoic	1.1±0.1	1.5±0.2	1.7±0.1	1.5±0.3	1.4±0.1	1.7±0.1	$P = 0.066$
C16:0 Palmitic	124.7±11.6	136.2±12.2	131.7±3.3	117.4±18.3	106.5±7.6	115.1±7.9	$P = 0.470$
C17:0 Margaric	2.1±0.3	2.7±0.4	2.8±0.1	2.5±0.4	2.4±0.1	3.0±0.2	$P = 0.391$
C18:0 Stearic	49.8±5.8	52.7±5.7	49.8±1.8	47.2±7.1	45.9±2.7	50.0±4.4	$P = 0.941$
C20:0 Arachidic	0.4±0.1^{c}	0.6±0.1^{bc}	0.6±0.1^{bc}	0.6±0.1^{bc}	0.9±0.1^{b}	1.4±0.1^{a}	$P < 0.001$
C22:0 Behenic	0.1±0.1^{b}	0.2±0.0^{b}	0.2±0.0^{b}	0.3±0.1^{b}	0.3±0.0^{b}	0.5±0.0^{a}	$P = 0.004$
C24:0 Lignoceric	5.2±0.4	5.4±0.5	5.3±0.2	5.0±0.7	4.8±0.4	5.5±0.4	$P = 0.868$
Total Saturated	187.8±19.1	204.9±19.4	198.6±5.5	179.8±28.1	167.1±10.9	183.7±13.3	$P = 0.709$
Mono-unsaturated Fatty Acids							
C16:1n7 Palmitoleic	11.5±2.1	13.3±2.0	12.4±0.6	10.0±2.7	7.8±0.6	7.7±0.2	$P = 0.149$
C18:1n9 Oleic	138.0±22.3	135.7±19.0	115.2±5.4	131.2±29.7	131.9±9.7	173.9±8.5	$P = 0.376$
C18:1n7 Vaccenic	14.1±1.9	16.2±2.5	15.3±0.6	15.2±3.0	15.1±1.1	19.1±1.2	$P = 0.561$
C20:1n9 Eicosenic	3.6±0.6^{b}	6.7±0.8^{ab}	9.6±0.8^{a}	6.7±1.7^{ab}	7.0±0.2^{ab}	9.2±0.6^{a}	$P = 0.007$
C22:1n9 Erucic	0.2±0.0^{c}	0.7±0.1^{b}	1.1±0.1^{a}	0.6±0.2^{b}	0.7±0.0^{b}	0.9±0.0^{ab}	$P < 0.001$
C24:1 Nervonic	1.2±0.1^{c}	2.0±0.0^{b}	2.6±0.1^{a}	2.1±0.2^{b}	2.1±0.1^{b}	2.4±0.1^{ab}	$P < 0.001$
Total Mono-unsaturated	168.5±27.1	174.6±24.5	156.2±7.5	166.0±37.5	164.4±11.6	213.2±10.5	$P = 0.567$
Poly-unsaturated Fatty Acids							
C18:2n6 Linoleic	74.6±7.4	64.7±8.9	45.4±1.1	61.8±11.9	65.0±5.4	83.5±4.1	$P = 0.052$
C18:3n6 Gamma-Linolenic	0.6±0.1	0.8±0.2	0.8±0.0	0.7±0.2	0.6±0.1	0.6±0.1	$P = 0.806$
C18:3n3 Alpha-Linolenic	7.2±0.9^{cd}	6.5±0.9^{cd}	4.9±0.1^{d}	10.3±2.5^{bc}	13.9±1.1^{b}	21.6±0.7^{a}	$P < 0.001$
C20:2n6 Eicosadienoic	2.7±0.3	2.5±0.2	2.1±0.2	2.6±0.5	2.6±0.3	3.2±0.3	$P = 0.379$
C20:3n6 Eicosatrienoic	1.8±0.1^{a}	1.6±0.2^{ab}	1.3±0.1^{ab}	1.3±0.2^{ab}	1.0±0.1^{b}	0.9±0.1^{b}	$P = 0.007$
C20:4n6 Arachidonic	21.1±1.2	23.0±2.0	23.1±0.6	20.9±2.6	19.0±1.9	21.4±1.7	$P = 0.634$
C20:5n3 Eicosapentaenoic	27.2±2.1^{b}	38.8±3.6^{ab}	45.3±0.9^{a}	36.2±5.6^{ab}	35.0±2.9^{ab}	40.9±3.3^{ab}	$P = 0.043$
C22:4n6 Docosatetraenoic	2.0±0.1	2.0±0.1	1.9±0.1	1.7±0.2	1.5±0.1	1.6±0.1	$P = 0.142$
C22:5n3 Docosapentaenoic	13.5±0.5^{b}	19.9±2.0^{ab}	22.2±0.7^{a}	19.0±2.7^{ab}	17.4±1.9^{ab}	19.7±1.4^{ab}	$P = 0.053$
C22:6n3 Docosahexaenoic	141.8±7.1^{b}	188.2±15.6^{ab}	217.2±3.4^{a}	179.8±22.4^{ab}	168.9±14.3^{ab}	194.2±14.4^{ab}	$P = 0.047$
Total Omega 3	189.8±10.6	253.5±22.0	289.7±4.1	245.3±33.1	235.2±19.8	276.4±19.5	$P = 0.057$
Total Omega 6	102.8±9.2	94.7±11.6	74.6±2.0	89.0±15.6	89.8±7.8	111.2±6.4	$P = 0.215$
Trans Fatty Acids							
Ct18:1n-9	1.5±0.1	1.5±0.2	1.5±0.1	1.3±0.2	1.0±0.1	1.0±0.1	$P = 0.057$
Ct18:1n-7	0.6±0.1^{b}	2.0±0.3^{a}	2.6±0.1^{a}	1.8±0.3^{a}	1.8±0.1^{a}	1.9±0.2^{a}	$P = 0.001$
Total Trans Fatty Acids	2.1±0.2	3.3±0.6	4.1±0.2	3.1±0.5	2.6±0.3	2.7±0.4	$P = 0.057$

[1] Values are mean ± SE; $n = 3$.

[2] Diet 1 - 0% fish oil + 100% poultry oil [added oil]; 1.42 g 100 g^{-1} LC n-3 PUFA, Diet 2 - 26.71% fish oil + 73.52% poultry oil [added oil]; 2.34 g 100 g^{-1} LC n-3 PUFA, Diet 3 - 50.35% fish oil + 50.06% poultry oil [added oil]; 3.33 g 100 g^{-1} LC n-3 PUFA, Diet 4 - 27.81% fish oil + 48.26% poultry oil + 24.13% canola oil [added oil], Diet 5 - 28.68% fish oil + 23.84% poultry oil + 47.68% canola oil [added oil], Diet 6 - 29.37% fish oil + 0% poultry oil + 70.86% canola oil [added oil].

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

Table 3.1.1.2.8. Fatty acid composition of red blood cells of Yellowtail Kingfish fed different fish oil, poultry oil and canola oil blend diets for 84 days.

Diet[1,2]	1	2	3	4	5	6	ANOVA[3]
Saturated Fatty Acids							
C14:0 Myristic	1.1±0.2	1.3±0.2	1.6±0.1	1.5±0.1	1.4±0.2	1.7±0.1	$P = 0.132$
C15:0 Pentadecanoic	1.1±0.3	1.1±0.3	1.5±0.2	1.4±0.3	1.2±0.3	1.5±0.3	$P = 0.721$
C16:0 Palmitic	60.6±6.5	56.8±5.0	63.1±3.2	66.0±2.1	64.0±6.4	57.1±3.1	$P = 0.673$
C17:0 Margaric	0.9±0.1^{b}	1.0±0.1^{ab}	1.2±0.1^{ab}	1.2±0.0^{ab}	1.3±0.1^{ab}	1.3±0.1^{a}	$P = 0.024$
C18:0 Stearic	37.4±5.3	34.4±2.1	37.9±1.1	40.0±1.4	41.6±4.2	37.5±2.6	$P = 0.697$
C20:0 Arachidic	0.5±0.0^{a}	0.5±0.1^{a}	0.7±0.1^{ab}	0.8±0.0^{b}	1.1±0.1^{a}	1.2±0.0^{a}	$P < 0.001$
C22:0 Behenic	0.2±0.0	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.1	0.3±0.0	$P = 0.099$
C24:0 Lignoceric	2.4±0.3	2.1±0.2	2.3±0.1	2.4±0.1	2.5±0.2	2.3±0.1	$P = 0.627$
Total Saturated	107.7±13.8	100.9±7.6	113.3±3.7	118.1±2.5	117.1±12.2	107.2±6.9	$P = 0.725$
Mono-unsaturated Fatty Acids							
C16:1n7 Palmitoleic	2.3±0.3	2.2±0.2	2.5±0.2	2.1±0.1	2.0±0.1	1.7±0.2	$P = 0.162$
C18:1n9 Oleic	26.7±2.7^{ab}	24.1±2.1^{b}	23.8±2.1^{b}	27.5±0.9^{ab}	30.7±1.2^{ab}	34.2±2.6^{a}	$P = 0.025$
C18:1n7 Vaccenic	5.0±0.5	4.7±0.4	5.4±0.3	5.5±0.1	6.2±0.5	6.2±0.1	$P = 0.067$
C20:1n9 Eicosenic	2.1±0.1^{c}	3.2±0.3^{b}	5.1±0.3^{a}	4.0±0.1^{ab}	4.6±0.4^{a}	4.5±0.1^{a}	$P < 0.001$
C22:1n9 Erucic	0.3±0.1^{b}	0.4±0.0^{a}	0.7±0.1^{a}	0.5±0.0^{a}	0.5±0.1^{a}	0.6±0.0^{a}	$P = 0.012$
C24:1 Nervonic	0.9±0.1^{c}	1.2±0.0^{b}	1.6±0.0^{a}	1.2±0.0^{ab}	1.4±0.1^{ab}	1.4±0.1^{ab}	$P = 0.001$
Total Mono-unsaturated	37.3±3.8	35.8±3.0	39.0±2.9	40.9±1.1	45.4±2.3	48.6±2.7	$P = 0.056$
Poly-unsaturated Fatty Acids							
C18:2n6 Linoleic	30.1±2.8^{a}	21.8±1.6^{b}	18.3±1.1^{b}	28.0±1.2^{a}	29.7±1.9^{a}	28.9±1.0^{a}	$P = 0.002$
C18:3n6 gamma-Linolenic	0.2±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.0	0.2±0.0	$P = 0.131$
C18:3n3 alpha-Linolenic	1.7±0.2^{d}	1.2±0.1^{e}	1.0±0.1^{e}	2.3±0.1^{c}	3.3±0.1^{b}	4.0±0.3^{a}	$P < 0.001$
C20:2n6 Eicosadienoic	0.9±0.1	0.8±0.1	0.9±0.0	1.0±0.0	1.0±0.1	1.1±0.0	$P = 0.055$
C20:3n6 Eicosatrienoic	1.0±0.1^{a}	0.8±0.1^{ab}	0.8±0.0^{ab}	0.8±0.0^{ab}	0.7±0.1^{ab}	0.6±0.0^{b}	$P = 0.014$
C20:4n6 Arachidonic	22.1±2.5	18.8±1.4	20.3±0.7	21.8±0.8	21.6±2.4	17.7±1.2	$P = 0.370$
C20:5n3 Eicosapentaenoic	21.8±1.9	23.4±2.2	27.4±1.6	27.7±1.6	27.5±2.4	23.0±1.1	$P = 0.128$
C22:4n6 Docosatetraenoic	0.9±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.6±0.0	$P = 0.272$
C22:5n3 Docosapentaenoic	5.8±0.4	6.3±0.6	7.1±0.5	7.4±0.4	7.4±0.4	6.5±0.3	$P = 0.095$
C22:6n3 Docosahexaenoic	64.1±6.4	69.2±5.4	82.6±5.7	79.7±3.1	83.5±6.8	78.0±4.4	$P = 0.145$
Total Omega 3	93.4±8.8	100.1±8.0	118.2±7.8	117.1±5.2	121.6±9.7	111.4±5.6	$P = 0.130$
Total Omega 6	55.2±5.6	43.2±3.2	41.4±2.0	52.7±2.0	54.0±4.6	49.1±2.0	$P = 0.072$
Trans Fatty Acids							
Ct18:1n9	0.4±0.0	0.4±0.0	0.4±0.0	0.3±0.0	0.3±0.0	0.3±0.0	$P = 0.318$
Ct18:1n7	0.4±0.0^{a}	0.3±0.0^{abc}	0.4±0.0^{ab}	0.2±0.0^{bc}	0.2±0.0^{abc}	0.2±0.0^{c}	$P = 0.009$
Total Trans Fatty Acids	0.8±0.0^{a}	0.6±0.0^{ab}	0.7±0.1^{ab}	0.6±0.1^{ab}	0.6±0.0^{b}	0.5±0.0^{b}	$P = 0.010$

[1] Values are mean ± SE; $n = 3$.

[2] Diet 1 - 0% fish oil + 100% poultry oil [added oil]; 1.42 g 100 g^{-1} LC n-3 PUFA, Diet 2 - 26.71% fish oil + 73.52% poultry oil [added oil]; 2.34 g 100 g^{-1} LC n-3 PUFA, Diet 3 - 50.35% fish oil + 50.06% poultry oil [added oil]; 3.33 g 100 g^{-1} LC n-3 PUFA, Diet 4 - 27.81% fish oil + 48.26% poultry oil + 24.13% canola oil [added oil], Diet 5 - 28.68% fish oil + 23.84% poultry oil + 47.68% canola oil [added oil], Diet 6 - 29.37% fish oil + 0% poultry oil + 70.86% canola oil [added oil].

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

Table 3.1.1.2.9.Visceral somatic parameters and gastrointestinal morphology of Yellowtail Kingfish fed different fish oil, poultry oil and canola oil blend diets for 84 days.

Diet[1,2]	1	2	3	4	5	6	ANOVA[3]
Visceral somatic parameters							
Intraperitoneal fat (%)	1.90±0.34	1.71±0.38	1.14±0.40	1.50±0.27	1.19±0.23	1.18±0.17	P = 0.438
Viscerosomatic index (VSI; %)	6.84±0.41	6.70±0.34	6.58±0.43	6.74±0.26	6.68±0.31	6.50±0.27	P = 0.985
Hepatosomatic index (HSI; %)	1.07±0.06	1.00±0.05	1.05±0.05	1.01±0.04	1.03±0.08	0.99±0.04	P = 0.869
Stomach morphology							
Gastric dilation score[4]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	0.22±0.11[a]	P = 0.023
Hindgut morphology							
Muscularis thickness	834±16	817±67	844±137	772±56	757±29	782±72	P = 0.941
Submucosa thickness	25±3	39±13	37±20	26±5	28±3	23±2	P = 0.818
Villi length	1254±55	1089±41	1268±139	1201±53	1153±53	1212±166	P = 0.796
Villi thickness	100±2	107±6	103±10	103±3	99±7	95±7	P = 0.833
Lamina propria thickness	16±2	19±3	18±5	17±1	14±2	16±2	P = 0.805
Lamina propria/villi thickness (%)	16±2	18±2	17±3	16±1	14±1	17±1	P = 0.765
Mucus cells per 100µm	4.42±0.27	5.73±1.12	5.02±0.38	3.79±0.56	4.67±0.78	5.38±0.46	P = 0.401
Eosinophilic droplets in epithelial cells	2±0	2±0	3±0	2±0	2±0	2±0	P = 0.156
Melanomacrophage centres	1±1	1±1	2±1	1±0	1±0	1±0	P = 0.944
Apparent digestibility coefficient (ADC; %)							
Dry matter	56.4±1.4	47.7±3.9	49.0±6.9	43.3±12.7	52.2±2.2	51.1±5.8	P = 0.809
Crude protein	77.2±0.3	75.4±3.5	75.2±3.1	72.4±3.7	75.9±1.1	74.3±2.5	P = 0.872
Gross energy	70.6±0.8	67.6±4.8	68.2±3.4	65.0±5.8	70.0±1.5	67.5±4.1	P = 0.920

[1] Values are mean ± SE; n = 3.

[2] Diet 1 - 0% fish oil + 100% poultry oil [added oil]; 1.42 g 100 g^{-1} LC n-3 PUFA, Diet 2 - 26.71% fish oil + 73.52% poultry oil [added oil]; 2.34 g 100 g^{-1} LC n-3 PUFA, Diet 3 - 50.35% fish oil + 50.06% poultry oil [added oil]; 3.33 g 100 g^{-1} LC n-3 PUFA, Diet 4 - 27.81% fish oil + 48.26% poultry oil + 24.13% canola oil [added oil], Diet 5 - 28.68% fish oil + 23.84% poultry oil + 47.68% canola oil [added oil], Diet 6 - 29.37% fish oil + 0% poultry oil + 70.86% canola oil [added oil].

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

[4] Gastric dilation score based on Chown (2015).

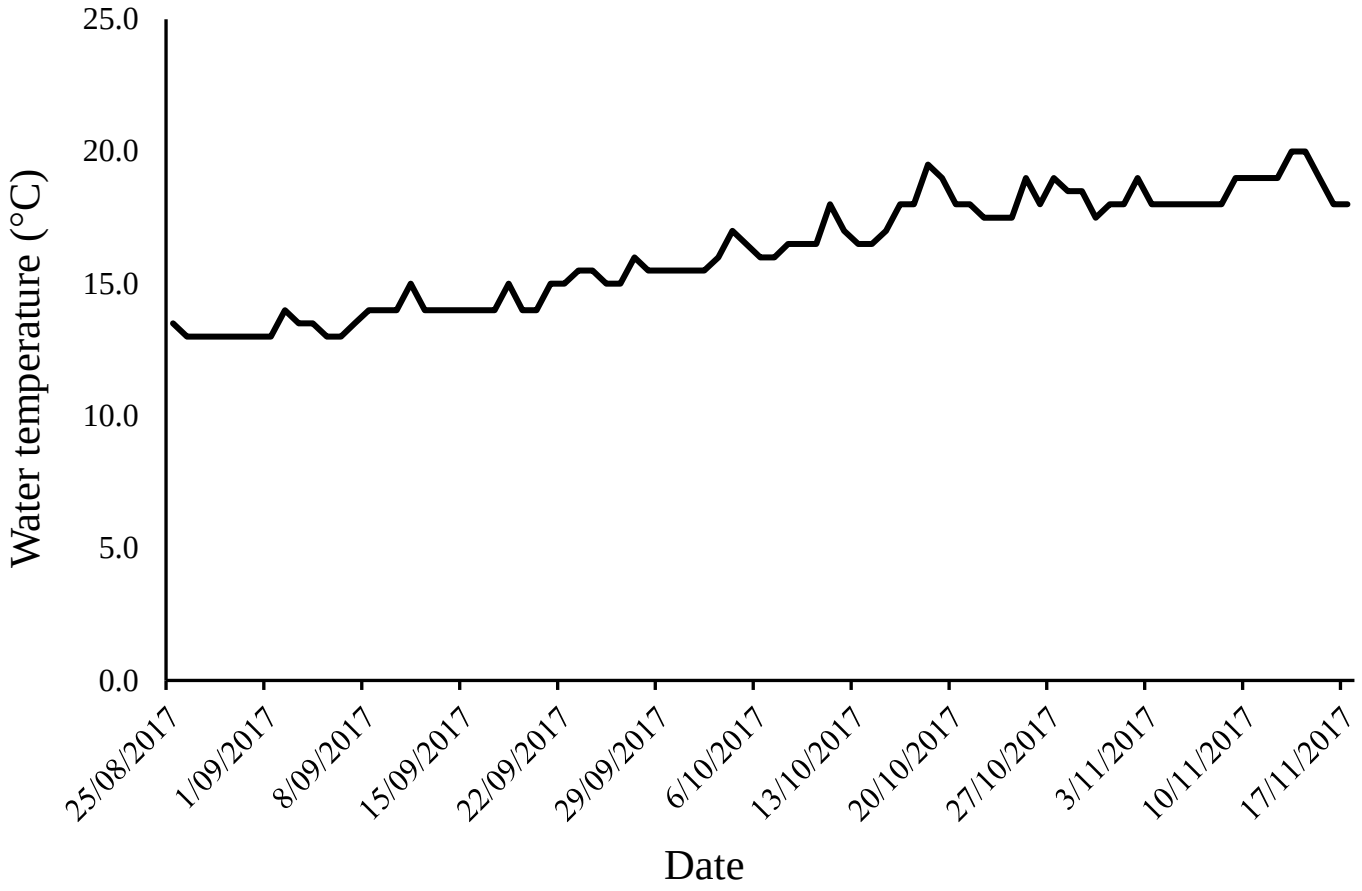


Figure 3.1.1.2.1. Water temperature profile during the 84 day experimental period (average 16.2 °C [range 13.0-20.0 °C]).

3.1.2. Chapter - Emulsifiers and protein and energy levels for large Yellowtail Kingfish.

3.1.2.1. Manuscript - Evaluation of dietary lipid levels and emulsifiers on growth and feed utilisation in large Yellowtail Kingfish (Seriola lalandi) at winter water temperatures.

David A.J. Stone [a, b, c, d], Matthew S. Bansemer [a], Paul Skordas [a], Nicole Ruff [e]

[a] South Australian Research and Development Institute, South Australian Aquatic Sciences Centre, West Beach, SA 5024, Australia.
[b] Flinders University, College of Science and Engineering, GPO Box 2100, Adelaide, SA 5001, Australia.
[c] University of Adelaide, School of Animal and Veterinary Sciences, Roseworthy, SA 5371, Australia
[d] Marine Innovation Southern Australia.
[e] Skretting Australia, Cambridge, TAS 7170, Australia.

This manuscript may be referenced as: Stone, D.A.J., Bansemer, M.S., Skordas, P. and Ruff, N. (2019). 3.1.2.1. Manuscript - Evaluation of dietary lipid levels and emulsifiers on growth and feed utilisation in large Yellowtail Kingfish (*Seriola lalandi*) at winter water temperatures (N3; Output 2b and 2e). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.67-83.

Abstract

Yellowtail Kingfish (*Seriola lalandi*; YTK) have a fast growth rate, and as a result, a high energy demand that should ideally be satisfied by dietary lipids. Recent research has highlighted the knowledge gap in dietary lipid requirements for large YTK at winter water temperatures. In this 84 day study, the effect of dietary lipid levels and the addition of a dietary emulsifier (LYSOFORTE® Liquid, Kemin Industries, Inc.) on the growth, feed utilisation, and gut health of YTK (> 1.5 kg) were investigated at winter water temperatures. This was a factorial experiment (2 × 2; n = 4 dietary treatments) which evaluated dietary lipid level (high, 30% and low, 20%) as the first factor and lipid emulsifier (with or without LYSOFORTE® Liquid) as the second factor. Fish were fed the 9 mm diameter pellets to apparent satiation once daily at 09:00 h. The specific growth rate (SGR; % d^{-1}) and protein deposition (PD) of YTK fed the 30% lipid diets were significantly higher than fish fed the 20% lipid diets. Dietary LYSOFORTE® Liquid inclusion or the interaction between dietary lipid level and LYSOFORTE® Liquid inclusion did not significantly influence SGR or PD Feed intake (% body weight d^{-1}) was not significantly influenced by dietary lipid level, dietary LYSOFORTE® Liquid inclusion or the interaction between the two variables. However, the feed conversion ratio (FCR) of fish fed the 30% lipid diet was significantly lower (improved) compared to those fed the 20% lipid. In contrast, dietary LYSOFORTE® Liquid inclusion did not significantly influence FCR. Intraperitoneal fat (%) and viscerosomatic index (VSI; %) were significantly affected by lipid level (30 > 20%). However, dress-out yields (gutted, head on and gills in) of processed fish were not significantly affected by lipid level. Results of the current study suggest that YTK may be fed a 30% dietary lipid level at winter water temperatures to improve growth and feed utilisation. However, given differences in whole weight versus dress-out yields weights, the use of high lipid diets to increase total final weight will ultimately come down to a marketing decision by YTK producers. We recommend further research investigating high (30%) dietary lipid diets under commercial conditions at winter temperatures before implementing this practice on-farm. In contrast, there was no apparent benefit to growth or feed utilisation by feeding a 40 mg kg $lipid^{-1}$dietary inclusion of LYSOFORTE® Liquid for YTK at winter water temperatures. After discussions with project participants, we do not recommend any further investigation of LYSOFORTE® Liquid for YTK at winter water temperatures.

Introduction

Yellowtail Kingfish (*Seriola lalandi*; YTK) are well suited to aquaculture due to their fast growth rate (reaching 3 kg in 12-18 months). As a result of their fast growth rate, YTK have a high energy demand (Pirozzi and Booth, 2009). Fish are able to deaminate protein for energy, however, this should be avoided as this reduces protein deposition and growth, and ultimately increases feed costs (Philips, 1972; Bowyer et al., 2013). As dietary carbohydrate has been reported to be poorly utilised by YTK (Booth et al., 2013), dietary lipids should ideally be used to satisfy the energy requirements (Booth et al., 2013; Stone et al., 2016). Recent research has identified that YTK may be fed a higher lipid, lower protein diet to achieve a protein sparing effect at warm summer water temperatures (19.5-26.0 °C) (Stone et al., 2016). The authors reported superior growth of YTK fed a 28% crude lipid (CL) and 42% crude protein (CP) compared to fish fed a 24% CL and 44% CP (Stone et al., 2016). However, cultured YTK in South Australia are exposed to fluctuating water temperatures that reach above 24 °C during summer and below 12 °C in winter (Miegel et al., 2010). YTK and other *Seriola* spp., may be less tolerant to high lipid diets at cool winter water temperatures compared to warm summer water temperatures (Talbot et al., 2000; Bowyer et al., 2012a; Bowyer et al., 2012b). Furthermore, high lipid diets, in addition to a number of other factors, have also been reported to be associated with the development of enteritis at cooler water temperatures (Sheppard, 2004; Bansemer et al., 2015).

YTK at sub-optimal winter water temperatures fed a commercial diet (24% CL [as fed]; 25.8% CL [dry]) to apparent satiation 6 d week^{-1} exhibited similar SGR to fish fed an Australian Sardine diet (4.1% [as fed]; 14.7% CL [dry]) to apparent satiation every second day (Stone et al., 2016). Furthermore, Mediterranean Yellowtail (*Seriola dumerilii*; 95 g) at sub-optimal water temperatures (18 °C) fed a high 30% CL diet had significantly increased visceral fat deposition (23.5% visceral fat), compared to those fed a low CL diet (18% CL level; 16% visceral fat) (Talbot et al., 2000). This trait is undesirable, as it contributes to reduced product yield and increased production costs (Talbot et al., 2000). To develop YTK diets to improve productivity, there is potential to improve our understanding of the effects of dietary lipid levels on growth performance, feed utilisation, product yield and gut health for YTK at winter water temperatures.

Improving lipid emulsification in the gastrointestinal tract has been suggested to improve the digestion and absorption of lipids in a number of aquaculture species (Ward and Carter, 2009; Perera and Simon, 2014; Zhao et al., 2015). Lipid absorption in YTK may be improved by the incorporation of emulsifiers in the diet. Emulsifiers are compounds that have a hydrophilic component that dissolves in a water phase, and also a hydrophobic component that dissolves in a lipid phase (Zhao et al., 2015). Due to their combined hydrophilic and hydrophobic properties, emulsifying agents may promote the dispersion of oil through a continuous phase of water ("oil-in-water") or water in a continuous phase of oil ("water-in-oil"). This beneficial chemical property may improve lipid and energy digestion. There are a number of commercially available emulsifiers including carboxymethylcellulose, polysorbate-80, mono- and di-glycerides, vegetable lecithins (corn, rapeseed, soy and sunflower), and also proprietary lysophospholipid products, including LYSOFORTE® and LYSOFORTE® Liquid (Kemin Industries, Inc., Singapore, Republic of Singapore) (Hung et al., 1997; Guiotto et al., 2015; Sugumar et al., 2015). In addition to being commercially available, LYSOFORTE® Liquid has been reported to be cost-effective and can be mixed with lipids prior to feed kernels top-coating (post-pelleting application), thus avoiding heat related losses of activity. Dietary inclusions of LYSOFORTE® Liquid have been reported to improve the growth performance and nutrient digestibility for Tra Catfish (*Pangasianodon hypophthalmus*) and White Shrimp (*Litopenaeus vanammei*) at optimal growing temperatures (Sugumar et al., 2014; Sugumar et al., 2015). However, the benefits of incorporating LYSOFORTE® Liquid in diets for YTK on diet digestibility and utilisation, gastrointestinal tract health and growth are unknown.

Aim

The aim of this study was to evaluate the effects of dietary lipid level, LYSOFORTE® Liquid inclusion and their interactive effects on diet digestibility and utilisation, gastrointestinal tract health and growth of large YTK at winter water temperatures.

Methods

Experimental design and diets

This was a factorial experiment (2 × 2; *n* = 4 dietary treatments) investigating lipid level and LYSOFORTE® Liquid inclusion. YTK were fed a 30% or 20% total lipid diet, with or without LYSOFORTE® Liquid (Kemin Industries, Inc., Singapore, Republic of Singapore). The pellet kernel (9 mm diameter), fish oil and poultry oil were supplied by Skretting Australia (Cambridge, Tasmania, Australia). The pellet kernel was formulated to contain 30% fish meal, and contained ~10% crude lipid. The pellet kernels were top coated at Aquafeeds Australia (Mount Barker, South Australia, Australia) with a blend of fish oil and poultry oil to achieve a total dietary lipid level of 20 or 30%. Diets were formulated to contain 2.12 g 100 g^{-1} dietary long chain omega-3 polyunsaturated fatty acids levels (∑LC n-3 PUFA; eicosapentaenoic acid [20:5n-3, EPA], docosapentaenoic acid [22:5n-3, DPA] and docosahexaenoic acid [22:6n-3, DHA]) to meet the requirements for optimal growth of large YTK at summer water temperatures (Stone et al., Manuscript 3.1.1.1). To ensure diets contained the desired total lipid level, poultry oil was selected as the alternative lipid source, as it is currently used in commercial YTK diets. With regard to the emulsifier, LYSOFORTE® Liquid was added at a concentration of 40 mg kg $lipid^{-1}$. This concentration was recommended for carnivorous fish by Kemin Industries (Chinnadurai Sugumar, Kemin Industries, Inc.; *personal communication*). LYSOFORTE® Liquid was added to the fish oil and poultry oil blend prior to top-coating kernels. The nutritional composition of the four experimental diets are displayed in Table 3.1.2.1.1.

Fish were fed to apparent satiation at 09:00 h daily, which involved feeding fish for four min $tank^{-1}$ or until a feed refusal response was observed. Feed intake was recorded daily, and tanks were cleaned every second day. This study ran for a total of 84 days.

Experimental fish

Experimental work was conducted in the pool-farm facility at SARDI SAASC. YTK (*n* = 276; 1.12 ± 0.11 kg; 426 ± 13 mm (fork length; mean ± standard deviation) were obtained from Clean Seas Seafood (Port Lincoln, South Australia, Australia). Upon arrival at the SARDI SAASC facility, YTK were transferred to 5000 L tanks supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated, aerated sea water at ambient temperature and held for ~2 months and fed a standard Ridley Pelagica diet (crude protein 46%; crude lipid 24%; gross energy 19.30 MJ kg^{-1}) once daily.

Fish treatment

Before the commencement, and during the experiment, YTK were treated for skin flukes (*Benedenia seriola*) and gill flukes (*Zeuxapta seriola*) with formalin (250 ppm for 30 min), and blood flukes (*Paradeontacylix* spp.) with in-feed praziquantel (~10-15 mg kg^{-1}). Fish were also treated for epitheliocystis with in-feed oxytetracycline (75 mg kg^{-1}) for 10 days during the experiment. Treatment was prescribed by Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd., Ballina, New South Wales, Australia).

Experimental stocking and intermediate weight checks

At the commencement of the current experiment (August 2016), YTK were anaesthetised in 5000 L tanks (total water volume 2500 L) using AQUI-S® (AQUI-S® New Zealand Ltd., Lower Hutt, New Zealand) at a concentration of 14 mg L^{-1} of seawater. Twenty three fish were removed from their tank, measured, weighed and stocked into one of the three replicate 5000 L tanks per treatment combination (*n* = 4 treatments; *n* = 12 tanks). Tanks were supplied with partial flow-through/recirculating (100% water exchange d^{-1}), sand filtered, UV treated sea water at ambient temperature. All tanks were supplied with aeration and oxygenation throughout the study. As required, mortalities were removed form the tanks, weighed, measured and recorded and replaced with tagged fish (T-tags) of a similar weight.

Tagged fish were included in biomass calculations for FCR (see Performance indice section), but excluded from all other analyses.

At four and eight weeks post-stocking, all fish were anaesthetised using AQUI-S® at a concentration of 14 mg L^{-1} of seawater and weighed and measured before being returned to their respective tanks.

Water quality analyses

Water quality parameters were measured daily at 10:30 h, and maintained at appropriate levels for acceptable growth of YTK throughout the study (Table 3.1.2.1.2). Water temperature was measured using a thermometer. Dissolved oxygen (mg L^{-1} and % saturation) was measured using a dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark). The pH was measured daily using a meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, Illinois, United States of America). Salinity (g L^{-1}) was measured weekly using a portable salinity refractometer (model RF20, Extech Instruments, Nashua, New Hampshire, United States of America).

Final harvest sampling

At day 84, all fish were anaesthetised using AQUI-S® at a concentration of 14 mg L^{-1} of seawater and weighed and measured. Four fish from each tank (n = 4 fish $tank^{-1}$; n = 12 tanks; n = 48 fish) were collected whole and stored frozen at -20 °C for analysis of proximate composition and fatty acids profile. Blood from three separate fish per tank (n = 3 fish $tank^{-1}$; n = 12 tanks; n = 36 fish) were collected using a 19 G needle with a 5 mL syringe, and transferred to two separate Vacuette® tubes (Z serum clot activator, EDTA or SST™ II advance tubes). Blood haematocrit was analysed at SARDI SAASC, and blood haematology and biochemistry at IDEXX (Unley, South Australia, Australia). These blood sampled fish were then dissected and the visceral, liver and visceral fat was weighed in order to calculate visceral index (VSI; %), hepatosomatic index (HSI; %) and intraperitoneal fat (%), respectively. In addition, a one cm^2 longitudinally opened midgut section were collected for histology. In brief, midgut samples were fixed in 10% seawater formalin for > 48 h, processed and embedded in paraffin wax. Tissue sections were cut using a microtome and floated onto Starfrost® glass slides and dried for > 24 h at room temperature before being stained. Midgut sections were stained with both hematoxylin and eosin (H and E) and high iron diamine/alcian blue pH 2.5 (HID/AB pH 2.5). Villus height, width and area, total goblet cell number and composition (sialylated and sulphated) were measured.

Apparent digestibility coefficients and nutrient digestion

At the conclusion of the 84 day growth experiment, a two week digestibility experiment was undertaken. After fish (n = 17 $tank^{-1}$) were weighed and measured they were returned to their tank, and fed their respective diet daily to apparent satiation for six days. After six days, fish were anaesthetised using AQUI-S® at a concentration of 20 mg L^{-1} of seawater (to enable handling and faecal matter collection), manually stripped and the faecal matter was collected. In brief, manual stripping involved placing the forefinger and thumb on either side of the fish abdomen at the pelvic fin. Moderate pressure was applied by the forefinger and thumb, and at the same time moved towards the anus, this process was repeated six times. Uncontaminated faecal samples (free from blood, urine and mucus) were collected in a 250 mL container and stored frozen at -20 °C. Fish were then revived in their respective tank, and fed their respective diet daily to apparent satiation for a further six days. Fish were manually stripped again to ensure adequate samples were collected. Faecal material from all fish from a tank from both stripping events were pooled for analysis.

Biochemical and histological analyses

The proximate composition analyses of diets, whole body tissue, and faeces were conducted according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardization (DIN) (2000). A one kg sample of each diet was collected, ground and analysed for proximate

composition (moisture, protein, fat, ash, carbohydrate and energy), fatty acids profile and cholesterol level. In addition, a total of twelve fish (n = 12 fish) at the start of the experiment, and four fish from each tank (n = 3 fish tank^{-1}; n = 12 tanks; n = 36 fish) at the conclusion of the experiment were collected and stored frozen at -20 °C. Whole fish samples were partially thawed, homogenised and analysed for proximate composition (moisture, protein, fat, ash, carbohydrate and energy) and fatty acid profile.

Performance indices

All data reported for each treatment for animal performance were based on the mean of the replicate tanks. All calculations using fish weight and diets were based on wet or as fed values, respectively:

- Weight gain = final weight - initial weight
- Biomass gain (kg tank^{-1}) = (final weight + ∑mortality weight) - (initial weight + ∑replacement weight)
- Specific growth rate (SGR, % d^{-1}) = ([ln final weight - ln initial weight] / d) × 100
- Length growth rate (mm d^{-1}) = (final fish length - initial fish length) / d
- Condition factor = (fish weight [g] / fish length [cm]^3) × 100
- Apparent feed conversion ratio (FCR) = feed consumed / fish weight gain
- Apparent protein deposition = ([final whole protein - initial whole protein] / protein intake) × 100
- Apparent energy deposition = ([final whole energy - initial whole energy] / energy intake) × 100
- Haematocrit count = red blood cell (mm) / total blood (red blood cell and plasma [mm]) 100 × 100
- Visceral index (VSI; %) = wet visceral wt × 100 / final wet fish wt
- Dress-out yield (gutted, head on and gills in) = (100% - VSI [%]) × final wet fish wt
- Hepatosomatic index (HSI; %) = wet liver wt × 100 / final wet fish wt

The apparent digestibility coefficient (ADC) for dietary dry matter, protein and energy was calculated using the following equation and methods described by Maynard and Loosli (1969) and Miegel et al. (2010):

$$ADC\ (\%) = 100 - (100 \times [\%M_{feed} / \%M_{faeces}] \times [\%N_{faeces} / \%N_{feed}])$$

Where M refers to inert marker (acid insoluble ash [AIA]); and N nutrient of interest.

Statistical analyses

IBM SPSS (version 24 for Windows; IBM SPSS Inc., USA) was used for all statistical analyses. Homogeneity of variances and normality among mean values were assessed using Levene's test for equality of variance errors and Shapiro-Wilk test, respectively. To assess the effect of lipid level (30 or 20%) and LYSOFORTE® Liquid inclusion (with or without) on YTK performance, data were analysed using two-factor ANOVA. A significance level of $P < 0.05$ was used for all statistical tests. All values are presented as means ± standard error (SE) of the mean unless otherwise stated.

Results

General observations

The nominal target dietary lipid levels for all diets were achieved (Table 3.1.2.1.1). For Diets 1 and 2, the dietary lipid levels were 30.0 and 28.6%, respectively, with protein levels of 41.9 and 41.0%, respectively (Table 3.1.2.1.1). For Diets 3 and 4, dietary lipid levels were 19.6 and 19.0%, respectively, with protein levels of 47.4 and 47.1%, respectively (Table 3.1.2.1.1).

There were no significant differences in the initial weight and fork length of YTK between treatments ($P > 0.05$; two-factor ANOVA; Table 3.1.2.1.3). The average initial weight and fork length were 1.12 ± 0.11 kg and 426 ± 13 mm (mean ± standard deviation; $n = 276$), respectively. Fish fed actively during the experiment with no apparent differences in behaviour were observed between treatments. The overall mortality for fish in the study was low (3.26%), and was not significantly influenced by dietary lipid level, dietary LYSOFORTE® Liquid inclusion or the interaction between these two factors ($P > 0.05$).

Growth performance

Final weight, biomass gain and SGR of YTK fed the 30% lipid diets were significantly higher than fish fed the 20% lipid diet ($P < 0.05$; two-factor ANOVA; Table 3.1.2.1.3). Dietary LYSOFORTE® Liquid inclusion or the interaction between dietary lipid level and LYSOFORTE® Liquid inclusion did not significantly influence final weight, biomass gain or SGR of fish ($P > 0.05$; two-factor ANOVA; Table 3.1.2.1.3).

Final fork length, length growth rate and final condition factor were not significantly affected by dietary lipid level, dietary LYSOFORTE® Liquid inclusion or the interaction between the two factors ($P > 0.05$; two-factor ANOVA; Table 3.1.2.1.3).

Feed utilisation

Apparent feed consumption (kg tank^{-1}) and feed intake (% BW d^{-1}) were not significantly influenced by dietary lipid level, dietary LYSOFORTE® Liquid inclusion or the interaction between the two factors ($P > 0.05$; two-factor ANOVA; Table 3.1.2.1.3). The fish fed the 30% lipid diets had a significantly lower (improved) apparent FCRs compared to those fed the 20% lipid diets ($P = 0.001$; two-factor ANOVA; Table 3.1.2.1.3). Dietary LYSOFORTE® Liquid inclusion or the interaction between dietary lipid level and LYSOFORTE® Liquid inclusion did not significantly influence the FCR of fish ($P > 0.05$; two-factor ANOVA; Table 3.1.2.1.3).

Whole fish proximate and energy composition

The moisture and protein contents of YTK fed the 20% lipid diets were significantly higher than those fed the 30% lipid diets ($P = 0.001$ and 0.006, respectively; two-factor ANOVA; Table 3.1.2.1.3). Dietary LYSOFORTE® Liquid inclusion or the interaction between dietary lipid level and LYSOFORTE® Liquid inclusion did not significantly influence moisture and protein contents ($P > 0.05$; two-factor ANOVA; Table 3.1.2.1.3). The lipid and energy contents of YTK fed the 30% lipid diets were significantly higher than those fed the 20% lipid diets ($P < 0.001$; two-factor ANOVA; Table 3.1.2.1.3). The lipid and energy contents of YTK were not significantly influenced by dietary LYSOFORTE® Liquid inclusion or the interaction between dietary lipid level and LYSOFORTE® Liquid inclusion ($P > 0.05$; two-factor ANOVA; Table 3.1.2.1.3). The ash and carbohydrate contents of fish were not affected by dietary lipid level, dietary LYSOFORTE® Liquid inclusion or the interaction between the two factors ($P > 0.05$; two-factor ANOVA; Table 3.1.2.1.3).

Nutrient retention

Dietary lipid level, dietary LYSOFORTE® Liquid inclusion or the interaction between the two factors did not significantly affect apparent protein deposition (18.11-20.45%; $P > 0.05$; two-factor ANOVA; Table 3.1.2.1.3). Apparent energy deposition was significantly higher for YTK fed the 30% lipid diets than those fed the 20% lipid diets ($P < 0.001$; two-factor ANOVA; Table 3.1.2.1.3). Dietary LYSOFORTE® Liquid inclusion or the interaction between dietary lipid level and LYSOFORTE® Liquid inclusion did not significantly influence apparent energy deposition ($P > 0.05$; Table 3.1.2.1.3).

Whole fish fatty acid composition

There were numerous significant differences of the whole fish fatty acid levels between dietary treatments ($P < 0.05$; one-factor ANOVA; Table 3.1.2.1.4). In general, a number of these fatty acids mirrored the differences observed in lipid levels between fish fed the different lipid level diets. For example, YTK fed the 30% lipid diet series had significantly higher Palmitic (C16:0), Stearic (C18:0), Myristoleic (C14:1), Palmitoleic (C16:1), Octadecenoic (C18:1n7), Oleic (C18:1n9), Linoleic (C18:2n6) and alpha-Linolenic (C18:3n3) levels than fish fed the 20% lipid diet series ($P < 0.05$; two-factor ANOVA; Table 3.1.2.1.4).

Blood biochemistry and haematology

Blood urea levels and platelets were significantly higher in YTK fed the 20% lipid diets than those fed the 30% lipid diets ($P < 0.05$; two-factor ANOVA; Table 3.1.2.1.5). YTK fed the 30% lipid diets had significantly higher blood alkaline phosphatase, triglyceride, and bile acid levels compared to fish fed the 20% lipid diets ($P < 0.05$; two-factor ANOVA; Table 3.1.2.1.5). These parameters were not significantly influenced by dietary LYSOFORTE® Liquid inclusion or the interaction between dietary lipid level and LYSOFORTE® Liquid inclusion ($P > 0.05$; Table 3.1.2.1.5). Furthermore, dietary lipid level, dietary LYSOFORTE® Liquid, or the interaction between these two factors did not significantly influence other blood haematology and biochemistry parameters measured ($P > 0.05$; two-factor ANOVA; Table 3.1.2.1.5).

Visceral somatic parameters and gastrointestinal tract morphology

Intraperitoneal fat, visceral index (VSI; %) and hepatosomatic index (HSI; %) of YTK fed the 30% dietary lipid diets were significantly higher than those fed the 20% lipid diets ($P = 0.004$, 0.006 and 0.009, respectively; two-factor ANOVA; Table 3.1.2.1.6). Dietary LYSOFORTE® Liquid inclusion and the interaction between dietary lipid level and LYSOFORTE® Liquid inclusion did not significantly influence intraperitoneal fat, VSI and HSI ($P > 0.05$). Dress-out yield (gutted, head on and gills in) was not significantly affected by dietary lipid level, dietary LYSOFORTE® Liquid inclusion or the interaction between the two factors ($P > 0.05$; two-factor ANOVA; Table 3.1.2.1.6).

Villus height, villus width, villus area, total goblet cell number, and sialylated goblet cell number in the midgut were not significantly affected by dietary lipid level, dietary LYSOFORTE® Liquid inclusion or the interaction between the two factors ($P > 0.05$; two-factor ANOVA; Table 3.1.2.1.6). While the number of sulphated goblet cell number was low (<1 cell per millimetre), fish fed diets containing LYSOFORTE® Liquid inclusions had significantly higher number of sulphated goblet cell number than those fed diets with no emulsifier ($P = 0.040$; two-factor ANOVA; Table 3.1.2.1.6). Dietary lipid level and the interaction between dietary lipid level and LYSOFORTE® Liquid inclusion did not significantly influence sulphated goblet cell number ($P > 0.05$; two-factor ANOVA; Table 3.1.2.1.6).

Apparent digestibility coefficients

The apparent dietary digestibility coefficients for crude protein for fish fed the 20% dietary lipids were significantly higher than those fed the 30% lipid diets ($P = 0.020$; two-factor ANOVA; Table 3.1.2.1.6).

Apparent dietary digestibility coefficients for crude protein were not significantly influenced by dietary LYSOFORTE® Liquid inclusion or the interaction between dietary lipid level and LYSOFORTE® Liquid inclusion ($P > 0.05$). Apparent dietary digestibility coefficient for dry matter and gross energy were not significantly affected by dietary lipid level, dietary LYSOFORTE® Liquid inclusion or the interaction between the two factors ($P > 0.05$; two-factor ANOVA; Table 3.1.2.1.6).

Discussion

Sparing dietary protein by increasing dietary energy (lipids or carbohydrates), has been successful in other aquaculture species including Silver Perch (*Bidyanus bidyanus*; Stone et al., 2003), Blunt Snout Bream (*Megalobrama amblycephala*; Li et al., 2012) and more recently in YTK at summer water temperatures (Stone et al., 2016). However, as cultured YTK in South Australia are exposed to seasonally fluctuating water temperatures that reach above 24 °C during summer and below 12 °C in winter (Miegel et al., 2010), there was a need to improve our understanding on the effects of dietary lipid level on the growth, feed utilisation and health of large YTK at winter water temperatures. We also investigated the effects of a dietary LYSOFORTE® Liquid inclusion as an emulsifying agent to improve lipid and energy digestibility and utilisation, feed utilisation, growth, and health of YTK at winter water temperatures.

In a recent study run at SARDI SAASC, Stone et al. (2016) reported superior growth of YTK fed a 28% crude lipid (CL) and 42% crude protein (CP) diet compared to fish fed a 24% CL and 44% CP diet at summer water temperatures (19.5-26.0 °C). The increased dietary lipid levels reported in the study of Stone et al. (2016) were achieved by increasing the dietary poultry oil inclusion levels, while keeping fish oil levels constant to balance LC n-3 PUFA levels. Similar to Stone et al. (2016), in the current study the 30% lipid levels in diets were achieved by increasing the poultry oil, relative to the levels used in the 20% lipid diets, while fish oil levels in both series of diets were held constant to balance LC n-3 PUFA levels. In the current study, it was hypothesised that large YTK at lower winter water temperatures (15.8 °C [range 14-20 °C]) may not be able to efficiently digest and utilise lipid and energy from high lipid/high poultry oil diets due to the following two factors: 1) decreased lipase activity of YTK during periods of cool water temperatures in winter compared to summer; and 2) the increased degree of fatty acid saturation of poultry oil compared to fish oil (Bowyer et al., 2012a; Bowyer et al., 2012b). Although we did not measure lipase activity in the current study, results for dietary energy digestibility, energy deposition, FCR and SGR indicate that large YTK are able to efficiently utilise energy from high lipid (30%)/high poultry oil diets at winter water temperatures. This finding is also consistent with subsequent positive results reported by Stone et al. (Manuscript 3.1.1.2) where high dietary inclusion levels of poultry oil (12.7% total poultry oil; 73.5% of total added lipid) supported good growth, feed utilisation and health of large (2 kg) YTK at cool water temperatures.

Upon closer scrutiny of results in the current study, the increased growth of fish fed the 30% lipid diets was in-part due to increased organ weight (VSI) combined with increased intraperitoneal fat levels, however, dress-out yields (gutted, head on and gills in) remained unchanged. This presents the YTK producer with several important production, processing and marketing decisions. It may be beneficial for producers to feed high fat diets to promote a higher total yield to target markets for whole fish. However, due to increased waste associated with processing, producers may choose to use diets containing lower lipid levels if targeting markets that require processed fish (heads on gills in, or steaks or fillets). Either way, this is ultimately a commercial decision for YTK producers.

Long chain omega-3 polyunsaturated fatty acids are essential for carnivorous fish to promote optimal growth and health, and biological functions (Tocher, 2010), and adequate levels must be provided in the diet. This experiment was undertaken utilising diets which were formulated to contain dietary LC n-3 PUFA level (2.12 g 100 g^{-1}) which promoted optimum growth of large (2.67 kg) YTK at summer water temperatures (Stone et al., Manuscript 3.1.1.1) and also for large (2 kg) fish at winter water temperatures (Stone et al., Manuscript 3.1.1.2). This was achieved in Diets 1, 3 and 4 (2.09-2.20 g 100 g^{-1}; Table 3.1.2.1.1), however, due to constraints with the commercial mixing and diet manufacture, the analysed LC n-3 PUFA level of Diet 2 was higher (2.78 g 100 g^{-1}). Stone et al. (Manuscript 3.1.1.1) reported that there was no benefit of increasing LC n-3 PUFA levels from 2.12 g 100 g^{-1}, up to 2.95 g 100 g^{-1} at summer water temperatures (up to 0.027% BW $d^{-1}$of LC n-3 PUFA; feed intake of ~0.90% BW d^{-1}; initial weight 2.67 kg). Additionally, there were no apparent differences in terms of growth performance,

feed utilisation, nutrient deposition or whole body composition for YTK fed Diet 1 or Diet 2 in the current study. Therefore, it is unlikely that discrepancies in LC n-3 PUFA levels impacted on results in this study.

In terms of fish health, there were no diet related alterations to digestive tract histology, or the majority of blood biochemical and haematological parameters. Interestingly, blood urea levels and platelets were significant higher in YTK fed the 20% lipid diets than those fed the 30% lipid diets, whereas, YTK fed the 30% lipid diets had significantly higher blood alkaline phosphatase, triglyceride, and bile acid levels compared to fish fed the 20% lipid diets. It is not unexpected for the high lipid diets to promote increases in blood triglyceride and bile acid levels, as increased lipid processing and transport is required to metabolise nutrients from the high fat diets. Differences in blood urea levels and platelets in YTK in response to the low lipid diets warrants further investigation.

Lipids need to be emulsified by gastric juice for proper digestion, particularly in species that exhibit higher lipase/esterase activity in the organ that synthesises these enzymes (phylic caeca) compared to the chyme (Perera and Simon, 2014). Lipase activity has been reported to be significantly higher in the phylic caeca of large YTK compared to other regions of the digestive tract (Doherty, 2016; Chapter 2.3.2). Improving emulsification in the gastrointestinal region may be improved by supplying exogenous emulsifying agents through inclusions in the diet (Sugumar et al., 2014; Sugumar et al., 2015). In the current study however, dietary LYSOFORTE® Liquid inclusion (40 mg kg lipid^{-1}) did not improve dietary digestibility, nutrient deposition, SGR or FCR. In contrast, in previous studies dietary inclusions of LYSOFORTE® liquid have been reported to improve growth performance and nutrient digestibility for Tra Catfish (*Pangasianodon hypophthalmus*) and White Shrimp (*Litopenaeus vanammei*) (Sugumar et al., 2014; Sugumar et al., 2015). However, these studies were conducted when the growth rates of these species were high (juvenile fish at optimal temperatures), as a result it is difficult to compare these results to the current study, which was run at sub-optimal winter water temperatures for YTK (Sugumar et al., 2014; Sugumar et al., 2015; Miegel et al., 2010). It should also be noted that the reports by Sugumar et al. (2014) and Sugumar et al. (2015) were non-peer reviewed in-house reports supplied by Kemin Inc., the producer of LYSOFORE Liquid®. There may be potential for the inclusion of emulsifiers at optimal growing temperatures for YTK and further research may be warranted in this area.

Conclusions and Recommendations

In conclusion, YTK grew well on all diets tested in this study. There were no diet related alterations to digestive tract histology, or the majority of blood biochemical and haematological parameters measured. With regard to lipid dietary inclusion level, YTK may be fed a diet containing 30% lipid to improve growth rates and feed utilisation, compared to feeding a 20% dietary lipid level at winter water temperatures. However, in terms of production and dietary lipid levels, target market needs to be considered as the weight increase was in-part related to increase in visceral and intraperitoneal fat weights and not dress-out yield (gutted, head on and gills in). YTK grew well during cooler water temperatures on diets containing high dietary inclusion levels of poultry oil (up to ~18%). This suggests poultry oil is a suitable lipid source to provide energy for commercial YTK diets for large fish at cool water temperatures. Increased poultry oil inclusion, to reduce the reliance on fish oil will lead to immediate diet cost reductions and provide feed manufacturers with greater feed formulating flexibility. We would also recommend future studies investigate the optimum lipid types and levels, and protein to lipid (energy) ratio at both summer and winter water temperatures for large YTK. This information would assist feed companies in providing YTK producers with more cost effective and sustainable diets.

The dietary inclusion of LYSOFORTE® Liquid at a concentration of 40 mg kg lipid^{-1} did not significantly influence the growth or feed utilisation parameters at both lipid levels (30 and 20%) investigated in the current study with large YTK at winter water temperatures. Based on results from the current study, we recommend further research under commercial conditions to investigate the effect of high dietary lipid (30%) and poultry oil levels at winter temperatures before implementing this nutritional strategy on-farm during winter. After discussions with project participants, we do not recommend any further investigation of LYSOFORTE® Liquid for YTK at winter water temperatures. However, further investigation of the potential use of emulsifiers to improve lipid utilisation at optimal growth rates at summer water temperatures may be warranted.

Findings

- Based on results from the current study, Stone et al. (2016) and also from Bowyer et al. (2012a), large YTK appear to be able to efficiently utilise dietary poultry oil at both winter and summer water temperatures, without negatively impacting digestive tract histology or health.
- Levels of up to ~18% poultry oil may be used successfully to provide energy in diets for large YTK. This will reduce our reliance on more valuable and limited supplies of fish oil, and in-turn, improve the economical and sustainable production of YTK
- High lipid diets (up to 30%) led to improvements in weight gain and FCR, while health was not negatively impacted.
- Apparent feed conversion ratio (FCR) was significantly influenced by lipid level at cool winter water temperatures, and was 2.08 in fish fed 30% lipid diets vs 2.37 in fish fed 20% lipid diets.
- An improvement in FCR based on the information provided within this Manuscript, will assist feed manufacturers in formulating commercial diets that achieve one of the overarching goals of the K4P project, which was to provide information to assist producers to achieve FCRs of < 2.2 for large YTK between 1.5-3.5 kg.
- High dietary lipid level also improved whole fish yield but not dress-out yield (gutted, head on and gills in). This has implications for dietary lipid/energy selection for production, processing and market selection.
- The incorporation of LYSOFORTE® Liquid at a concentration of 40 mg kg lipid^{-1} in high or low lipid/energy diets did not improve lipid utilisation for YTK at cool water temperatures.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The authors would also like to acknowledge the support of the South Australian Research and Development Institute (SARDI) and Marine Innovation Southern Australia for their financial support of Assoc. Prof. David Stone and the provision of the SARDI SAASC experimental facilities at West Beach, South Australia. We would also like to thank Dr Matthew Bransden of Skretting Australia for his input into experimental design, experimental diet formulation and pellet kernel manufacture, and Dr Richard Smullen and Dr Michael Salini of Ridley, and Dr Craig Foster and Dr Trent D'Antignana of Clean Seas Seafood for their input into experimental design. Thanks to Mr. Joel Scanlon of Aquafeeds Australia for the use of the facilities for post-pelleting oil application. Thanks to Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd.) for the diagnosis and prescription of skin and gill fluke treatments. We also thank Leigh Kuerschner, Krishna-Lee Currie, Jessica Buss, Nicole Thompson, Filipa Isabel, Samantha Chown, Mark Purvis and Aaron Teoh for their technical assistance during the experiment.

References

Bansemer, M.S., Forder, R.E.A., Howarth, G.S., Suitor, G.M., Bowyer, J., Stone, D.A.J., 2015. The effect of dietary soybean meal and soy protein concentrate on the intestinal mucus layer and the development of sub-acute enteritis in yellowtail kingfish (*Seriola lalandi*) at suboptimal water temperature. Aquaculture Nutrition 21, 300-310.

Booth, M.A., Moses, M.D., Allan, G.L., 2013. Utilisation of carbohydrate by yellowtail kingfish *Seriola lalandi*. Aquaculture 376-379, 151-161.

Bowyer, J.N., Qin, J.G., Smullen, R.P., Stone, D.A.J., 2012a. Replacement of fish oil by poultry oil and canola oil in Yellowtail Kingfish (*Seriola lalandi*) at optimal and suboptimal temperatures. Aquaculture 356-357, 211-222.

Bowyer, J.N., Qin, J.G., Adams, L.R., Thomson, M.J.S., Stone, D.A.J., 2012b. The response of digestive enzyme acitivties and gut histology in yellowtail kingfish (*Seriola lalandi*) to dietary fish oil substitution at different temperatures. Aquaculture 368-369, 19-28.

Bowyer J.N., Qin J.G., Smullen R.P., Adams L.R., Stone D.A.J., 2013. The use of a soy product in Yellowtail Kingfish (*Seriola lalandi*) feeds at different water temperatures: 1. Solvent extracted soybean meal. Aquaculture 384-387, 35-45.

Guiotto, E.N., Tomás, M.C., Diehl, B.W.K., 2015. Sunflower Lecithin. Polar Lipids, 57-75.

Hung, S.S., Berge, G.M., Storebakken, T., 1997. Growth and digestibility effects of soya lecithin and choline chloride on juvenile Atlantic salmon. Aquaculture Nutrition 3, 141-144.

Li, X., Jiang, Y., Liu, W., Ge, X., 2012. Protein-sparing effect of dietary lipid in practical diets for blunt snout bream (*Megalobrama amblycephala*) fingerlings: effects on digestive and metabolic responses. Fish Physiol. Biochem. 38, 529-541.

Perera, E., Simon, C., 2014. Digestive physiology of spiny lobsters: implications for formulated diet development. Reviews in Aquaculture 6, 1-19.

Philips, A.M., 1972. Calorie and energy requirements. In: J.E. Halver (ed.) Fish Nutrition, pp. 2-29. Academic Press, London.

Sheppard, M., 2004. A Photographic Guide to Diseases of Yellowtail (*Seriola*) Fish. Quadra Printers Ltd, Nanaimo, BC, Canada.

Stone, D.A.J., Allan, G.L., Anderson, A.J., 2003. Carbohydrate utilization by juvenile silver perch, *Bidyanus bidyanus* (Mitchell). III. The protein-sparing effect of wheat starch-based carbohydrates. Aquaculture Research 34, 123-134.

Stone, D.A.J., D'Antignana, T., Bansemer, M.S., 2016. Final Report. Refining Yellowtail Kingfish feeds and feed management. Prepared by the South Australian Research and Development Institute (Aquatic Sciences), Adelaide. AS-CRC Project No. 2013/730. 144pp.

Sugumar, C., Bindhu, L.V., Chandrasekar, K.V., 2014. Lysoforte saves feed cost for aqua producers by partially replacing soy-lecithin in shrimp diets. Technical report produced by Kemin (Asia Pacific Regional Headquarters, Singapore, Singapore). Report no. TL-14-00026. 8pp.

Sugumar, C., Bindhu, L.V., Chandrasekar, K.V., 2015. Partial replacement of fish oil in tra catfish (*Pangasianodon hypophthalmus*) diet by different levels of lysoforte® dry. Technical literature produced by Kemin (Asia Pacific Regional Headquarters, Singapore, Singapore). Report no. TL-15-00011. 6pp.

Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquaculture Research 41, 717-732.

Ward, L.R., Carter, C.G., 2009. An evaluation of the nutritional value of alternative lipid sources to juvenile southern rock lobster, *Jasus edwardsii*. Aquaculture 296, 292-298.

Zhao, P.Y., Li, H.L., Hossain, M.M., Kim, I.H., 2015. Effect of emulsifier (lysophospholipids) on growth performance, nutrient digestibility and blood profile in weanling pigs. Animal Feed Science and Technology 207, 190-195.

Table 3.1.2.1.1. Analysed composition of the experimental diets containing high or low lipid levels, with or without an emulsifier (LYSOFORTE® Liquid).

Diet[1]	**1**	**2**	**3**	**4**
Nominal dietary lipid level (%)	30	30	20	20
Dietary emulsifier (mg lipid kg^{-1})	0	40	0	40
Analysed proximate composition (g 100 g^{-1})				
Moisture	4.8	7.7	6.6	7.5
Crude protein	41.9	41.0	47.4	47.1
Crude lipid	30.0	28.6	19.6	19.0
Ash	9.7	9.7	11.0	10.9
Carbohydrate[2]	13.6	13.0	15.4	15.5
Gross energy (MJ kg^{-1})	20.50	19.80	17.90	17.70
Analysed fatty acids (mg 100 g^{-1})				
Saturated Fatty Acids				
C4:0 Butyric	<10	<10	<10	<10
C6:0 Caproic	<10	<10	<10	<10
C8:0 Caprylic	<10	<10	<10	<10
C10:0 Capric	<10	<10	<10	<10
C12:0 Lauric	<10	<10	<10	<10
C13:0 Trisdecanoic	<10	<10	<10	<10
C14:0 Myristic	660	810	570	560
C15:0 Pentadecanoic	69	80	58	57
C16:0 Palmitic	6370	6010	4030	3920
C17:0 Margaric	70	110	74	66
C18:0 Stearic	1920	1790	1320	1300
C20:0 Arachidic	54	44	43	46
C22:0 Docosanoic	31	32	33	34
C24:0 Tetracosanoic	22	22	16	26
Mono-unsaturated Fatty Acids				
C10:1 Decenoic	<10	<10	<10	<10
C14:1 Myristoleic	62	56	38	38
C15:1 Pentadecenoic	<10	<10	<10	<10
C16:1 Palmitoleic	1680	1660	1080	1030
C17:1 Heptadecenoic	85	120	90	83
C18:1n-6 Octadecenoic	<10	<10	<10	<10
C18:1n-7 Octadecenoic	770	750	510	490
C18:1n-9 Oleic	10780	9330	5940	5720
C20:1n-9 Eicosenoic	180	180	130	120
C20:1n-11,13 Eicosenoic	57	59	43	42
C20:1 Eicosenoic (total)	240	240	170	160
C22:1n-9 Docosenoic	20	24	20	20
C22:1n-11,13 Docosenoic	<10	<10	<10	<10
C24:1 Tetracosenoic	45	59	48	49
Poly-unsaturated Fatty Acids				
C18:2n-6 Linoleic	3270	2850	1940	1910
C18:2n 9c 11t Octadecadienoic Conjugate	<10	<10	<10	<10
C18:3n-6 Gamma Linolenic	46	44	28	26
C20:2n-6 Eicosadienoic	35	22	23	27
C20:3n-6 Dihomo-gamma-linoleic	39	34	30	28
C20:4n-6 Arachidonic	180	190	160	160
C22:4n-6 Docosatetraenoic	35	48	51	38
C22:5n-6 Docosapentaenoic	46	61	43	48
C18:3n-4 Octadectrenoic acid	<10	<10	<10	<10
C18:3n-3 Alpha Linolenic	530	480	330	320
C18:4n-3 Steridonic	150	190	140	130
C20:3n-3 Eicosatrienoic	<10	<10	<10	<10
C20:4n-3 Eicosatetraenoic	75	93	79	74
C20:5n-3 Eicosapentanaeoic	1050	1450	1090	1040
C21:5n-3 Heneicosapentaenoic	<10	<10	<10	<10
C22:5n-3 Docosapentaenoic	170	220	180	170
C22:6n-3 Docosahexaenoic	870	1110	930	900
∑LC n3 PUFA	2090	2780	2200	2110
Other (mg 100 g^{-1})				
Cholesterol	293	297	277	275

[1] The kernels, fish oil and poultry oil used to make diets were supplied by Skretting Australia (Cambridge, Tasmania, Australia).
[2] Carbohydrate = 100 - (moisture + lipid + protein + ash).

Table 3.1.2.1.2. Summary of measured water quality parameters throughout the 84 day study.

Item[1]	Temperature (°C)	Dissolved oxygen (mg L^{-1})	Dissolved oxygen (% saturation)	pH	Salinity (mg L^{-1})	Ammonia (ppm)	CO_2 (mg L^{-1})
Mean	15.8 ± 1.4	7.8 ± 0.4	100.2 ± 3.9	7.8 ± 0.1	38 ± 1	0.00 ± 0.00	1 ± 0
Range	14.0 - 20.0	6.6 - 9.4	88.0 - 118.0	7.5 - 8.3	36 - 38	0.00 - 0.00	0 - 2

Values means ± standard deviation.

Table 3.1.2.1.3. Growth performance, feed utilisation, proximate composition and nutrient retention of Yellowtail Kingfish fed different dietary lipid levels, with or without an emulsifier for 84 days at winter water temperatures.

Diet[1]	1	2	3	4			
Nominal dietary lipid level (%)	30		20		Two factor ANOVA[2]		
Dietary emulsifier (mg lipid kg^{-1})	0	40	0	40	Lipid level (A)	Emulsifier (B)	A × B
Growth performance							
Initial weight (kg)	1.12±0.01	1.13±0.00	1.12±0.01	1.12±0.00	0.837	0.929	0.846
Final weight (kg)	1.48±0.02	1.51±0.01	1.46±0.02	1.44±0.02	0.031 (30% > 20%)	0.796	0.273
Biomass gain (kg tank^{-1})[4]	8.44±0.62	8.83±0.27	7.87±0.27	7.33±0.53	0.038 (30% > 20%)	0.866	0.328
SGR (% d^{-1})	0.34±0.02	0.35±0.01	0.32±0.01	0.30±0.02	0.048 (30% > 20%)	0.756	0.355
Initial fork length (mm)	426±1	426±1	424±2	426±1	0.515	0.352	0.709
Final fork length (mm)	452±3	457±1	453±1	452±1	0.529	0.543	0.117
Length growth rate (mm d^{-1})	0.31±0.03^{b}	0.36±0.02^{a}	0.35±0.01^{a}	0.31±0.02^{b}	0.750	0.750	0.050
Final Condition factor	1.61±0.03	1.59±0.01	1.57±0.00	1.56±0.02	0.079	0.666	0.682
Feed utilisation (as fed)							
Apparent feed consumption (kg tank^{-1})	17.64±0.95	18.16±0.29	18.10±0.10	17.79±0.47	0.935	0.846	0.474
Apparent feed intake (% BW d^{-1})	0.78±0.04	0.79±0.01	0.80±0.01	0.79±0.01	0.408	1.000	0.651
Apparent FCR	2.10±0.05	2.06±0.04	2.30±0.07	2.44±0.12	0.001 (30% < 20%)	0.380	0.064
Whole proximate composition (wet basis)[3]							
Moisture (%)	64.7±0.6	63.8±0.2	66.7±0.8	66.5±0.2	0.001 (30% < 20%)	0.296	0.513
Protein (%)	18.9±0.3	19.2±0.4	20.0±0.2	20.1±0.3	0.006 (30% < 20%)	0.437	0.831
Lipid (%)	14.2±0.5	14.5±0.4	11.9±0.3	11.9±0.1	<0.001 (30% > 20%)	0.578	0.658
Ash (%)	2.2±0.0	2.1±0.0	2.0±0.1	2.1±0.1	0.237	0.431	0.237
Carbohydrate (%)	<1.5	<1.5	<1.5	<1.5	NA	NA	NA
Energy (MJ kg^{-1})	8.55±0.07	8.65±0.10	7.79±0.10	7.88±0.04	<0.001 (30% > 20%)	0.259	0.937
Nutrient retention[4]							
Apparent PD	18.11±1.70	20.45±1.76	18.52±0.26	18.31±0.66	0.513	0.411	0.345
Apparent ED	35.10±1.23	37.31±1.13	29.81±1.37	30.26±0.42	<0.001 (30% > 20%)	0.250	0.447

[1] Values are mean ± SE; $n = 3$.

[2] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$), NA not statistically analysed.

[3] Initial fish proximate composition (wet basis): Moisture 68.9%, protein 19.9%, lipid 8.2%, ash 2.3%, carbohydrate < 1.5%, energy 6.42 MJ kg^{-1}.

[4] ED = energy deposition; PD = protein deposition.

Table 3.1.2.1.4. Fatty acid composition (mg 100 g^{-1}) of whole Yellowtail Kingfish fed different dietary lipid levels, with or without an emulsifier for 84 days at winter water temperatures.

Diet[1,2]	1	2	3	4			
Nominal dietary lipid level (%)	30		20		Two factor ANOVA[3]		
Dietary emulsifier (mg lipid kg^{-1})	0	40	0	40	Lipid level (A)	Emulsifier (B)	A × B
Saturated Fatty Acids							
C14:0 Myristic	343±13[b]	390±10[a]	350±15[b]	340±6[b]	0.100	0.155	0.041
C15:0 Pentadecanoic	44±4	47±2	45±2	45±2	0.782	0.563	0.706
C16:0 Palmitic	2157±91	2260±89	1943±80	1903±33	0.003 (30% > 20%)	0.688	0.379
C17:0 Margaric	47±4	52±3	50±2	55±5	0.490	0.184	0.965
C18:0 Stearic	783±32	817±32	713±32	697±7	0.005 (30% > 20%)	0.768	0.394
C20:0 Arachidic	24±3	27±2	26±1	24±1	0.777	0.621	0.226
C24:0 Lignoceric	29±1	35±0	30±1	32±0	0.152	0.006 (0 < 40)	0.053
Mono-unsaturated Fatty Acids							
C14:1 Myristoleic	19±0	20±1	17±0	18±1	0.001 (30% > 20%)	0.078	0.715
C16:1 Palmitoleic	857±28	877±17	710±31	703±15	<0.001 (30% > 20%)	0.776	0.588
C17:1 Heptadecenoic	34±1[b]	42±1[a]	34±1[b]	35±1[b]	0.002	0.001	0.002
C18:1n7 Octadecenoic	430±15	447±12	370±17	370±6	<0.001 (30% > 20%)	0.534	0.549
C18:1n9 Oleic	4700±219	4517±124	3490±165	3473±110	<0.001 (30% > 20%)	0.532	0.618
C20:1 Eicosenic	293±34	310±12	260±46	247±41	0.164	0.962	0.684
C20:1n9 Eicosenoic	170±15	180±6	163±9	157±7	0.135	0.867	0.423
C20:1n-11,13 Gadoleic	127±18	133±7	130±6	123±9	0.743	1.000	0.554
C22:1n9 Docosenoic	21±3	23±1	22±1	20±1	0.630	0.927	0.327
C24:1 Nervonic	51±8	49±4	44±1	43±3	0.209	0.730	0.896
Poly-unsaturated Fatty Acids							
C18:2n6 Linoleic	1410±61	1333±29	1047±37	1070±35	<0.001 (30% > 20%)	0.552	0.270
C18:3n3 alpha-Linolenic	227±9	220±6	177±7	173±3	<0.001 (30% > 20%)	0.434	0.803
C18:4n3 Steridonic	93±4[b]	110±0[a]	93±3[b]	95±2[b]	0.016	0.007	0.023
C20:2n6 Eicosadienoic	31±2	31±1	28±2	28±1	0.024	0.898	0.904
C20:3n6 Eicosatrienoic	21±1	23±1	19±1	19±1	0.011	0.519	0.195
C20:4n3 Eicosatetraenoic	173±20	190±6	187±9	180±10	0.896	0.680	0.377
C20:4n6 Arachidonic	137±7	153±3	140±6	137±3	0.282	0.278	0.081
C20:5n3 Eicosapentaenoic	570±21[b]	683±13[a]	570±20[b]	580±6[b]	0.013	0.005	0.013
C22:4n6 Docosatetraenoic	55±6	57±2	55±2	54±3	0.698	0.782	0.662
C22:5n3 Docosapentaenoic	247±17	273±9	257±15	247±7	0.528	0.542	0.176
C22:6n3 Docosahexaenoic	1060±95	1183±35	1107±41	1097±47	0.747	0.347	0.293
∑LC n3 PUFA	1877±129	2140±57	1933±72	1923±54	0.402	0.189	0.140

[1] Values are mean ± SE; $n = 3$.

[2] Values for the following fatty acids < 10 mg 100 g-1 and were excluded from the table: C4:0 Butyric, C6:0 Caproic, C8:0 Caprylic, C10:0 Capric, C12:0 Lauric, C13:0 Trisdecanoic, C22:0 Behenic, C10:1 Decenoic, C15:1 Pentadecanoic, C18:3n6 gamma-Linolenic, C20:3n3 Eicosatrienoic.

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$). NA = not analysed.

Table 3.1.2.1.5. Blood haematology and biochemistry of Yellowtail Kingfish fed different dietary lipid levels, with or without an emulsifier for 84 days at cool water temperatures.

Diet[1]	1	2	3	4			
Nominal dietary lipid level (%)	30		20		Two factor ANOVA[2]		
Dietary emulsifier (mg lipid kg^{-1})	0	40	0	40	Lipid level (A)	Emulsifier (B)	A × B
Biochemistry[3]							
Sodium (mmol L^{-1})	195±1	192±1	194±3	195±1	0.488	0.615	0.418
Potassium (mmol L^{-1})	4.1±0.2	4.6±0.2	4.8±0.5	5.6±0.5	0.055	0.078	0.755
Urea (mmol L^{-1})	6.0±0.7	6.7±0.3	8.0±1.0	8.1±0.7	0.029 (30% < 20%)	0.538	0.718
Creatinine (mmol L^{-1})	0.03±0.01	0.03±0.00	0.02±0.00	0.03±0.01	0.395	0.784	0.430
Calcium (mmol L^{-1})	2.9±0.1	2.9±0.0	3.0±0.2	3.0±0.0	0.197	0.669	1.000
Protein (g L^{-1})	34±2	34±1	34±1	36±0	0.317	0.481	0.495
Albumin (g L^{-1})	10±1	9±0	9±0	10±0	0.664	0.678	0.217
Globulin (g L^{-1})	24±1	25±1	24±2	26±0	0.676	0.317	0.691
Total Bilirubin (mmol L^{-1})	2±0	5±2	2±0	2±0	0.265	0.176	0.257
ALT (IU L^{-1})	9±1	12±3	9±2	12±3	0.837	0.292	0.949
ALP (IU L^{-1})	35±6	40±3	30±2	27±0	0.022 (30% > 20%)	0.820	0.307
Magnesium (mmol L^{-1})	1.9±0.0	1.8±0.1	1.8±0.1	1.9±0.1	0.729	0.742	0.189
Cholesterol (mmol L^{-1})	4.3±0.3	5.0±0.1	4.8±0.3	4.7±0.3	0.712	0.259	0.175
Triglyceride (mmol L^{-1})	5.0±0.5	5.1±0.3	4.1±0.4	3.4±0.1	0.007 (30% > 20%)	0.424	0.296
Bile Acids (mmol L^{-1})	29.9±12.8	16.9±5.2	9.7±3.2	5.0±1.4	0.047 (30% > 20%)	0.230	0.574
Haematology[4]							
RBC (×10^{12})	2.3±0.2	2.0±0.1	1.8±0.2	2.3±0.1	0.692	0.555	0.039*
HGB (g L^{-1})	95±5	86±12	93±2	101±3	0.346	0.923	0.237
PCV (L L^{-1})	0.42±0.03	0.38±0.03	0.36±0.06	0.43±0.02	0.849	0.718	0.213
MCV (fl)	181±2	176±2	178±7	182±3	0.626	0.938	0.291
MCH (pg)	42±2	50±6	51±8	45±3	0.752	0.764	0.210
MCHC (g L^{-1})	232±12	287±36	282±57	245±23	0.924	0.805	0.232
WBC (×10^{9})	5.5±0.2	5.3±0.1	5.6±0.1	5.5±0.1	0.318	0.460	1.000
Granulocytes (%)	8±1	7±0	9±1	8±0	0.231	0.219	0.461
Lymph (%)	91±1	91±1	90±1	90±0	0.082	1.000	1.000
Mono (%)	1±0	2±1	1±1	2±0	0.700	0.065	0.715
Eosin (%)	0±0	0±0	0±0	0±0	-	-	-
Baso (%)	0±0	0±0	0±0	0±0	-	-	-
Platelets (×10^{9})	17±3	16±0	26±1	22±0	0.001 (30% < 20%)	0.236	0.527

[1] Values are mean ± SE; n = 3. SE less than 0.01 are reported as "0.00".
[2] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$). * A significant interaction was detected; however, no differences between treatments were observed using post-hoc tests (Student-Newman-Keuls tests).
[3] ALT = alanine aminotransferase; ALP = alkaline phosphatase;
[4] Blood smear content: red and white cell normal; Baso = basophil; Eosin = eosinophil; HGB = haemoglobin; Lymph = lymphocytes; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; MCV = mean corpuscular volume; Mono = monocytes; PCV = packed cell volume; RBC = red blood cell count; WBC = white blood cell count.

Table 3.1.2.1.6. Visceral somatic parameters, midgut morphology and apparent digestibility coefficient for Yellowtail Kingfish fed different dietary lipid levels, with or without an emulsifier for 84 days at winter water temperatures.

Diet[1]	1	2	3	4			
Nominal dietary lipid level (%)	30		20		Two factor ANOVA[2]		
Dietary emulsifier (mg lipid kg^{-1})	0	40	0	40	Lipid level (A)	Emulsifier (B)	A × B
Visercal somatic parameters							
Intraperitoneal fat (%)	2.53±0.34	2.41±0.32	1.53±0.23	1.25±0.38	0.004 (30% > 20%)	0.521	0.802
Viscerosomatic index (VSI; %)	7.83±0.30	8.18±0.53	6.73±0.25	6.83±0.41	0.006 (30% > 20%)	0.564	0.758
Hepatosomatic index (HSI; %)	1.44±0.05	1.35±0.12	1.15±0.06	1.12±0.11	0.009 (30% > 20%)	0.485	0.759
Dress-out yield (kg)	1.37±0.02	1.39±0.00	1.37±0.01	1.34±0.01	0.108	1.000	0.093
Midgut morphology							
Villus height (µm)	1109.9±13.6	1204.4±56.9	1147.7±44.7	1165.3±68.5	0.990	0.257	0.466
Villus width (µm)	518.7±31.2	517.1±19.9	467.7±29.0	524.7±85.3	0.655	0.533	0.564
Villus area ($µm^2$)	264970±9633	277956±24144	238186±7576	277604±62780	0.686	0.420	0.709
Total goblet cell number[3]	141.3±22.1	124.2±14.4	121.9±12.2	156.0±13.8	0.728	0.621	0.150
Sialylated goblet cell number[4]	141.0±22.1	123.3±14.3	121.6±12.1	155.5±13.9	0.722	0.637	0.147
Sulphated goblet cell number[5]	0.3±0.0	0.8±0.2	0.3±0.0	0.5±0.2	0.376	0.040 (0 < 40)	0.199
Apparent digestibility coefficient (ADC; %)							
Dry matter	36.2±6.6	42.4±4.0	53.9±2.2	46.1±7.6	0.077	0.896	0.241
Protein	65.2±3.2	67.8±3.2	77.4±1.2	72.2±3.9	0.020 (30% < 20%)	0.697	0.240
Energy	48.6±4.4	54.6±2.0	56.9±3.2	53.0±7.0	0.461	0.832	0.304

[1] Values are mean ± SE; $n = 3$.
[2] A significance level of $P < 0.05$ was used for all statistical tests.
[3] Expressed as the sum of goblet cells observed in samples stained with PAS/AB pH 2.5 and HID/AB pH 2.5 per millimetre villus height.
[4] Expressed as total number of sialylated goblet cells per millimetre height.
[5] Expressed as total number of sulphated goblet cells per millimetre villus height.

3.1.2.2. Manuscript - Dietary lipid and protein levels influences the growth and feed utilisation in large Yellowtail Kingfish (Seriola lalandi) at warm water temperatures.

David A.J. Stone [a, b, c, d], Matthew S. Bansemer [a], Krishna-Lee Currie [a], Paul Skordas [a], Michael Salini[e]

[a] South Australian Research and Development Institute, South Australian Aquatic Sciences Centre, West Beach, SA 5024, Australia
[b] Flinders University, College of Science and Engineering, GPO Box 2100, Adelaide, SA 5001, Australia
[c] University of Adelaide, School of Animal and Veterinary Sciences, Roseworthy, SA 5371, Australia
[d] Marine Innovation Southern Australia
[e] Ridley, Narangba, QLD 4504, Australia

This manuscript may be referenced as: Stone, D.A.J., Bansemer, M.S., Currie, K.L., Skordas, P. and Salini, M. (2019). 3.1.2.2. Manuscript - 3.1.2.2. Dietary lipid and protein levels influences the growth and feed utilisation in large Yellowtail Kingfish (*Seriola lalandi*) at warm water temperatures (FS3; Output 2b). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.84-105.

Abstract

Growth is initially limited by dietary protein availability and digestion, and also amino acid profile and availability. Yellowtail Kingfish (*Seriola lalandi*; YTK) have a fast growth rate, and as a result, a high energy demand that should ideally be satisfied by dietary lipids. The aim of this experiment was to determine the optimum dietary protein and lipid levels for large YTK production at warm water temperatures. In this 84 day experiment (3 × 2 factorial design), the growth, feed utilisation and health of YTK (2.13 kg) fed three nominal crude protein (CP) levels (40, 44 and 48%) at two nominal gross energy (GE) levels (20 MJ kg^{-1} [25% crude lipid; CL] and 21 MJ kg^{-1} [30% CL]) were investigated. The health of fish was not significantly affected by CP or CL levels. Generally, fish fed the 25% CL diets grew better than those fed the 30% CL diets. The specific growth rate of fish was significantly affected by the interaction between dietary CP and CL level. This interaction was primarily driven by the significant increase in growth for fish fed Diet 1 (40% CP × 25% CL) and Diet 2 (44% CP × 25% CL) compared to their respective 30% CL diets (Diet 4 [40% CP × 30% CL] and Diet 5 [44% CP × 30% CL]). The growth of fish fed Diet 3 (48% CP × 25% CL) and Diet 6 (48% CP× 30% CL) was not significantly different. Apparent feed conversion ratio (FCR) was not significantly influenced by diet, but tended to be improved in fish fed the 44% CP × 25% CL diet, compared to other diets. Typically, fish fed the 25% CL diet series consumed more feed than those fed the 30 CL diet series. Fish fed the 25% CL series increased their feed intake with decreasing dietary CP level, to potentially compensate for reduced dietary CP. In contrast, fish fed the 30% CL series did not. Crude protein intake was higher when fish were fed the 48% CP diets, and was reduced when fed the 40% and 44% CP diets. This response was more pronounced in the 30% CL diet series. Based on these results, we recommend that diets for 2.0-3.5 kg YTK at warm water temperatures contain 44% CP (37% digestible protein [DP]), 25% CL (24% digestible lipid [DL]), 20.5 MJ kg^{-1} GE (16.9 MJ kg^{-1} digestible energy [DE]) and a CP to GE ratio of 21.6 g MJ^{-1} (21.8 g DP MJ^{-1} DE). In terms of actual protein and energy intake required to promote optimal growth, there appears to be a "sweet spot" of 5.2 g CP^{-1} kg BW^{-1} d^{-1} (4.5 g DP^{-1} kg BW^{-1} d^{-1}) and 242 KJ^{-1} GE kg BW^{-1} d^{-1} (207 KJ^{-1} DE kg BW^{-1} d^{-1}) for large YTK (2-3 kg). High dietary lipid level (30% CL) impacted feed, protein and energy intake, feed utilisation and ultimately growth. Further research in pilot scale commercial trials are needed before implementing diets containing the recommended levels of protein, lipid and energy on-farm.

Introduction

In Australia, Yellowtail Kingfish (*Seriola lalandi*; YTK) are cultured in South Australia, Western Australia and New South Wales. YTK are well suited to aquaculture due to their excellent flesh quality and fast growth rate and may reach > 3 kg in 12-18 months (Stone et al., 2016). Dietary protein and lipid play major roles in the nutritional value of aquafeeds. Growth is initially limited by dietary protein availability and digestion, and also amino acid profile and availability. However, protein is an expensive dietary macronutrient. Dietary protein levels of 45-55% are considered ideal for optimal growth in the grow-out phase of sub-adult temperate marine species such as juvenile YTK, European Seabass (*Dicentrarchus labrax*), Japanese Yellowtail (*Seriola quinqueradiata*), Mediterranean Yellowtail (*Seriola dumerilii*) and Gilthead Seabream (*Sparus aurata*) (Alvarez et al. 1998; Peres and Oliva-Teles 1999; Koven 2002; Masumoto 2002; Booth et al., 2010). When provided in excess to their requirements, fish are able to deaminate protein to supply energy for metabolism rather than tissue growth. However, as protein is expensive this should be avoided as it increases feed costs, reduces protein deposition and growth and increase ammonia production (Molina-Poveda, 2016). To reduce protein deamination, the minimum dietary protein level required to promote optimal growth should be provided to ensure protein is used for tissue growth rather than energy. To spare protein and reduce diet costs, dietary energy should be provided in the form of either lipid or carbohydrate (NRC, 2011), the degree of which is dependent on the target species. YTK have a limited ability to digest dietary carbohydrates for energy, and reduced growth performance is typically observed at dietary inclusions of > 10% carbohydrate (Booth et al., 2013). Therefore, dietary lipids, rather than carbohydrate, should ideally be used to satisfy the energy requirements of YTK (Booth et al., 2010; Stone et al., 2016).

When formulating diets to provide optimum levels of protein and energy there are several important production issues to consider. A diet with deficient levels of energy will result in decreased growth due to a proportion of the protein being used for energy rather than body protein synthesis. On the other hand, a diet with excess energy may result in reduced feed intake and also excessive fat deposition in fish (Talbot et al., 2000; Masumoto, 2002; Johansen et al., 2003; Oliva-Teles, 2012). Previous research, on other marine fish species and the closely related Japanese Yellowtail, indicated that dietary crude lipid (CL) levels of 15-20% were considered ideal for optimal growth (Peres and Oliva-Teles, 1999; Koven, 2002; Masumoto, 2002). Currently commercial diets for Australian YTK contain ~25% lipid (Stone et al., 2016). However, Pirozzi and Booth (2009) suggested that due to their fast growth rate and high energy demand, YTK may have the ability to utilise higher lipid (energy) diets.

There is a trend in aquaculture to use high lipid, energy dense, diets to improve growth rates and feed conversion ratios and ultimately productivity (NRC, 2011). The use of such diets with fish is size and species dependent. Studies on salmonids such as Rainbow Trout (*Oncorhynchus mykiss*) and Atlantic Salmon (*Salmo salar*), have indicated that high lipid intake results in increases lipid content in muscle and intestinal tissue (Jobling, 1998; Refstie et al., 2001; Jobling et al., 2002). However, Atlantic Salmon were able to tolerate higher levels of dietary lipid than Rainbow Trout, as measured by higher fillet lipid levels and reduced levels of visceral fat. Stone et al. (2016) reported that large YTK fed a low protein (42% crude protein [CP]), high lipid diet (28% CL) at warm water temperatures (19.5-26.0 °C) exhibited higher energy deposition and tended to have higher body fat levels, suggesting increased levels of muscle and visceral fat compared to those fed a 44% CP 24% CL diet. More recently, results from Bansemer et al. (Manuscript 3.1.2.1) have indicated large YTK exhibited increased visceral fat when fed high lipid diets at cool water temperatures. Increased fat deposition in visceral tissues may ultimately lead to reductions in production yields during processing (Talbot et al., 2000; Weihe et al., 2018; Bansemer et al., Manuscript 3.1.2.1).

Typically, in a range of fish species the dietary protein to energy ratio decreases inversely with increasing body weight (NRC, 2011; Molina-Poveda, 2016). This also appears to be the case for small YTK (size range: 50 g to < 2 kg) at warm water temperatures (21-24 °C) (Booth et al., 2010). As such, it was hypothesised that YTK > 2 kg would continue to follow this trend. Further research to investigate dietary protein and lipid levels required to promote optimum growth of large YTK at warm water temperatures is needed.

Aim

The aim of this experiment was to determine the optimum dietary protein and lipid levels for large YTK production at warm water temperatures.

Methods

Experimental design and diets

This study used a 3 × 2 factorial design, with crude protein level as the first factor (40, 44 and 48%) and crude lipid (25% CL [20 MJ kg^{-1}] and 30% CL [21 MJ kg^{-1}]) level as the second factor. The six diets were formulated with input from all project participants, and then manufactured by Ridley (Narangba, Queensland, Australia). Diets were formulated to contain highly palatable and digestible ingredients at realistic commercial inclusion levels. Analysed diet composition is displayed in Table 3.1.2.2.1 and Table 3.1.2.2.2. Fish were fed to apparent satiation daily at 09:00 h, which involved feeding fish for four min $tank^{-1}$ or until a feed refusal response was observed. Feed input was measured daily. Tanks were cleaned every second day.

Experimental fish

Experimental work was conducted in the pool-farm facility at the South Australian Research and Development Institute, South Australian Aquatic Science Centre (SARDI SAASC; West Beach, South Australia, Australia). YTK (n = 360; 2.13 ± 0.23 kg; 504 ± 19 mm fork length; mean ± standard deviation) were obtained from Clean Seas Seafood (Port Lincoln, South Australia, Australia). Upon arrival at the SARDI SAASC facility, YTK were transferred to 5000 L tanks supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated, aerated sea water at ambient temperature and held for ~1 month. During this period fish were fed a standard Ridley Pelagica diet (CP 46%; CL 24%; GE 19.30 MJ kg^{-1}).

Gill fluke treatment

Upon arrival at SARDI SAASC, YTK were inspected, and were observed to have a low burden of gill flukes (*Zeuxapta seriola*). Treatment was deemed necessary, and was prescribed by Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd., Ballina, New South Wales, Australia).

Experimental stocking and intermediate weight checks

At the commencement of the experiment (January 2018), YTK were anaesthetised in 5000 L tanks (total water volume 2500 L) using AQUI-S® (AQUI-S® New Zealand Ltd., Lower Hutt, New Zealand) at a concentration of 14 mg L^{-1} of seawater. Twenty fish were removed from their tank, measured, weighed and stocked into one of the three replicate 5000 L tanks per treatment combination (n = 6 diet treatments; n = 18 tanks).

Tanks were supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated sea water at ambient temperature. All tanks were supplied with aeration and oxygenation throughout the study.

As required, mortalities were removed form the tanks, weighed, measured and recorded and replaced with tagged fish (T-tags) of a similar weight. Tagged fish were included in biomass calculations for FCR (see Performance indice section), but excluded from all other analyses.

At 4 weeks and 8 weeks post-stocking, all fish were anaesthetised using AQUI-S® at a concentration of 14 mg L^{-1} of seawater. YTK were weighed and measured, and returned back to their respective tanks. Feeding commenced the following day.

Water quality analyses

Water quality parameters were measured daily at 12:30 h, and maintained at appropriate levels for acceptable growth of YTK throughout the study (Table 3.1.2.2.3). Water temperature was measured using a thermometer (Figure 3.1.2.2.1). Dissolved oxygen (mg L^{-1} and % saturation) was measured using a dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark). The pH was measured daily using a meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, Illinois, United States of America). Salinity (g L^{-1}) was measured weekly using a portable salinity refractometer (model RF20, Extech Instruments, Nashua, New Hampshire, United States of America).

Final harvest sampling

At day 84, all fish were anaesthetised using AQUI-S® at a concentration of 14 mg L^{-1} of seawater and weighed and measured. Three fish from each tank (n = 3 fish $tank^{-1}$; n = 18 tanks; n = 54 fish) were collected whole and stored frozen at -20 °C for biochemical analysis. Blood samples from three separate fish per tank (n = 3 fish $tank^{-1}$; n = 18 tanks; n = 54 fish) were collected using a 19 G needle with a 5 mL syringe. Blood samples were transferred to two separate Vacuette® or BD vacutainer® tubes (Z serum clot activator or EDTA tubes). Blood serum was analysed for biochemistry parameters and whole blood was analysed for blood haematology parameters conducted by IDEXX (Unley, South Australia, Australia). These blood sampled fish were then dissected and the visceral organs, liver and visceral fat were weighed in order to calculate visceral index (VSI; %), hepatosomatic index (HSI; %) and intraperitoneal fat (%), respectively. The stomach from these fish were opened longitudinally, and were subjectively scored for gastric dilation (Chown, 2015). Briefly, Stage 0 is defined as having pronounced/well defined folds throughout the pylorus, anterior and distal stomach, while Stage 1 is defined as having minimal or absent folds throughout the pylorus and anterior stomach, but has pronounced/well defined folds in the distal stomach (Chown, 2015). In addition, one cm^2 longitudinally opened hindgut sections were collected from blood sampled fish for histology. In brief, hindgut samples were fixed in 10% seawater formalin for > 48 h, processed and embedded in paraffin wax. Tissue sections were cut using a microtome and floated onto Starfrost® glass slides and dried for > 24 h at room temperature before being stained with hematoxylin and eosin (H and E) and periodic acid-schiff alcian blue (PAS/AB pH 2.5). Gastrointestinal morphological parameters in the hindgut including muscle and serosa thickness, villi length and thickness, lamina propria thickness, total goblet cell number, eosinophilic droplets in epithelial cells and melanomacrophage centres were measured.

Biochemical and histological analyses

The proximate composition analyses of diets and whole body tissue were conducted according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardization (DIN) (2000). A one kg sample of each diet was collected, ground and analysed for proximate composition (moisture, protein, fat, ash, carbohydrate and energy), rancidity (p-anisidine value and peroxide value), amino acid profile, taurine, cholesterol, minerals and fatty acid profile. In addition, a total of twelve fish (n = 12 fish) at the start of the experiment, and three fish from each tank (n = 3 fish $tank^{-1}$; n = 18 tanks; n = 54 fish) at the conclusions of the experiment were collected and stored frozen at -20 °C. Whole fish samples were partially thawed, homogenised and analysed for proximate composition (moisture, protein, fat, ash, carbohydrate and energy) and fatty acid profile.

Performance indices

All data reported for each treatment for animal performance were based on the mean value of the replicate tanks. All calculations using fish weight and diets were based on wet or as fed values, respectively:

- Biomass gain (kg $tank^{-1}$) = (final weight + ∑mortality weight) - (initial weight + ∑replacement weight)
- Specific growth rate (SGR, % d^{-1}) = ([ln final weight - ln initial weight] / d) × 100

- Length growth rate (mm d^{-1}) = (final fish fork length - initial fish fork length) / d
- Condition factor = (fish weight [g] / fish fork length $[cm]^3$) × 100
- Apparent feed conversion ratio (FCR) = feed consumed / fish weight gain
- Dressed weight (head on, gills out and gutted) = (gill weight + visceral wt) / final wet fish wt
- Intraperitoneal fat (%) = wet intraperitoneal fat wt / final wet fish wt × 100
- Visceral index (VSI; %) = wet visceral wt / final wet fish wt × 100
- Hepatosomatic index (HSI; %) = wet liver wt / final wet fish wt × 100

Statistical analyses

The IBM SPSS (version 24 for Windows; IBM SPSS Inc., USA) statistical program was used for all statistical analyses. Homogeneity of variances and normality among mean values were assessed using Levene's test for equality of variance errors and Shapiro-Wilk test, respectively. Data was analysed using two-factor ANOVA, with CP level as the first factor (40, 44 and 48%) and CL (25% and 30%) level as the second factor. When significant effects were observed, the Student-Newman-Keuls post-hoc test was used to detect significant differences between all treatments. When a significant interaction was observed, one-factor ANOVA was used to determine the difference between all treatments for a given variable. A significance level of $P < 0.05$ was used for all statistical tests. All values are presented as means ± standard error (SE) of the mean, unless otherwise stated.

Results

General observations

There were no significant differences in the initial weight and fork length of YTK between treatments in the current study ($P > 0.05$; two-factor ANOVA; Table 3.1.2.2.4). The average initial weight and fork length were 2.13 ± 0.23 kg and 504 ± 19 mm (mean ± standard deviation; $n = 360$), respectively. Fish fed actively during the experiment, with no apparent differences observed between dietary treatments. The overall mortality for fish in the study was low (1.94%), and there were no apparent signs of disease.

Growth performance

Dietary CP level did not significantly influence growth parameters (final weight, biomass gain, specific growth rate, length growth rate and final condition factor) ($P > 0.05$; two-factor ANOVA; Table 3.1.2.2.4). However, these growth parameters were significantly influenced by CL level and the interaction between CP level and CL level ($P < 0.05$; two-factor ANOVA; Table 3.1.2.2.4). Generally, the growth for fish fed the 25% CL diets was higher than those fed the 30% CL diets The interaction between CP and CL level was primarily driven by the significant increase in growth for fish fed Diet 1 (40% CP × 25% CL) and Diet 2 (44% CP × 25% CL) compared to their respective 30% CL diets (Diet 4 [40% CP × 30% CL] and Diet 5 [44% CP × 30% CL]). In contrast, the growth of fish fed Diet 3 (48% CP × 25% CL) and Diet 6 (48% CP × 30% CL) was not significantly different. Final fork length was significantly affected by CL level ($P = 0.029$; two-factor ANOVA; 25 < 30% CL; Table 3.1.2.2.4), but not by CP level or the interaction between the two factors ($P > 0.05$; two-factor ANOVA; Table 3.1.2.2.4).

Feed utilisation

Apparent feed intake (% BW d^{-1}) of YTK was significantly influenced by CP level ($P = 0.01$), CL level ($P < 0.001$), and their interaction ($P = 0.003$; two-factor ANOVA; Table 3.1.2.2.4). Generally, apparent feed intake for fish fed the 25% CL diets was higher than those fed the 30% CL diets. The interaction between CP and lipid level was primarily driven by the significant increased feed intake for fish fed Diet

1 (40% CP × 25% CL) and Diet 2 (44% CP × 25% CL) compared to fed Diet 4 (40% CP × 30% CL) and Diet 5 (44% CP × 30% CL), respectively. In contrast, there was no significant difference between the feed intake of fish fed Diet 3 (48% CP × 25% CL) and Diet 6 (48% CP × 30% CL).
For the 25% dietary CL series, there was also a significant negative linear relationship between analysed dietary CP level and feed intake (% BW d^{-1}) (y = -0.016x + 1.924, R^2 = 0.646, P = 0.012; Figure 3.1.2.2.2). For the 30% dietary CL series, there was no significant linear relationship between dietary CP level and feed intake (P = 0.364; Figure 3.1.2.2.2). With regards to dietary protein intake (g protein kg $fish^{-1}$ d^{-1}), for both the 25 and 30% dietary CL series, there were significant positive linear relationships between analysed dietary CP level and CP intake (25% CL series, y = 0.057x + 2.832, R^2 = 0.583, P = 0.017; 30% CL series, y = 0.141x - 1.334, R^2 = 0.772, P = 0.002; Figure 3.1.2.2.4). The response was more pronounced in fish fed the 30% dietary CL series.
The apparent feed conversion ratio (FCR) of YTK was not significantly influenced by CP level (P = 0.242), CL level (P = 0.557), or their interaction (P = 0.147; two-factor ANOVA; Table 3.1.2.2.4). The apparent FCR of fish fed Diet 2 (44% CP × 25% CL) tended to be superior to those fed other diets.

Whole fish proximate and energy composition

Crude protein level, CL level, and the interaction between the two factors did not significantly influence moisture (59.2-61.4%), protein (19.6-20.6% wet), lipid (16.6-18.8% wet), ash (2.2-2.8% wet), carbohydrate (< 1% wet) or energy (9.6-10.4 MJ kg^{-1} wet) content (P > 0.05; two-factor ANOVA; Table 3.1.2.2.4).

Nutrient deposition

Apparent protein deposition (22.08-24.72%) and apparent energy deposition (27.51-31.67%) of YTK were not significantly affected by CP level, CL level or the interaction between the two factors (P > 0.05; two-factor ANOVA; Table 3.1.2.2.4).

Whole fish fatty acid composition

Total mono trans fatty acid levels were significantly higher in fish fed the 30% lipid diet series than those fed the 25% lipid diet series (P = 0.017; two-factor ANOVA; Table 3.1.2.2.5). Dietary CP level or the interaction between CP level and CL level did not significantly influence total mono trans fatty acid level (P < 0.05; two-factor ANOVA; Table 3.1.2.2.5). Other whole fish fatty acid levels were not significantly influenced by CP level, CL level or the interaction between these two factors (P > 0.05; two-factor ANOVA; Table 3.1.2.2.5).

Blood biochemistry and haematology

There were a number of minor, but significant differences detected in blood biochemistry and haematology. Sodium, urea and haemoglobin levels were significantly higher in fish fed the 25% CL diet series compared to those fed the 30% CL diet series (P < 0.05; two-factor ANOVA; Table 3.1.2.2.6). Crude protein level or the interaction between CP level and CL level did not significantly influence sodium, urea and haemoglobin levels (P > 0.05; two-factor ANOVA; Table 3.1.2.2.6). Total bilirubin level was significantly higher in fish fed 44% CP diets than those fed 48% CP diets (P < 0.05), while the total bilirubin level in fish fed the 40% CP diets were statistically similar to those fed the 44% and 48% CP diets (P > 0.05). CL level or the interaction between CP level and CL level did not significantly influence total bilirubin levels (P > 0.05; two-factor ANOVA; Table 3.1.2.2.6). Other blood biochemistry and haematology parameters measured were not significantly influenced by CP level, CL level or the interaction between these two factors (P > 0.05; two-factor ANOVA; Table 3.1.2.2.6).

Liver and gastrointestinal tract morphology

The hepatosomatic index (1.22-1.44%) of YTK were not significantly influenced by CP level, CL level or the interaction between these two factors ($P > 0.05$; two-factor ANOVA; Table 3.1.2.2.7).

Dietary CP level, CL level or the interaction between these two factors did not significantly affect the gastric dilation score ($P > 0.05$; two-factor ANOVA; Table 3.1.2.2.7). All fish, except for two which were scored as Stage 1 (one fed 40% CP × 25% CL, and one fed 48% CP × 25% CL), were determined to be Stage 0 (healthy/no gastric dilation). Muscularis and submucosa thickness, villi length and thickness, lamina propria thickness, total goblet cells number, eosinophilic droplets in epithelial cells and melanomacrophage centres in the hindgut were not significantly affected by CP level, CL level or the interaction between these two factors ($P > 0.05$; two-factor ANOVA; Table 3.1.2.2.7).

Dressed weight, intraperitoneal fat and visceral index

The dressed weight (89.72-90.40%), intraperitoneal fat (1.47-1.99%) or visceral index (6.96-7.80%) of YTK were not significantly influenced by CP level, CL level or the interaction between these two factors ($P > 0.05$; two-factor ANOVA; Table 3.1.2.2.7).

Discussion

The aim of this experiment was to determine the optimum dietary protein and lipid levels for large YTK production at warm water temperatures. In the current study, YTK readily accepted and consumed all experimental diets. In terms of health, there were some minor differences in blood biochemical (sodium, urea and bilirubin) and haematological (haemoglobin) parameters. All other health parameters remained unaffected by diet.

In the current study, the best growth of large YTK was observed in fish fed the 44% CP × 25% CL diet. This diet contained 42.9% CP, 25.3% CL and 19.90 MJ GE kg^{-1} with a protein to energy ratio of 21.6 g MJ^{-1} (Table 3.1.2.2.1). On a calculated digestible basis, this equated to 36.9% DP, 24.4% DL and 16.9 MJ DE kg^{-1} with a digestible protein to energy ratio of 21.8 g DP MJ^{-1} DE. (Tables 3.1.2.2.1 and Table 3.1.2.2.8). In terms of the actual protein and energy intake required to promote optimal growth, there appears to be a "sweet spot" of 5.2 g CP^{-1} kg BW^{-1} d^{-1} (4.5 g DP^{-1} kg BW^{-1} d^{-1}) and 242 KJ^{-1} GE kg BW^{-1} d^{-1} (207 KJ^{-1} DE kg BW^{-1} d^{-1}) for large YTK (2-3 kg) (Figure 3.1.2.2.4). Results are in line with data derived from the model developed by Booth et al. (2010) using smaller YTK cultured under a similar temperature regime (Table 3.1.2.2.8). Booth et al. (2010) reported decreasing dietary DP, increasing dietary DE and decreasing dietary DP:DE ratios as fish weight increased to 2 kg (Table 3.1.2.2.8).

Booth et al. (2010) estimated the DP and DE requirements for juvenile and small sub-adult YTK (size range: 50 g to 2 kg) at warm water temperatures (21-24 °C) using two commercial diets (Table 3.1.2.2.8). Booth et al. (2010) reported the daily amount of dietary DP required for growth was up to five times greater for smaller 50 g YTK (22.8 g DP. kg^{-1} BW d^{-1}) in comparison to the DP maintenance requirements (4.2 g DP. kg^{-1} BW d^{-1}). The current study did not attempt to measure the maintenance requirements of large YTK at warm water temperatures. However, there were large differences in the daily amount of dietary DP required for optimal growth between the 50 g fish used in the study of Booth et al. (2010) and the larger 2-3 kg fish in the current study (4.5 g CP^{-1} kg BW^{-1} d^{-1}). Differences may be explained by the higher metabolic rate and protein requirement of the smaller fish (Pirozzi and Booth, 2009; Booth et al. 2010). The differences in results emphasises the importance of evaluating the nutritional requirements for a species based on a range of fish sizes relevant to the entire production cycle. Further research is needed in this area.

In the current study feed intake, protein intake and energy intake were all influenced by dietary protein and lipid inclusion levels (Figure 3.1.2.2.2; Table 3.1.2.2.4). For the 25% CL diet series, fish increased their feed intake as dietary CP level decreased. The increased feed intake was likely due to fish fed this series of diets attempting to compensate to meet their daily protein requirements. Interestingly, in the 25% CL diet series, even though fish consumed more food when fed the lower protein diets, equivalence in protein intake was not achieved (Figure 3.1.2.2.3). In contrast, fish fed the 30% CL diets did not appear to regulate their feed intake based on dietary protein level (Figure 3.1.2.2.2). This is likely due

to the supply of excess dietary energy in the 30% CL diets suppressing feed intake, which consequently negatively impacted daily protein intake, especially in fish fed the 40 and 44% CP diets (Figure 3.1.2.2.3). The reduced daily protein intake may have contributed to the reduced growth performance observed in large YTK fed these diets (Table 3.1.2.2.4). It appears that in order to increase gross energy intake, fish fed the 25% CL series (nominal GE level 20.5 MJ kg^{-1}) consumed more feed than fish fed the 30% CL series (nominal GE level 21.5 MJ kg^{-1}) (Figure 3.1.2.2.2). Generally, with the exception for fish fed the 48% CP × 30% lipid diet, this resulted in higher energy intake rates for fish fed the 25% CL diets compared to those fed the 30% CL diets (Figure 3.1.2.2.4).

When fish were cultured at warm water temperatures in the current experiment, dressed weight, visceral somatic index, hepatosomatic index, and intraperitoneal fat levels were not altered in response to dietary lipid level. This is contrary with results from previous studies with large YTK fed high lipid diets at warm (Stone et al., 2016) or cool water temperatures (Bansemer et al., Manuscript 3.1.2.1). In the previous studies high lipid level diets tended to improve weight gain. However, the results reported by Bansemer et al. (Manuscript 3.1.2.1), where dress-out weight was measured, suggested the increased growth may have been, in part due, to increased levels of intraperitoneal fat and increased visceral mass. This suggests that large YTK are able to utilize lipid as an energy source for growth more efficiently during periods of warm water temperatures as opposed to cool water temperatures (Manuscript 3.1.2.1). However, Stone et al. (2016), also reported higher energy deposition, suggesting increased levels of muscle and visceral fat, in large YTK fed a 28% lipid diet at warm water temperatures. Diets in all studies were formulated to contain sufficient levels nutrients, including long chain n-3 fatty acids and taurine. Differences in responses observed between studies may have been due to differences metabolism at different water temperatures, or differences in dietary lipid contents between studies. Thus, when assessing overall growth performance and feed efficiency it may be beneficial to use the carcass weight as a biometric measurement of dietary effects. Processing method and target markets should also be taken into consideration.

The diets in this experiment were formulated using commercially available ingredients and manufactured using the standard industrial practice of cooking extrusion. Oil was then applied to the pellet kernels using vacuum infusion coating. Practically speaking, the high lipid level used in this study presented several problems. Initially, due to formulation constraints associated with energy density it was very difficult to formulate the diet containing 48% CP and 30% lipid. In fact, although it was required for experimental purposes, the feed company involved suggested this formulation was not commercially viable. The second problem was associated with pellet oil leakage in diets of the 30% lipid series. In the current study this was dealt with by storing the feeds in the freezer prior to feeding. This is not a viable commercial practice and steps need to be made to optimise the pellet structure if levels of 30% CL are to be successfully used in commercial feeds for YTK production.

Diets for large YTK in the current study were formulated using specific nutritional information derived from limited information pertaining to large YTK and a range of related and non-related species (Stone and Bellgrove, 2013). The methionine content of the diets for large YTK in the current study were formulated to be 1.3%, with analysed levels ranging from 1.3-1.7% (Table 3.1.2.2.1). Throughout the K4P project, and after we commenced the current study, new amino acid requirement information for juvenile YTK was developed. Booth et al. (Manuscript 3.1.5.3) investigated the methionine requirements of juvenile YTK and based on growth performance and feed utilisation estimated it to be ~2% of the diet. Regardless, of the lower dietary levels methionine used in the current study, overall growth rates of fish were good (Table 3.1.2.2.4). Similar to other fin fish species (NRC, 2011), new evidence that suggests that cysteine can spare a significant proportion of essential methionine for juvenile YTK has also come to light (Booth et al., Manuscript 3.1.5.3). Given the higher nutritional requirements for faster growing smaller fish (NRC, 2011), it is possible that the methionine requirement of large fish may be lower than those reported for small fish by Booth et al. (Manuscript 3.1.5.3). This amino acid requirement may have been satisfied by the combination of dietary methionine and cysteine in the current study. Regardless, we should acknowledge that recommended protein levels may be further reduced by optimising dietary amino acid profiles (e.g. methionine, lysine and histidine) based on new information obtained from this project and also into the future. This demonstrates the importance of our ongoing quest to improve our understanding of the nutrient requirements for YTK at all stages of development.

Conclusions and Recommendations

Based on results from the current experiment, on a practical basis we recommend that diets for large (2.0-3.5 kg) YTK at warm water temperatures contain a CP level of 43% (DP 37%), a CL level of 25% (DL 24%), a GE level of 20 MJ kg^{-1} (DE 17 MJ kg^{-1}) with a CP:GE ratio of 21.6 g CP MJ^{-1} GE (21.8 g DP MJ^{-1} DE). Based on the current feed intake rates, this provided fish with 5.2 g CP^{-1} kg BW^{-1} d^{-1} (4.5 g DP^{-1} kg BW^{-1} d^{-1}) and 242 KJ^{-1} GE kg BW^{-1} d^{-1} (207 KJ^{-1} DE kg BW^{-1} d^{-1}). Further research in pilot scale commercial trials are needed before implementing these diet specification recommendations on-farm.

We do not recommend the use of high lipid levels (30% CL) in commercial diets for large (>2 kg) YTK at warm water temperatures. High dietary lipid levels (30% CL) appear to interfere with daily feed, protein and energy intake rates and feed utilisation and ultimately growth, especially at lower dietary protein levels. It is also commercially impractical to formulate and manufacture diets containing high CP and CL levels (~48% CP, 30% CL).

Dietary lipid level did not affect product yield of large YTK at warm water temperatures. This suggests that fish are able to utilize lipid as an energy source for growth more efficiently during periods of warm water temperatures as opposed to periods of cool water temperatures (Manuscript 3.1.2.1). More research is required in this area of physiology for large YTK.

Findings

- Based on results from the current experiment, on a practical basis we recommend that diets for large (2.0-3.5 kg) YTK at warm water temperatures contain a CP level of 43% (DP 37%), a CL level of 25% (DL 24%), a GE level of 20 MJ kg^{-1} (DE 17 MJ kg^{-1}) with a CP:GE ratio of 21.6 g CP MJ^{-1} GE (21.8 g DP MJ^{-1} DE).
- This information improves our knowledge of the protein, lipid and energy requirements for large YTK cultured at warm water temperatures.
- Results confirm that current commercial diets are adequately formulated, in terms of protein, lipid and energy levels, for optimal growth of large YTK at warm water temperatures. However, further gains in growth performance may be achieved with advancements in our knowledge of specific essential amino acid requirements.
- All FCRs in the current Manuscript ranged from 2.18 down to 1.95. Apparent feed conversion ratio (FCR) was not significantly influenced by diet, but tended to be improved in fish fed the 44% CP × 25% CL diet (1.95), compared to other diets (2.09-2.18).
- An improvement in FCR based on the information provided within this Manuscript, will assist feed manufacturers in formulating commercial diets that achieve one of the overarching goals of the K4P project, which was to provide information to assist producers to achieve FCRs of < 2.2 for large YTK between 1.5-3.5 kg.

Publications

No publications have resulted from this R&D to date.

Acknowledgements

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The authors would also like to acknowledge the South Australian Research and Development Institute (SARDI) and Marine Innovation Southern Australia for their financial support of Assoc. Prof. David Stone and the provision of the SARDI SAASC experimental facilities at West Beach, South Australia, Australia. We would also like to thank Dr Trent D'Antignana of Nutrisea for his input into experimental design. Dr Richard Smullen of Ridley for his input into experimental design, experimental diet formulation and manufacture, and Dr Leo Nankervis of Skretting Australia for his input into experimental design. Thanks to Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd.) and Dr Marty Deveney for the diagnosis and advice pertaining to gill fluke treatments. We also thank Leigh Kuerschner, Krishna-Lee Currie, Mark Purvis, Jessica Buss, Jackson Doherty, Nicole Thompson, Ben Crowe and Samantha Chown for their technical assistance during the experiment.

References

Alvarez, M.J., Lopez-Bote, C.J., Diez, A., Corraze, G., Arzel, J., Dias, J., 1998. Dietary fish oil and digestible protein modify susceptibility to lipid peroxidation in the muscle of rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchus labrax*). British Journal of Nutrition 80, 281-289.

Biswas, B.K., Ji, S.C., Biswas, A.K., Seoka, M., Kim, Y.S., Kawasaki, K.I., Takii, K., 2009. Dietary protein and lipid requirements for the Pacific bluefin tuna *Thunnus orientalis* juvenile. Aquaculture 288, 114-119.

Booth, M.A., Allan, G.L., Pirozzi, I., 2010. Estimation of digestible protein and energy requirements of yellowtail kingfish *Seriola lalandi* using a factorial approach. Aquaculture, 247-259.

Booth, M.A., Moses, M.D., Allan, G.L., 2013. Utilisation of carbohydrate by yellowtail kingfish *Seriola lalandi*. Aquaculture 376, 151-161.

Chown, S., 2015. Impact of diet retention on gastric evacuation time, growth and the development of gastric dilation in Yellowtail Kingfish (*Seriola lalandi*). Honours thesis, Flinders University, Adelaide, Australia. 85 pp.

Jobling, M., 1998. Influence of dietary fat level and increased adiposity on growth and fat deposition in rainbow trout, *Oncorhyncus mykiss* (Walbaum). Aquaculture Research 29, 601-607.

Jobling, M., Larsen, A.V., Andreassen, B., Olsen, R.L., 2002. Adiposity and growth of post-smolt Atlantic salmon *Salmo salar* L. Aquaculture Research 33, 533-541.

Johansen, S.J.S., Sveier, H., Jobling, M., 2003. Lipostatic regulation of feed intake in Atlantic salmon *Salmo salar* L. defending adiposity at the expense of growth. Aquaculture Research 34, 317-331.

Koven, W., Kolkovski, S., Hadas, E., Gamsiz, K., Tandler, A., 2001. Advances in the development of micro diets for gilthead seabream, *Sparus aurata*: a review. Aquaculture 194, 107-121.

National Research Council (NRC), 2011. Nutrient Requirements of Fish and Shrimp, National Academy Press, Washington, DC, pp. 1-376.

Masumoto, T., 2002. Yellowtail, Seriola quinqueradiata. In: Webster, C.D., Lim, C.E., (Eds), Nutrient Requirements and Feeding of Finfish for Aquaculture. CABI Publishing, Oxon, UK, pp. 131-146.

Molina-Poveda, C., 2016. Nutrient requirements. In: Nates, S.F., (Ed), Aquafeed Formulation. Academic Press, pp. 75-216.

Oliva-Teles, A., 2012. Nutrition and health of aquaculture fish: review article. Journal of Fish Disease 35, 83-108.

Peres, H., Oliva-Teles, A., 1999. Influence of temperature on protein utilization in juvenile European seabass (*Dicentrarchus labrax*). Aquaculture 170, 337-348.

Pirozzi, I., Booth, M.A., 2009. The routine metabolic rate of mulloway (*Argyrosomus japonicus: Sciaenidae*) and Yellowtail Kingfish (*Seriola lalandi: Carangidae*) acclimated to six different temperatures. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 152, 586-592.

Refstie, S., Storebakken, T., Baeverfjord, G., Roem, A.J., 2001. Long-term protein and lipid growth of Atlantic salmon (*Salmo salar*) fed diets with partial replacement of fish meal by soy products at medium or high lipid level. Aquaculture 193, 91-106.

Stone, D.A.J., Bellgrove E., 2013. A literature review: the current status of knowledge of the nutritional requirements of yellowtail kingfish. In: Stone, D.A.J. and Bowyer, J.N. (Eds). Final Report. Sustainable Feeds and Feed Management for Yellowtail Kingfish (*Seriola lalandi*). South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2013/000200-1. SARDI Research Report Series No. 751. pp. 92-121.

Stone, D.A.J., D'Antignana, T., Bansemer, M.S., 2016. Final Report. Refining Yellowtail Kingfish feeds and feed management. Prepared by the South Australian Research and Development Institute (Aquatic Sciences), Adelaide. AS-CRC Project No. 2013/730. 144 pp.

Talbot, C., Garcia-Gomez, A., De la Gandara, F., Muraccioli, P., 2000. Food intake, growth, and body composition in Mediterranean yellowtail (*Seriola dumerili*) fed isonitrogenous diets containing different lipid levels. Cahiers Options Mediterraneennes 47, 249-266.

Weihe, R., Dessen, J-E., Arge, R., Thomassen, M.S., Hatlen, B., Rørvik, K-A., 2018 Improving production efficiency of farmed Atlantic salmon (*Salmo salar* L.) by isoenergetic diets with increased dietary protein-to-lipid ratio. Aquaculture Research 49, 1441-1453.

Table 3.1.2.2.1. Analysed proximate composition, rancidity tests, amino acid and mineral composition of the six experimental diets formulated to contain two nominal crude lipid levels and three nominal crude protein levels.

Diet	1	2	3	4	5	6
Nominal crude lipid level (%)	25			30		
Nominal crude protein level (%)	40	44	48	40	44	48
Analysed proximate composition (g 100 g^{-1})						
Moisture	6.8	5.7	6	5.6	4.6	4.5
Crude protein	38.5	42.9	46.4	40.5	44.2	46.9
Calculated digestible protein	34.2	36.9	40.0	33.8	37.0	40.1
Crude lipid	25.4	25.3	25.7	29.3	32.0	29.0
Calculated digestible lipid	24.9	24.4	24.3	29.8	29.8	29.8
Ash	7.6	7.3	6.9	6.9	6.8	6.6
Carbohydrate[1]	21.7	18.8	15.0	17.7	12.4	13.0
Gross energy (MJ kg^{-1})	19.70	19.90	19.90	20.80	21.40	20.90
Calculated digestible energy (MJ kg^{-1})	16.89	16.94	17.21	18.60	18.79	19.00
Protein:energy ratio (g CP MJ^{-1} GE)	19.5	21.6	23.3	19.5	20.7	22.4
Calculated digestible Protein:digestible energy ratio (g DP MJ^{-1} DE)	20.3	21.8	23.2	18.2	19.7	21.1
Rancidity test						
p-Anisidine Value	5.3	5.5	4.7	5.2	5.7	5.1
Peroxide Value (mEqO2 kg^{-1})	4.3	5.7	4.2	5.4	6.6	5.4
Analysed amino acids (g 100 g^{-1})						
Alanine	2.30	2.50	2.70	2.30	2.70	2.60
Arginine	2.50	2.50	2.60	2.30	2.40	2.40
Aspartic Acid	3.90	4.10	4.30	3.60	4.30	4.10
Glutamic Acid	7.20	8.10	8.60	6.90	7.90	7.80
Glycine	2.10	2.20	2.30	2.10	2.30	2.20
Histidine	1.00	1.10	1.30	1.10	1.30	1.40
Hydroxyproline	0.27	0.26	0.21	0.21	0.22	0.22
Isoleucine	1.30	1.30	1.40	1.20	1.30	1.30
Leucine	2.90	3.10	3.50	2.90	3.40	3.30
Lysine	2.80	3.10	3.20	2.70	3.20	3.10
Methionine	1.30	1.30	1.70	1.30	1.40	1.40
Phenylalanine	2.10	2.30	2.60	2.20	2.70	3.00
Proline	2.00	2.20	2.40	2.00	2.30	2.20
Serine	1.90	2.10	2.20	1.90	2.10	2.10
Threonine	1.30	1.40	1.50	1.30	1.50	1.50
Tyrosine	1.20	1.30	1.40	1.20	1.50	1.60
Valine	2.10	2.30	2.50	2.20	2.50	2.40
Taurine	1.20	1.30	1.40	1.20	1.40	1.30
Cholesterol (mg 100 g^{-1})	290	250	280	270	280	330
Analysed minerals (mg kg^{-1})						
Calcium	12000	11000	9900	10000	10000	10000
Copper	9.5	8.1	7.7	7.2	7.6	7.5
Iron	290	310	480	280	370	450
Magnesium	1700	1700	1600	1500	1500	1500
Manganese	46	45	33	36	44	36
Phosphorus	15000	14000	14000	15000	15000	14000
Potassium	5300	4900	5100	5100	4800	4600
Selenium	2.5	2.8	3.2	3.1	3.2	3.2
Sodium	7800	8200	8300	7700	8300	7800
Zinc	130	130	130	130	130	140

[1] Carbohydrate = 100 - (moisture + lipid + protein + ash).

Table 3.1.2.2.2. Analysed fatty acid composition of the six experimental diets formulated to contain two nominal crude lipid levels and three nominal crude protein levels.

Diet	1	2	3	4	5	6
Nominal crude lipid level (%)	25			30		
Nominal crude protein level (%)	40	44	48	40	44	48
Analysed fatty acids (mg 100 g^{-1})						
Saturated Fatty Acids						
C4:0 Butyric	<10	<10	<10	<10	<10	<10
C6:0 Caproic	<10	<10	<10	<10	<10	<10
C8:0 Caprylic	<10	<10	<10	<10	<10	<10
C10:0 Capric	51	51	26	29	64	58
C12:0 Lauric	<10	<10	<10	<10	<10	<10
C14:0 Myristic	686	734	771	791	864	783
C15:0 Pentadecanoic	76	76	77	88	96	87
C16:0 Palmitic	5537	5541	5603	6446	7072	6438
C17:0 Margaric	102	101	103	117	128	116
C18:0 Stearic	1397	1392	1414	1612	1760	1624
C20:0 Arachidic	51	51	51	59	64	58
C22:0 Behenic	51	51	51	29	32	29
C24:0 Lignoceric	<10	<10	<10	<10	<10	<10
Total Saturated	8026	8020	8147	9259	10144	9222
Mono-unsaturated Fatty Acids						
C14:1 Myristoleic	51	25	26	59	64	58
C16:1 Palmitoleic	1473	1493	1491	1699	1888	1711
C17:1 Heptadecenoic	<10	<10	<10	<10	<10	<10
C18:1 Oleic	8255	8071	8070	9698	10688	9686
C18:1 Vaccenic	610	582	591	703	736	667
C20:1 Eicosenic	229	228	231	264	256	261
C22:1 Cetoleic	102	101	129	147	128	116
C22:1 Docosenoic	25	25	26	29	32	<10
C24:1 Nervonic	51	51	51	59	64	29
Total Mono-unsaturated	10795	10601	10666	12599	13856	12557
Poly-unsaturated Fatty Acids						
C18:2n6 Linoleic	3277	3137	3161	3575	3904	3509
C18:3n6 gamma-Linolenic	25	51	26	29	32	29
C18:3n3 alpha-Linolenic	432	430	437	498	544	493
C20:2n6 Eicosadienoic	51	51	51	29	32	29
C20:3n6 Eicosatrienoic	25	25	26	29	32	29
C20:3n3 Eicosatrienoic	<10	<10	<10	<10	<10	<10
C20:4n6 Arachidonic	178	202	206	205	224	203
C20:5n3 Eicosapentaenoic	991	1139	1182	1172	1248	1131
C22:2n6 Docosadienoic	<10	<10	<10	<10	<10	<10
C22:4n6 Docosatetraenoic	25	25	26	<10	<10	<10
C22:5n3 Docosapentaenoic	203	202	206	205	224	203
C22:6n3 Docosahexaenoic	991	1088	1182	1172	1248	1131
∑LC n-3 PUFA	2184	2429	2570	2549	2720	2465
Total Poly-unsaturated	6401	6502	6708	7237	7776	7047
Omega 6 Fatty Acids	3607	3466	3495	3956	4288	3857
Omega 3 Fatty Acids	2769	3011	3187	3252	3456	3161
Total Mono Trans Fatty Acids	76	51	51	59	96	58
Total Poly Trans Fatty Acids	102	127	129	117	128	116
P:M:S Ratio	0.8:1.3:1	0.8:1.3:1	0.8:1.3:1	0.8:1.4:1	0.8:1.4:1	0.8:1.4:1

Table 3.1.2.2.3. Summary of water quality parameters measured throughout the 84 day experiment.

Item[1]	Temperature (°C)	Dissolved oxygen (mg L^{-1})	Dissolved oxygen (% saturation)	pH	Salinity (mg L^{-1})	Ammonia (ppm)	CO_2 (mg L^{-1})
Mean	21.7 ± 1.5	6.9 ± 0.6	97.3 ± 6.9	7.94 ± 0.12	38 ± 0	0.03 ± 0.09	1 ± 1
Range	19.0 - 25.5	5.6 - 9.0	81.0 - 120.0	7.64 - 8.26	37 - 38	0.00 - 0.25	0 - 2

[1] Values means ± standard deviation.

Table 3.1.2.2.4. Growth performance, feed utilisation, proximate composition and nutrient deposition of Yellowtail Kingfish fed different dietary protein and lipid levels at warm water temperatures for 84 days.

Dietary crude lipid level	25			30			Two-factor ANOVA[2]		
Dietary crude protein level[1]	40	44	48	40	44	48	Lipid (A)	Protein (B)	A × B
Growth performance									
Initial weight (kg)	2.13±0.01	2.13±0.03	2.13±0.03	2.13±0.02	2.13±0.01	2.13±0.02	*P* = 0.714	*P* = 0.995	*P* = 0.996
Final weight (kg)	3.44±0.08^{ab}	3.50±0.04^{a}	3.31±0.01^{abc}	3.21±0.05^{bc}	3.15±0.06^{c}	3.30±0.08^{abc}	*P* = 0.001	*P* = 0.930	*P* = 0.042
Biomass gain (kg tank^{-1})	26.20±1.60^{a}	27.32±0.40^{a}	23.55±0.33^{ab}	21.60±0.82^{b}	20.39±0.91^{b}	23.44±1.18^{ab}	*P* < 0.001	*P* = 0.902	*P* = 0.013
SGR (%$^{-1}$)	0.57±0.03^{a}	0.58±0.01^{a}	0.52±0.01^{ab}	0.49±0.01^{b}	0.46±0.02^{b}	0.52±0.02^{ab}	*P* < 0.001	*P* = 0.956	*P* = 0.013
Initial fork length (mm)	506±1	500±3	504±4	504±2	506±2	503±1	*P* = 0.579	*P* = 0.511	*P* = 0.307
Final fork length (mm)	589±3	582±4	580±3	578±3	576±2	579±2	*P* = 0.029 (>)	*P* = 0.324	*P* = 0.229
Length growth rate (mm d^{-1})	0.97±0.04^{a}	0.97±0.02^{a}	0.90±0.02^{ab}	0.87±0.03^{ab}	0.83±0.01^{b}	0.89±0.01^{ab}	*P* < 0.001	*P* = 0.574	*P* = 0.034
Final Condition factor	1.69±0.01^{b}	1.77±0.02^{a}	1.70±0.02^{ab}	1.66±0.01^{b}	1.65±0.03^{b}	1.70±0.03^{ab}	*P* = 0.013	*P* = 0.191	*P* = 0.021
Feed utilisation (as fed)									
Apparent feed intake (% BW d^{-1})	1.31±0.03^{a}	1.22±0.01^{b}	1.19±0.02^{bc}	1.11±0.01^{cd}	1.05±0.01^{d}	1.16±0.01^{bc}	*P* < 0.001	*P* = 0.010	*P* = 0.003
Apparent FCR	2.18±0.07	1.95±0.01	2.13±0.03	2.13±0.07	2.13±0.08	2.09±0.08	*P* = 0.557	*P* = 0.242	*P* = 0.147
Proximate composition (wet basis)[3]									
Moisture (%)	59.20.7	60.9±1.1	61.4±0.5	60.0±0.8	61.0±0.2	60.2±0.1	*P* = 0.871	*P* = 0.092	*P* = 0.343
Protein (%)	19.8±0.3	20.1±0.3	20.6±0.4	19.6±0.2	20.2±0.4	20.4±0.4	*P* = 0.609	*P* = 0.058	*P* = 0.787
Lipid (%)	18.8±1.2	18.0±1.5	16.6±0.2	18.6±1.1	18.1±0.6	16.8±0.2	*P* = 0.951	*P* = 0.086	*P* = 0.984
Ash (%)	2.5±0.4	2.2±0.4	2.4±0.2	2.8±0.2	2.5±0.3	2.5±0.2	*P* = 0.282	*P* = 0.576	*P* = 0.914
Carbohydrate (%)	<1	<1	<1	<1	<1	<1	NA	NA	NA
Energy (MJ kg^{-1})	10.4±0.4	10.1±0.6	9.6±0.0	10.2±0.4	10.1±0.3	9.7±0.1	*P* = 0.935	*P* = 0.151	*P* = 0.964
Nutrient deposition[4]									
Apparent PD	23.95±1.67	24.72±0.78	22.61±1.07	22.43±1.30	22.71±2.03	22.08±1.00	*P* = 0.205	*P* = 0.573	*P* = 0.862
Apparent ED	31.67±1.78	32.67±3.48	28.07±0.52	31.37±3.66	30.17±2.79	27.51±0.99	*P* = 0.568	*P* = 0.212	*P* = 0.892

[1] Values are mean ± SE; $n = 3$.

[2] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments. Values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$). For lipid ANOVA *P* values, the > symbol indicates the 25% lipid level response was significantly greater than the 30% lipid level response. When a significant interaction was observed, one-factor ANOVA was used to determine the difference between all treatments for a given variable ($n = 3$).

[3] Initial fish proximate composition (wet basis): Moisture 64.8%, protein 19.7%, lipid 13.8%, ash 2.1%, carbohydrate < 1%, energy 8.40 MJ kg^{-1}.

[4] PD = protein deposition; ED = energy deposition.

Table 3.1.2.2.5. Fatty acid composition (mg 100 g^{-1}) of Yellowtail Kingfish fed different dietary protein and lipid levels at warm temperatures for 84 days.

Dietary crude lipid level	25			30			Two-factor ANOVA[3]		
Dietary crude protein level[1,2]	40	44	48	40	44	48	Lipid (A)	Protein (B)	A × B
Saturated Fatty Acids									
C14:0 Myristic	568±76	510±46	492±1	504±36	506±18	454±8	*P* = 0.277	*P* = 0.282	*P* = 0.760
C15:0 Pentadecanoic	56±4	54±5	50±0	56±3	54±2	50±1	*P* = 0.959	*P* = 0.083	*P* = 0.991
C16:0 Palmitic	3345±155	3301±285	3060±56	3377±183	3264±127	3046±32	*P* = 0.960	*P* = 0.125	*P* = 0.977
C17:0 Margaric	68±3	72±6	66±1	75±4	66±7	62±6	*P* = 0.706	*P* = 0.309	*P* = 0.416
C18:0 Stearic	899±47	894±76	830±8	911±39	878±15	829±14	*P* = 0.958	*P* = 0.131	*P* = 0.942
C20:0 Arachidic	38±2	36±3	33±0	30±5	36±1	28±6	*P* = 0.146	*P* = 0.298	*P* = 0.555
C22:0 Docosanoic	19±1	18±2	11±6	<10	12±6	11±6	*P* = 0.110	*P* = 0.683	*P* = 0.425
Total Saturated Fat	5036±277	4897±424	4560±59	5002±263	4853±170	4514±47	*P* = 0.827	*P* = 0.111	*P* = 1.000
Mono-unsaturated Fatty Acids									
C16:1 Palmitoleic	1180±58	1146±95	1046±19	1182±86	1150±43	1081±6	*P* = 0.773	*P* = 0.116	*P* = 0.956
C18:1 Oleic	6276±268	6208±542	5590±120	6527±380	6322±199	5942±86	*P* = 0.326	*P* = 0.099	*P* = 0.928
C18:1 Vaccenic	550±34	535±50	482±13	540±32	536±21	504±5	*P* = 0.848	*P* = 0.152	*P* = 0.860
C20:1 Eicosenoic	312±66	246±22	243±3	255±20	265±4	230±14	*P* = 0.487	*P* = 0.301	*P* = 0.480
C22:1 Cetoleic	200±59	138±14	144±10	144±14	163±6	134±17	*P* = 0.550	*P* = 0.479	*P* = 0.355
C22:1 Docosenoic	45±9	36±3	33±0	37±2	36±1	34±0	*P* = 0.465	*P* = 0.199	*P* = 0.560
C24:1 Tetracosenoic	51±9	36±3	33±0	37±2	42±5	34±0	*P* = 0.549	*P* = 0.130	*P* = 0.110
Mono Unsaturated Fat	8626±496	8369±729	7599±147	8747±532	8532±279	7963±80	*P* = 0.528	*P* = 0.097	*P* = 0.958
Poly-unsaturated Fatty Acids									
C18:2n-6 Linoleic	2370±95	2295±174	2141±14	2392±146	2306±82	2162±27	*P* = 0.828	*P* = 0.082	*P* = 0.998
C18:3n-3 Alpha Linolenic	333±34	288±24	277±3	311±24	301±15	269±3	*P* = 0.744	*P* = 0.062	*P* = 0.701
C18:3n-6 Gamma Linolenic	19±1	18±2	17±0	19±1	24±6	17±0	*P* = 0.332	*P* = 0.263	*P* = 0.531
C20:2n-6 Eicosadienoic	38±2	36±3	33±0	37±2	36±1	34±0	*P* = 0.940	*P* = 0.107	*P* = 0.965
C20:3n-6 Dihomo-gamma-linoleic	19±1	18±2	17±0	19±1	18±1	17±0	*P* = 0.879	*P* = 0.088	*P* = 0.921
C20:4n-6 Arachidonic	99±4	108±9	88±5	112±7	96±6	90±6	*P* = 0.902	*P* = 0.051	*P* = 0.189
C20:5n-3 Eicosapentaenoic	644±98	564±47	531±12	566±39	543±29	487±8	*P* = 0.221	*P* = 0.156	*P* = 0.845
C22:4n-6 Docosatetraenoic	19±1	18±2	17±0	19±1	12±6	17±0	*P* = 0.363	*P* = 0.364	*P* = 0.321
C22:5n-3 Docosapentaenoic	252±32	234±20	216±8	224±13	211±12	202±2	*P* = 0.124	*P* = 0.232	*P* = 0.916
C22:6n-3 Docosahexaenoic	951±124	837±54	796±35	882±52	820±45	734±26	*P* = 0.333	*P* = 0.070	*P* = 0.912
∑LC n3 PUFA	1847±253	1635±120	1543±53	1671±104	1574±86	1423±33	*P* = 0.248	*P* = 0.111	*P* = 0.908
Poly Unsaturated Fat	4953±424	4596±350	4314±45	4747±295	4543±199	4195±67	*P* = 0.547	*P* = 0.083	*P* = 0.961
Total Omega 3	2294±302	2031±153	1924±58	2076±133	1972±103	1787±40	*P* = 0.269	*P* = 0.119	*P* = 0.882
Total Omega 6	2589±110	2511±192	2335±11	2603±159	2505±94	2352±30	*P* = 0.927	*P* = 0.084	*P* = 0.994
Trans Fatty Acids									
Total Mono Trans Fat Acids	38±2	41±3	33±0	50±7	48±7	45±6	*P* = 0.017 (<)	*P* = 0.440	*P* = 0.864
Total Poly Trans Fatty Acids	108±20	90±8	94±5	93±5	96±6	90±5	*P* = 0.573	*P* = 0.592	*P* = 0.582

[1] Values for the following fatty acids < 10 mg 100 g^{-1} and were excluded from the table: C4:0 Butyric, C6:0 Caproic, C8:0 Caprylic, C10:0 Capric, C12:0 Lauric, C24:0 Tetracosanoic, C14:1 Myristoleic, C17:1 Heptadecenoic and C20:3n-3 Eicosatrienoic.

[2] Values are mean ± SE; $n = 3$.

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments. Values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$). For lipid ANOVA *P* values, the < symbol indicates the 30% lipid level response was significantly greater than the 25% lipid level response.

Table 3.1.2.2.6. Blood biochemistry of serum and blood haematology on whole blood of Yellowtail Kingfish fed different dietary protein and lipid levels at warm water temperatures for 84 days.

Dietary crude lipid level	25			30			Two factor-ANOVA[2]		
Dietary crude protein level[1]	40	44	48	40	44	48	Lipid (A)	Protein (B)	A × B
Biochemistry[3]									
Sodium (mmol L^{-1})	197.07±2.75	203.65±1.09	202.17±3.02	194.99±0.73	195.21±1.93	197.98±3.05	*P* = 0.024 (>)	*P* = 0.201	*P* = 0.397
Potassium (mmol L^{-1})	2.22±0.27	2.16±0.04	2.32±0.16	2.49±0.22	2.41±0.07	2.45±0.34	*P* = 0.186	*P* = 0.879	*P* = 0.929
Urea (mmol L^{-1})	3.7±0.1	3.6±0.3	3.4±0.1	3.3±0.2	2.8±0.2	2.9±0.1	*P* = 0.001 (>)	*P* = 0.110	*P* = 0.525
Creatinine (mmol L^{-1})	0.042±0.017	0.027±0.003	0.029±0.008	0.022±0.002	0.023±0.002	0.032±0.011	*P* = 0.338	*P* = 0.700	*P* = 0.504
Calcium (mmol L^{-1})	3.24±0.08	3.28±0.07	3.18±0.06	3.14±0.06	3.17±0.03	3.23±0.02	*P* = 0.204	*P* = 0.800	*P* = 0.351
Protein (g L^{-1})	39±1	39±1	36±1	36±1	38±0	37±1	*P* = 0.384	*P* = 0.309	*P* = 0.306
Albumin (g L^{-1})	12±1	12±1	11±0	11±1	11±0	12±0	*P* = 0.138	*P* = 1.000	*P* = 0.272
Globulin (g L^{-1})	27±1	27±1	25±1	25±1	26±0	26±0	*P* = 0.385	*P* = 0.116	*P* = 0.414
Total Bilirubin (mmol L^{-1})[4]	2±0	2±0	1±0	1±0	1±0	1±0	*P* = 0.605	*P* = 0.046	*P* = 0.783
ALT (IU L^{-1})	7±2	9±0	8±1	11±2	10±2	9±2	*P* = 0.083	*P* = 0.821	*P* = 0.616
ALP (IU L^{-1})	19±1	19±1	22±2	19±1	22±5	24±2	*P* = 0.356	*P* = 0.161	*P* = 0.800
Magnesium (mmol L^{-1})	1.31±0.02	1.50±0.08	1.36±0.03	1.29±0.03	1.35±0.04	1.46±0.06	*P* = 0.626	*P* = 0.074	*P* = 0.072
Cholesterol (mmol L^{-1})	3.5±0.3	3.6±0.2	3.4±0.1	3.9±0.2	3.9±0.1	3.5±0.3	*P* = 0.122	*P* = 0.370	*P* = 0.673
Triglyceride (mmol L^{-1})	1.40±0.04	1.49±0.07	1.85±0.12	1.50±0.07	1.62±0.40	1.86±0.05	*P* = 0.573	*P* = 0.063	*P* = 0.944
Bile Acids (mmol L^{-1})	2.6±0.6	8.1±2.4	10.3±3.7	9.1±1.6	10.0±5.0	17.4±6.5	*P* = 0.132	*P* = 0.126	*P* = 0.768
Haematology[5]									
RBC ($\times 10^{12}$)	3.13±0.17	3.12±0.17	2.84±0.43	3.02±0.05	3.17±0.04	2.73±0.25	*P* = 0.732	*P* = 0.222	*P* = 0.916
HGB (g L^{-1})	110±4	111±3	111±5	104±3	110±1	97±5	*P* = 0.032 (>)	*P* = 0.244	*P* = 0.237
PCV (L L^{-1})	0.52±0.01	0.55±0.02	0.49±0.07	0.47±0.05	0.55±0.01	0.48±0.05	*P* = 0.565	*P* = 0.235	*P* = 0.833
MCV (fl)	174.0±4.1	165.2±11.8	173.4±1.8	173.2±1.9	174.4±0.6	177.3±1.3	*P* = 0.323	*P* = 0.543	*P* = 0.645
MCH (pg)	35.2±1.1	35.8±1.2	36.8±1.8	34.6±0.4	34.7±0.5	42.0±7.7	*P* = 0.666	*P* = 0.307	*P* = 0.573
MCHC (g L^{-1})	203±2	203±4	212±9	200±4	199±3	233±39	*P* = 0.727	*P* = 0.296	*P* = 0.683
WBC ($\times 10^{9}$)	6.7±0.1	6.9±0.1	7.0±0.0	6.9±0.1	6.9±0.2	6.9±0.1	*P* = 1.000	*P* = 0.342	*P* = 0.443
Granulocytes (%)	7±4	5±0	11±5	3±1	9±3	3±0	*P* = 0.348	*P* = 0.856	*P* = 0.162
Lymph (%)	85±8	91±1	78±6	90±2	84±6	89±1	*P* = 0.429	*P* = 0.683	*P* = 0.223
Mono (%)	6±4	4±1	9±4	5±1	5±2	5±1	*P* = 0.556	*P* = 0.417	*P* = 0.698
Eosin (%)	2±1	1±1	3±1	2±1	2±1	2±1	*P* = 0.722	*P* = 0.574	*P* = 0.763
Baso (%)	0±0	0±0	0±0	0±0	0±0	0±0	NA	NA	NA
Platelets ($\times 10^{9}$)	85±6	85±3	74±11	75±6	82±2	84±11	*P* = 0.869	*P* = 0.815	*P* = 0.414

[1] Values are mean ± SE; $n = 3$. SE less than 0.01 are reported as "0.00".

[2] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments. Values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$). For lipid ANOVA *P* values, the > symbol indicates the 25% lipid level response was significantly greater than the 30% lipid level response. NA = not statistically analysed due to zero values.

[3] ALT = alanine aminotransferase; ALP = alkaline phosphatase.

[4] Bilirubin Two factor-ANOVA results level were significantly affected by crude protein inclusion (44% CP^{a}, 40% CP^{ab}, 48% CP^{b}).

[5] Smear content: red and white cell normal; Baso = basophil; Eosin = eosinophil; HGB = haemoglobin; Lymph = lymphocytes; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; MCV = mean corpuscular volume; Mono = monocytes; PCV = packed cell volume; RBC = red blood cell count; WBC = white blood cell count.

Table 3.1.2.2.7. Visceral somatic parameters, stomach morphology and gastrointestinal morphology of Yellowtail Kingfish fed different dietary protein and lipid levels at warm water temperatures for 84 days.

Dietary crude lipid level	25			30			Two-factor ANOVA[2]		
Dietary crude protein level[1]	40	44	48	40	44	48	Lipid (A)	Protein (B)	A × B
Visceral somatic parameters									
Dressed weight (%)	90.13±0.30	89.73±0.53	89.79±0.16	90.40±0.28	89.89±0.28	89.72±0.61	$P = 0.694$	$P = 0.332$	$P = 0.907$
Visceral somatic index (VSI; %)	7.40±0.38	7.80±0.55	7.43±0.19	6.96±0.22	7.58±0.33	7.70±0.57	$P = 0.683$	$P = 0.392$	$P = 0.669$
Hepatosomatic index (HSI; %)	1.42±0.12	1.34±0.05	1.22±0.04	1.44±0.03	1.43±0.11	1.27±0.13	$P = 0.443$	$P = 0.105$	$P = 0.912$
Intraperitoneal fat (%)	1.60±0.11	1.85±0.23	1.92±0.22	1.47±0.12	1.99±0.21	1.81±0.13	$P = 0.812$	$P = 0.064$	$P = 0.705$
Stomach morphology									
Gastric dilation score[3]	0.11±0.11	0.00±0.00	0.11±0.11	0.00±0.00	0.00±0.00	0.00±0.00	$P = 0.150$	$P = 0.595$	$P = 0.619$
Hindgut morphology									
Muscularis thickness	900±29	900±29	863±10	838±110	820±2	899±57	$P = 0.390$	$P = 0.925$	$P = 0.527$
Submucosa thickness	22.0±4.2	23.7±8.3	20.6±1.2	21.1±1.9	19.1±1.4	18.8±1.3	$P = 0.407$	$P = 0.862$	$P = 0.888$
Villi length	1388±57	1412±105	1350±87	1392±110	1200±36	1301±126	$P = 0.252$	$P = 0.633$	$P = 0.497$
Villi thickness	90±8	92±3	89±6	92±5	84±5	91±3	$P = 0.735$	$P = 0.827$	$P = 0.582$
Lamina propria thickness	10±2	10±1	10±1	11±1	10±2	9±1	$P = 0.779$	$P = 0.667$	$P = 0.732$
Lamina propria/villi thickness (%)	10.24±1.21	11.18±1.32	10.42±0.68	12.18±0.83	12.10±2.04	9.53±0.84	$P = 0.517$	$P = 0.372$	$P = 0.528$
Mucus cells per 100µm	2.80±0.48	3.36±0.19	3.64±0.51	3.74±0.31	3.44±0.13	3.50±0.68	$P = 0.390$	$P = 0.783$	$P = 0.437$
Eosinophilic droplets in epithelial cells	3±0	3±0	3±0	2±0	3±0	2±0	$P = 0.372$	$P = 0.830$	$P = 0.848$
Melanomacrophage centres	1±0	2±0	2±0	2±0	2±1	3±0	$P = 0.138$	$P = 0.443$	$P = 0.546$

[1] Values are mean ± SE; $n = 3$.
[2] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).
[3] Gastric dilation score based on Chown (2015).

Table 3.1.2.2.8. Estimated optimal dietary digestible energy (DE) and digestible protein (DP) levels and digestible protein to energy ratios for Yellowtail Kingfish of different sizes.

Item[1]	Fish weight (g)[2,3]								
	50	100	200	300	600	900	1000	2000	2000-3000
Estimated DE (MJ DE kg^{-1} diet)[4]	12.0	12.0	12.0	15.0	15.0	15.0	18.0	18.0	16.9
Estimated DP (%)[5]	45.6	45.6	45.6	46.5	46.5	46.5	43.2	43.2	37.0
DP:DE (g DP: MJ DE^{-1})[6]	38.0	38.0	38.0	31.0	31.0	31.0	24.0	24.0	21.8

[1] Means (n = 3).
[2] Data for fish ranging in size from 50 to 2000g from Booth et al. (2010).
[3] Data for fish sized between 2000 to 3000 g derived from the current study.
[4] DE = digestible energy.
[5] DP = digestible protein.
[6] DP:DE = digestible protein to digestible energy ratio.

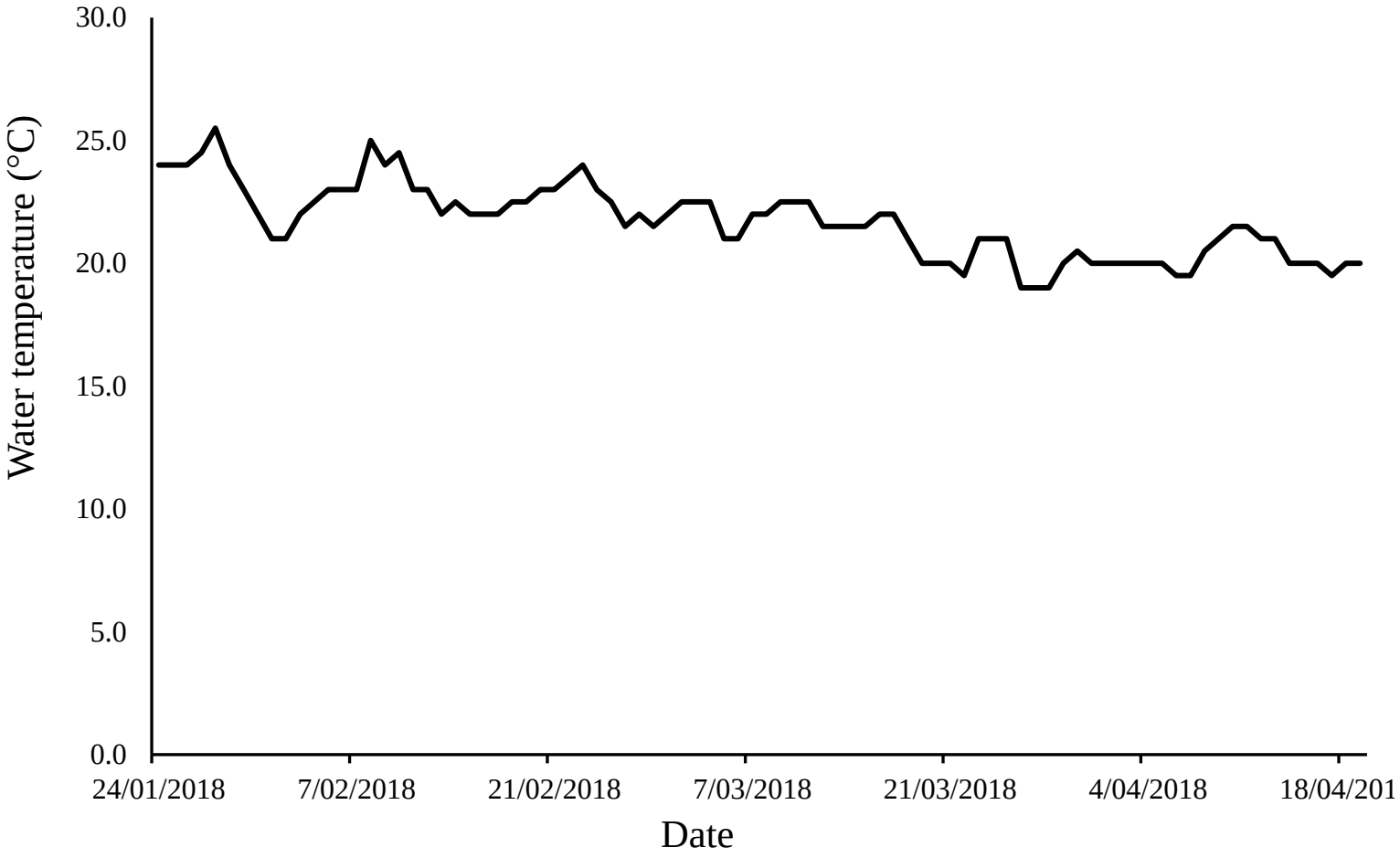


Figure 3.1.2.2.1. Water temperature profile for the duration of the 84 day experimental period. (Average 21.7 °C [range 19.0-25.5°C]).

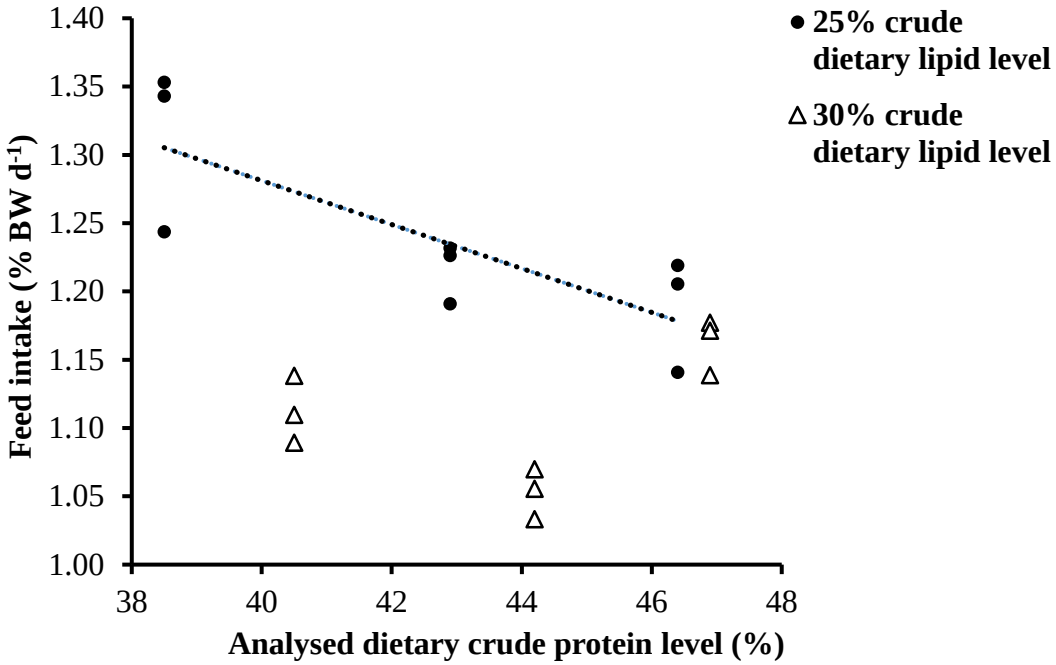


Figure 3.1.2.2.2. Feed intake (% BW d^{-1}) of Yellowtail Kingfish fed graded nominal dietary crude protein (40, 44 and 48%) and dietary crude lipid (25 and 30%) levels at warm water temperatures for 84 days. Values are tank means, n = 3 tanks per treatment combination. Linear relationship: 25% crude dietary lipid series, y = -0.016x + 1.924, R^2 = 0.646, P = 0.012; 30% crude dietary lipid series, P = 0.364.

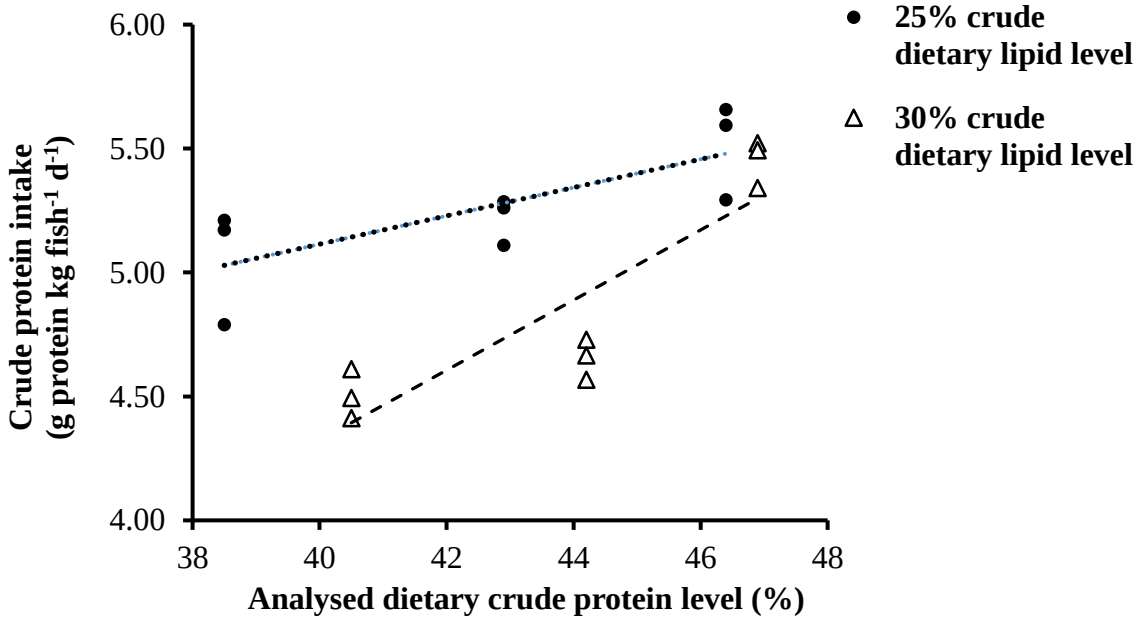


Figure 3.1.2.2.3. Crude protein intake (g protein kg $fish^{-1}$ d^{-1}) of Yellowtail Kingfish fed graded nominal dietary crude protein (40, 44 and 48%) and dietary crude lipid (25 and 30%) levels at warm water temperatures for 84 days. Values are tank means, n = 3 tanks per treatment combination. Linear relationships: 25% crude dietary lipid series, y = 0.057x + 2.832, R^2 = 0.583, P = 0.017; 30% crude dietary lipid series, y = 0.141x - 1.334, R^2 = 0.772, P = 0.002.

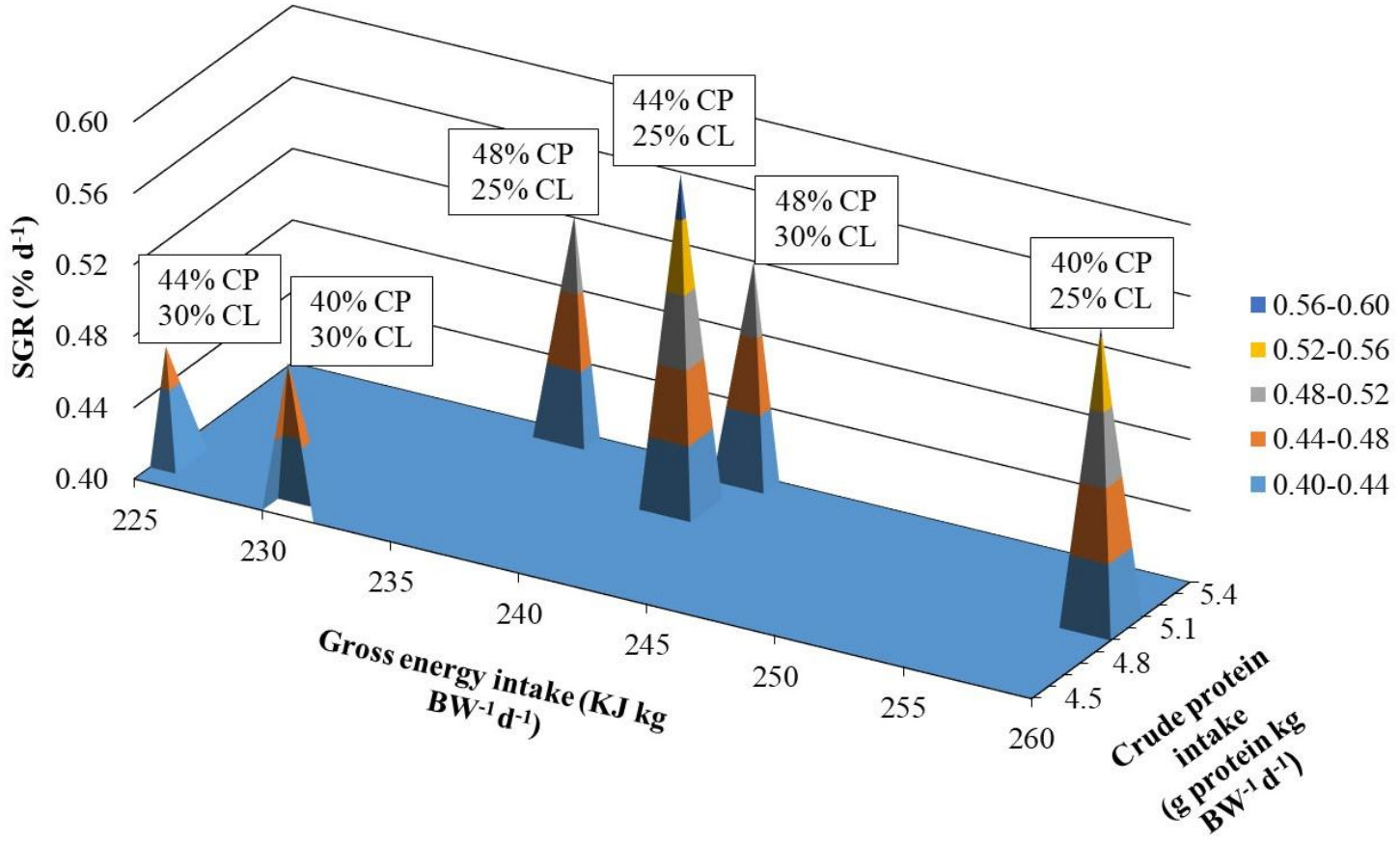


Figure 3.1.2.2.4. Effect of daily crude protein intake (g protein kg BW^{-1} d^{-1}) and gross energy intake (kJ energy kg BW^{-1} d^{-1}) on the specific growth rate of Yellowtail Kingfish feed nominal dietary crude protein (40, 44 and 48%) and dietary crude lipid (25 and 30%) levels at warm water temperatures for 84 days. Values are tank means, *n* = 3 tanks per treatment combination.

3.1.3. Chapter - Wild derived fish meal replacement for large Yellowtail Kingfish.

3.1.3.1. Manuscript - Reducing dietary wild derived fish meal inclusion levels in production diets for large Yellowtail Kingfish (Seriola lalandi).

Matthew S. Bansemer [a], David A.J. Stone [a, b, c, d], Paul Skordas [a], Krishna Lee Currie [a, b], Benjamin Crowe [b], Leo Nankervis [e], Michael Salini [f]

[a] South Australian Research and Development Institute, South Australian Aquatic Sciences Centre, West Beach, SA 5024, Australia
[b] Flinders University, College of Science and Engineering, GPO Box 2100, Adelaide, SA 5001, Australia
[c] University of Adelaide, School of Animal and Veterinary Sciences, Roseworthy, SA 5371, Australia
[d] Marine Innovation Southern Australia
[e] Skretting Australia, Cambridge, TAS 7170, Australia
[f] Ridley, Narangba, QLD 4504, Australia

This manuscript may be referenced as: Bansemer, M.S., Stone, D.A.J., Skordas, P., Currie, K.L., Crowe, B., Nankervis, L.and Salini, M. (2019). 3.1.3.1. Manuscript - Reducing dietary wild derived fish meal inclusion levels in production diets for large Yellowtail Kingfish (*Seriola lalandi*) (N5/N2; Output 2a). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027),* Adelaide, June. pp.106-131.

Abstract

Further research to understand dietary wild derived fish meal (WD FM) substitution with commercially relevant alternative ingredients for large Yellowtail Kingfish (*Seriola lalandi*; YTK) was needed. This 36 week study was designed to investigate the effects of replacing dietary inclusions of WD FM with alternative protein rich ingredients (Poultry meal, PM; Soy protein concentrate, SPC; and FM by-product) on the growth performance, feed utilisation, and health of YTK (2.52 kg) at ambient water temperatures (average 16.6 °C; range 23.5-13.0 °C). Six diets were formulated on a digestible basis to contain 39% digestible protein (~45-47% crude protein), 24% digestible lipid (~25% crude lipid), and a digestible energy level of 16.9 MJ kg^{-1} (~20.1 MJ kg^{-1} gross energy level). Fish were fed to apparent satiation once daily at 10:00 h. Diet did not significantly influence fish growth, feed utilisation, gastrointestinal health, or blood hematology and biochemistry indices measured. Indices associated with bile acid metabolism and liver histology also indicated no significant effects of WD FM substitution. Results from the current study are encouraging and provide valuable commercially relevant information to reduce the dietary WD FM inclusion levels and costs of diets and improve the sustainable production of large YTK. The inclusion of the alternative protein sources resulted in improvements in the fish in-fish out ratios of between 4.8 to 17.9% and 25.4 to 35.1%, respectively, for fish fed diets where WD FM was substituted by 33.3% or 66.7%. We recommend that when using SPC, diets contain no less than 20% WD FM. When using PM, we may recommend that diets contain no less than 20% FM (WD or FM by-product). When using FM by-product, we may recommend that diets contain a total of 30% FM, where at least 10% is derived from wild stocks, and no more than 20% is FM by-product. These recommendations are dependent on the changing cost of raw materials. This is a commercial decision for YTK producers and the feed manufacturers. The available information points toward flexibility in formulation. We recommend that trends with some of the alternatives

to WD FM be followed up with further pilot scale commercial on-farm trials before the full formulation flexibility is realised.

Introduction

As aquaculture production increases, increased demand for wild derived fish meal (WD FM) and fish oil may result in substantial increases in price, while further demand for these ingredients may exceed supply (Gatlin et al., 2007). Yellowtail Kingfish (*Seriola lalandi*; YTK) are cultured globally, and are currently typically fed commercial diet formulations that contain ~30% WD FM inclusion levels. To improve the sustainability and reduce diet costs, alternative ingredients will be required to reduce dietary WD FM inclusion levels in production diets for YTK (Gatlin et al., 2007; Stone and Bowyer, 2013; Stone et al., 2016). Currently however, little published information relating to the effect of reducing dietary WD FM levels with the inclusion of alternative ingredients on production is available for large YTK (Stone et al., 2016).

A number of studies have investigated the potential of alternative dietary ingredients to reduce WD FM levels in aquaculture diets for fish, including land animal protein by-products (e.g. poultry meal (PM), meat meal, feather meal and blood meal), land plant proteins (e.g. soy protein concentrate (SPC), solvent extracted soybean meal (SE SBM), wheat and corn gluten meal, de hulled lupin meal and faba bean meal/concentrate), and also fish meal (FM) by-products (Gatlin et al., 2007; Ouraji et al., 2013; Bowyer et al, 2013a, Bowyer et al., 2013b; Stone and Bowyer, 2013; Bansemer et al., 2015; Skretting Australia, 2015; Davidson et al., 2016; De Santis et al., 2016). These studies have met with varying levels of success. Based on the aforementioned studies, and following discussions with Australian Aquafeed companies, YTK producers and research providers associated with the K4P project, PM, SPC and FM by-product were identified to have the greatest potential to partially replace dietary inclusions of WD FM in production diets for large YTK. These alternative ingredients also have the added benefit of being considerably cheaper (PM, ~$1000; SPC, ~$1200-1700; and FM by-products ~$2000 tonne^{-1}) than WD FM (> $2300 tonne^{-1}) (Mr Joel Scanlon, Aquafeeds Australia, Mount Barker, South Australia, Australia; *personal communication*; Dr Nicole Ruff, Skretting Australia, Cambridge, Tasmania, Australia; *personal communication*).

Poultry meal is high in protein (~65%), has an excellent amino acid profile and has been successfully used to reduce dietary WD FM inclusions for a number of aquaculture species (Sealey et al., 2011; Zhou et al., 2011; Davidson et al., 2016). Juvenile Cobia (*Rachycentron canadum*; 5.8 g) fed a 50% WD FM diet had a similar growth rate as fish fed a 35% WD FM and 15% PM (Zhou et al., 2011). However, further reductions in dietary WD FM inclusion level to 20% (30% PM) led to reduced growth rates compared to fish fed the 50% WD FM control diet (Zhou et al., 2011). In contrast, juvenile Atlantic Salmon (*Salmo salar*; 281 g) fed a 0% WD FM + 30% PM diet exhibited inferior growth, compared to those fed a 19.5% WD FM diet (Davidson et al., 2016). While these studies have successfully used PM as protein source, a species-dependent response to replacing WD FM with PM is apparent. Current commercial Australian YTK diets contain varying levels of PM as a protein source; however, the effect of replacing WD FM with PM in diets for large YTK is not clearly understood (Bowyer et al., 2013a; Bowyer et al., 2013b; Stone and Bowyer, 2013; Stone et al., 2016) and further research was required.

Dietary inclusions of soy products in aquafeeds for a range of finfish species has received considerable attention (van den Ingh et al., 1991; Baeverfjord and Krogdahl 1996; Barrows et al., 2007; Gatlin et al., 2007; Bowyer et al., 2013a; Bowyer et al., 2013b; Bansemer et al., 2015; Stone et al., 2018). Dietary inclusions of SE SBM in YTK diets has been reported to reduce growth, feed utilisation, and also led to the development of sub-acute enteritis (Bowyer et al., 2013a; Bansemer et al., 2015; Stone et al., 2018). As such, recommendations from the previous studies have suggested that SE SBM should be excluded from YTK diets (Stone and Bowyer, 2013; Bowyer et al., 2013a; Bansemer et al., 2015; Stone et al., 2016; Stone et al., 2018), In contrast, Bowyer et al. (2013b) reported that the growth rate and nutrient utilisation of juvenile YTK (initial weight 22 g) fed a 20% dietary inclusion of SPC was similar to a fish meal control diet. Soy protein concentrate, a highly

refined and more expensive product derived from SBM, has undergone extensive processing via heat and alcohol extraction to remove and reduce certain types and levels of antinutritional factors (Gatlin et al., 2007; Bowyer et al., 2013a; Bowyer et al., 2013b; Bansemer et al., 2015). While the inclusion of SPC in diets for fingerling YTK has met with success (Bowyer et al., 2013b; Bansemer et al., 2015), the effect of replacing dietary WD FM with SPC for large YTK (> 1.5 kg) required further research.

The fish oil component inherent in fish meal, although variable, may contain appreciable levels of the essential long chain omega-3 highly polyunsaturated fatty acids (LC n-3 PUFA), eicosapentaenoic acid [20:5n-3, EPA], docosapentaenoic acid [22:5n-3, DPA] and docosahexaenoic acid [22:6n-3, DHA]), while alternative ingredients derived from terrestrial animal or plant sources typically lack these LC n-3 PUFA (Higgs et al., 2006; Bowyer et al., 2012a). While, fish meal by-products obtained from seafood processing wastes may contain appreciable levels of LC n-3 PUFA. Fish meal by-products are not only less expensive than WD FM, they also have the added marketing benefit of being considered as sustainable ingredients as they are excluded from the fish in-fish out ratio calculation (Tacon and Metian, 2008; Jackson, 2009; Terpstra, 2015). Hernández et al. (2014) reported high apparent lipid (~99%) and protein (~80%) digestibility for spotted rose snapper (*Lutjanus guttatus*) fed a 26% dietary inclusion of FM by-product, compared to fish fed a WD FM control diet (lipid and protein digestibility were 86 and 98%, respectively). However, the higher ash content of FM by-product meal may be problematic in diet formulations (Hernandez et al., 2014).

Aim

Research investigating dietary inclusions of alternative ingredients to replace WD FM have been positive for juvenile YTK (< 1 kg), and other aquaculture species. However, little published information is available regarding reducing WD FM levels in commercial diets for large YTK (> 1.5 kg). The aim of the current study was to investigate the effects of replacing dietary inclusions of WD FM with alternative protein rich ingredients (PM, SPC and FM by-product) on the growth performance, feed utilisation, and health of YTK over an extended period.

Methods

Experimental design and diets

Wild derived FM and three alternative protein source ingredients (PM, SPC and FM by-product meal) were investigated in this study. The biochemical composition of the four protein source ingredients are displayed in Tables 3.1.3.1.1 and 3.1.3.1.2. The fish meal by-product, PM and SPC ingredients were included into a control diet (30% wild derived fish meal diet) by reducing wild derived fish meal levels to 20% and 10%. This resulted in six separate diets in this study:

- Diet 1: Control
    - Contained 30% wild derived fish meal diet
- Diet 2: 20% wild derived fishmeal diet plus ~10% FM by-product
    - Replaced 33.33% (= 10.00% wild derived fish meal dietary inclusion level) of digestible wild derived fish meal protein with digestible fish meal by-product protein (= 10.70% of fish meal by-product dietary inclusion level)
- Diet 3: 10% wild derived fishmeal diet plus ~20% FM by-product
    - Replaced 66.67% (= 20.00% wild derived fish meal dietary inclusion level) of digestible wild derived fish meal protein with digestible fish meal by-product protein (= 21.40% of fish meal by-product dietary inclusion level)
- Diet 4: 20% wild derived fishmeal diet plus ~10% PM

    - Replaced 33.33% (= 10.00% wild derived fish meal dietary inclusion level) of digestible wild derived fish meal protein with digestible poultry meal protein (= 11.32% of poultry meal dietary inclusion level)
- Diet 6: 10% wild derived fishmeal diet plus ~10% PM + ~10% FM by-product
    - Replaced 66.67% (= 20.00% wild derived fish meal dietary inclusion level) of digestible wild derived fish meal protein with digestible fish meal by-product protein (= 10.70% of fish meal by-product dietary inclusion level) and digestible poultry meal protein (= 11.32% of poultry meal dietary inclusion level)
- Diet 7: 20% wild derived fishmeal diet plus ~10% SPC
    - Replaced 33.33% (= 10.00% wild derived fish meal dietary inclusion level) of digestible wild derived fish meal protein with digestible soy protein concentrate protein (= 10.88% of soy protein concentrate dietary inclusion level)

The biochemical composition of the six experimental diets are displayed in Tables 3.1.3.1.3 and 3.1.3.1.4 The six diets were formulated on a digestible basis, based on protein and energy digestibility data reported for YTK (Booth et al., 2010; Stone and Bowyer, 2013), to contain 39% digestible protein (~45-47% crude protein), 24% digestible lipid (~25% crude lipid), and a digestible energy level of 16.9 MJ kg^{-1} (~20.1 MJ kg^{-1} gross energy level). Diets were also formulated to contain highly palatable and digestible ingredients at realistic commercial inclusion levels.

The experimental diets (9 mm pellet diameter) were manufactured by Skretting Australia using cooking extrusion technology. Fish were fed to apparent satiation daily at 09:00 h. Apparent satiation feeding was achieved by providing feed to the tank and monitoring feed intake of fish over a period of four min $tank^{-1}$. Care was taken to minimise waste by dispersing feed evenly and slowly across each tank. Once small quantities of uneaten feed were observed on the tank bottom, fish were judged to have reached apparent satiation. Feed inputs were recorded daily.

Experimental fish

Experimental work was conducted in the pool-farm facility at the South Australian Research and Development Institute, South Australian Aquatic Science Centre (SARDI SAASC; West Beach, South Australia, Australia). YTK (n = 306; 2.52 ± 0.25 kg; 546 ± 20 mm (fork length; mean ± standard deviation) were obtained from Clean Seas Seafood (Port Lincoln, South Australia, Australia). Upon arrival at the SARDI SAASC facility, YTK were transferred to 5000 L tanks supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated, aerated sea water at ambient temperature and held for ~1 month and fed a standard Ridley Pelagica diet (crude protein 46%; crude lipid 24%; gross energy 19.30 MJ kg^{-1}).

Skin and gill fluke treatment

Upon arrival at SARDI SAASC, YTK were inspected, and were observed to have a low burden of skin flukes (*Benedenia seriola*) and gill flukes (*Zeuxapta seriola*). Treatment was deemed necessary, and was prescribed by Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd., Ballina, New South Wales, Australia).

Experimental stocking and intermediate weight checks

At the commencement of the experiment (March 2017), YTK were anaesthetised in 5000 L tanks (total water volume 2500 L) using AQUI-S® (AQUI-S® New Zealand Ltd., Lower Hutt, New Zealand) at a concentration of 14 mg L^{-1} of seawater. Seventeen fish were removed from their tank, measured, weighed and stocked into one of the three replicate 5000 L tanks treatment $combination^{-1}$ (n = 6 diets; n = 18 tanks). Tanks were supplied with partial flow-through/recirculating (100%

system water exchange d^{-1}), sand filtered, UV treated sea water at ambient temperature. All tanks were supplied with aeration and oxygenation throughout the study. As required, mortalities were removed, weighed, measured and recorded required and replaced with tagged fish (T-tags) of a similar weight. Tagged fish were included in biomass calculations for FCR (see Performance indice section), but excluded from all other analyses. This study ran for a total of 84 days.

At 4, 8, 12, 16, 20, 24, 28 and 32 weeks post-stocking, all fish were anaesthetised using AQUI-S® at a concentration of 14 mg L^{-1} of seawater. YTK were measured, weighed, visually inspected for skin and gill flukes, and returned back to their respective tanks.

Water quality analyses

Water quality parameters were measured daily at 12:00 h, and maintained at appropriate levels for acceptable growth of YTK throughout the study (Table 3.1.3.1.5). Water temperature was measured using a thermometer. Dissolved oxygen (mg L^{-1} and % saturation) was measured using a dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark). The pH was measured daily using a meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, Illinois, United States of America). Salinity (g L^{-1}) was measured weekly using a portable salinity refractometer (model RF20, Extech Instruments, Nashua, New Hampshire, United States of America).

Final harvest sampling

At 36 weeks (252 days), all fish were anaesthetised using AQUI-S® at a concentration of 14 mg L^{-1} of seawater and weighed and measured. Three fish from each tank (n = 3 fish $tank^{-1}$; n = 18 tanks; n = 54 fish) were whole collected and stored frozen at -20 °C for biochemical analysis. Blood from three separate fish per tank (n = 3 fish $tank^{-1}$; n = 18 tanks; n = 54 fish) were collected using a 19 G needle with a 5 mL syringe, in two separate Vacuette® or BD vacutainer ® tubes (Z serum clot activator or EDTA tubes). Serum was analysed for blood biochemistry and whole blood was analysed for blood haematology conducted by IDEXX (Unley, South Australia, Australia). These blood sampled fish were then dissected and the viscera, liver and visceral fat was weighed in order to calculate visceral index (VSI; %), hepatosomatic index (HSI; %) and intraperitoneal fat (%), respectively. The stomach from these fish were opened longitudinally, and were subjectively scored for gastric dilation (Chown, 2015). Briefly, Stage 0 is defined as having pronounced/well defined folds throughout the pylorus, anterior and distal stomach, while Stage 1 is defined as having minimal or absent folds throughout the pylorus and anterior stomach, but has pronounced/well defined folds in the distal stomach (Chown, 2015). In addition, one cm^2 longitudinally opened hindgut sections were collected from blood sampled fish for histology. In brief, hindgut samples were fixed in 10% seawater formalin for > 48 h, processed and embedded in paraffin wax. Tissue sections were cut using a microtome and floated onto Starfrost® glass slides and dried for > 24 h at room temperature before being stained with hematoxylin and eosin (H and E) and periodic acid-schiff alcian blue (PAS/AB pH 2.5). Gastrointestinal morphological parameters in the hindgut including muscularis and submucosa thickness, villus length and thickness, lamina propria thickness, total goblet cell number, eosinophilic droplets in epithelial cells and melanomacrophage centres.

Biochemical and histological analyses

The proximate composition analyses of diets and whole body tissue were conducted according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardization (DIN) (2000). A one kg sample of each diet was collected, ground and analysed for proximate composition (moisture, protein, fat, ash, carbohydrate and energy), amino acid profile, taurine level, fatty acids profile and rancidity (p-anisidine and peroxide value). In addition, a total of twelve fish (n = 12 fish) at the start of the experiment, and three fish from each replicate tank at the conclusions

of the experiment were collected and stored frozen at -20 °C. Whole fish samples were partially thawed, homogenised and analysed for proximate composition, fatty acids profile, amino acids profile, taurine and mineral composition.

Performance indices

All data reported for each treatment for animal performance were based on the mean of the three replicate tanks. All calculations using fish weight and diets were based on wet or as fed values, respectively:

- Biomass gain (kg $tank^{-1}$) = (final weight + ∑mortality weight) - (initial weight + ∑replacement weight)
- Specific growth rate (SGR, % d^{-1}) = ([ln final weight - ln initial weight] / d) × 100
- Length growth rate (mm d^{-1}) = (final fish fork length - initial fish fork length) / d
- Condition factor = (fish weight [g] / fish fork length $[cm]^3$) × 100
- Apparent feed conversion ratio (FCR) = feed consumed / fish weight gain
- Apparent protein deposition = ([final soft body protein - initial soft body protein] / protein intake × 100
- Apparent energy deposition = ([final soft body energy - initial soft body energy] / energy intake × 100
- Intraperitoneal fat (%) = wet intraperitoneal fat wt × 100 / final wet fish wt
- Visceral index (VSI; %) = wet visceral wt × 100 / final wet fish wt
- Hepatosomatic index (HSI; %) = wet liver wt × 100 / final wet fish wt
- Fish in-fish out ratio (FI-FO) = FCR × 0.75 × 0.5 × [(% fish meal in feed / 22.5) + ((% fish oil in feed - 0.08 × % fish meal in feed) / 5)]

Where the FI-FO ratio is expressed in reduction fish equivalent and FCR is the feed conversion ratio (kg feed kg^{-1} fish). The yield of reduction fish is 22.5 % WD fish meal and 5 % fish oil. The factor 0.75 takes into account that about 25% of the WD fishmeal and fish oil is nowadays produced from fish processing by-products, and the factor 0.08 takes into account that WD fish meal contains ~8 % fish oil (Terpstra, 2015).

Statistical analyses

The IBM SPSS software package (version 24 for Windows; IBM SPSS Inc., USA) was used for all statistical analyses. Homogeneity of variances and normality among mean values were assessed using Levene's test for equality of variance errors and Shapiro-Wilk test, respectively. Data were compared across all treatments using a one-factor ANOVA. When significant effects were observed, the Student-Newman-Keuls post-hoc test was used to detect significant differences between all treatments. A significance level of $P < 0.05$ was used for all statistical tests. All values are presented as means ± standard error (SE) of the mean unless otherwise stated.

Results

General observations

There were no significant differences in the initial weight and fork length of YTK between treatments ($P > 0.05$; one-factor ANOVA; Table 3.1.3.1.6). The average initial weight and fork

length were 2.52 ± 0.25 kg and 546 ± 20 mm (mean ± standard deviation; n = 306), respectively. YTK fed actively during the experiment, and there were no apparent differences in feeding activity observed between diets. Throughout the experiment, fish appeared healthy and in good condition. The mortality rate was low (< 1%). The water temperature profile throughout the experiment is displayed in Figure 3.1.3.1.1 (average water temperature was 16.6 °C [range 23.5-13.0 °C]).

Growth performance

Final weight (P = 0.321), biomass gain (P = 0.157), specific growth rate (SGR; P = 0.120), final fork length (P = 0.368), length growth rate (P = 0.163) and final condition factor (P = 0.272) of YTK were not significantly influenced by diet (one-factor ANOVA; Table 3.1.3.1.6).

Feed utilisation

Apparent feed intake (% BW d^{-1}; P = 0.409) and apparent feed consumption rate (kg $tank^{-1}$; P = 0.235) were not significantly affected by diet (one-factor ANOVA; Table 3.1.3.1.6). Fish fed Diet 1 (30% wild derived fishmeal) and Diet 2 (20% wild derived fishmeal + 10.70% fish meal by-product) had numerically lower feed intake rates than fish fed the other diets.

Feed conversion ratio (FCR) of YTK was not significantly influenced by diet (P = 0.193; one-factor ANOVA; Table 3.1.3.1.6). Fish fed Diet 1 (30% wild derived fishmeal) and Diet 7 (20% wild derived fishmeal + 10.88% soy protein concentrate) had a numerically superior FCR than those fed other diets. In contrast, fish fed Diet 3 (10% wild derived fish meal + 21.40% fish meal by-product) had a numerically lower FCR compared to those fed other diets.

Whole fish proximate, energy, fatty acid, amino acid and mineral composition

Tissue moisture (62.4-64.5%), protein (19.1-19.8% wet), lipid (15.2-15.8% wet) ash (1.9-2.5% wet), carbohydrate (< 1% wet) and energy (8.97-9.20 MJ kg^{-1} wet) contents of fish were not significantly different between diets (P > 0.05; one-factor ANOVA; Table 3.1.3.1.6). The fatty acid and amino acid composition of fish were also not significantly influenced by diet (P > 0.05; one-factor ANOVA; Table 3.1.3.1.7 and 3.1.3.1.8, respectively). With regard to the mineral composition, the potassium content of fish fed Diet 3 (10% WD FM + 21.40% FM by-product) was significantly lower (6.3-7.3%) than those fed Diet 1 (30% WD FM), Diet 2 (20% WD FM + 10.70% FM by-product) and Diet 7 (20% WD FM + 10.88 SPC) (P = 0.019; one-factor ANOVA; Table 3.1.3.1.9). Potassium content was within normal ranges for similar fish from previous experiments (Stone et al., 2016). Diet did not significantly influence other mineral levels measured (calcium, copper, iron, magnesium, manganese, phosphorus, selenium, sodium, zinc (P > 0.05; one-factor ANOVA; Table 3.1.3.1.9).

Nutrient retention

Diet had no significant effect on apparent protein deposition (20.58-22.74%) or apparent energy deposition (21.02-23.04%) of fish (P > 0.05; one-factor ANOVA; Table 3.1.3.1.6).

Blood haematology and biochemistry

All measured blood haematology and biochemistry parameters were not significantly affected by diet (P > 0.05; Table 3.1.3.1.10).

Visceral somatic parameters and gastrointestinal morphology

Intraperitoneal fat (1.52-1.78%), visceral index (5.86-6.67%) and hepatosomatic index (0.83-0.91%) of fish were not significantly influenced by diet ($P > 0.05$; one-factor ANOVA; Table 3.1.3.1.11). Diet did not affect gastric dilation ($P = 0.458$; one-factor ANOVA; Table 3.1.3.1.11). All fish, except for one fed Diet 1 (Stage 1), were determined to be Stage 0 (healthy/no gastric dilation; Table 3.1.3.1.9). Muscularis and submucosa thickness, villi length and thickness, lamina propria thickness, total goblet cells, eosinophilic droplets in epithelial cells and melanomacrophage centres in the hindgut were not significantly affected by diet ($P > 0.05$; one-factor ANOVA; Table 3.1.3.1.11).

Apparent digestibility coefficients

Apparent digestibility coefficients (ADC) for diet dry matter and protein were significantly affected by diet ($P = 0.023$ and 0.016, respectively; one-factor ANOVA; Table 3.1.3.1.11). Dry matter and protein ADC was significantly higher for fish fed Diet 2 (20% WD FM + 10.70% FM by-product) and Diet 6 (10% WD FM + 10.70% FM by-product + 11.32% PM) than those fed Diet 3 (10% WD FM + 21.4% FM by-product). Dry matter and protein ADC for fish fed Diet 1 (30% WD FM), Diet 4 (20% WD FM + 11.32% PM) and Diet 7 (20% WD FM + 10.88 SPC) were statistically similar, and statistically similar to those fed Diet 2 (20% WD FM + 10.70% FM by-product), Diet 3 (10% WD FM + 21.4% FM by-product) and Diet 6 (10% WD FM + 10.70% FM by-product + 11.32% PM) ($P > 0.05$; one-factor ANOVA; Table 3.1.3.1.11). Gross energy ADC was not significantly affected by diet ($P = 0.055$; one-factor ANOVA; Table 3.1.3.1.11).

Discussion

The aim of the current study was to investigate the effects of replacing dietary inclusions of WD FM with alternative protein rich ingredients (PM, SPC and FM by-product) on the growth performance, feed utilisation, and health of YTK at ambient water temperatures. The ultimate outcome was to provide information to improve the sustainability of current diet formulations and economic viability for YTK production by reducing/optimising dietary WD FM inclusion levels. In order to achieve this aim and outcome, YTK were fed diets that had WD FM replaced, with either FM by-product, poultry meal (PM) or soy protein concentrate (SPC), or a combination of two alternative ingredients in a series of six diets. Over the course of the 36 week experiment, there were no significant differences in any of the growth, feed utilisation or blood hematology and biochemistry or visceral somatic parameters, digestive tract morphology or hindgut histology indices measured between the six diets tested. Indices associated with bile acid metabolism (total bile acid in synthesis, storage, or excretion) and liver function and histology (unsaturated neutral lipid storage within hepatocytes, total lipid storage within hepatocytes, hepatocyte vacuolisation) also indicated no significant effects of WD FM substitution (Crowe et al., 2018).

The maximum inclusion levels of PM (11.32%) and SPC (10.88%) used in diet for large YTK in the current study supported good growth. These results are in agreement for a range of other carnivorous freshwater and marine species, such as Rainbow Trout (*Oncorhynchus mykiss*) (Sealey et al., 2011), Atlantic Salmon (*Salmo salar*) (Davidson et al., 2016), Cobia (*Rachycentron canadum*) (Zhou et al., 2011) and juvenile YTK (Bowyer et al., 2013b). With regards to FM by-product, even though no significant differences for growth performance or feed utilisation were observed in this study, there were tendencies for the performance to decline when YTK were fed this ingredient at high levels (Diet 3, 10% WD FM + 21.4% FM by-product) compared to all other diets. This finding is consistent with results from Kim et al. (2018) who reported the growth and feed utilisation of Korean Rockfish (*Sebastes schlegeli*) also tended to be reduced as high ash tuna by-product FM replaced WD FM as levels exceeded 50%. The ash content of the tuna by-product FM was 21.4% as opposed to 13.7 for the WD FM (Kim et al., 2018).

As FM by-product is derived from fish which have been processed to recover the edible portion of flesh, bone and hence ash contents are typically high (Aksnes and Mundheim 1997; Caballero et al., 1999; Kim et al., 2018), as is the case in the current study (~20%; Table 3.1.3.1.1). Gatlin et al. (2007) recommended an optimum target level of 4-8% ash content for ingredients to be used as fishmeal replacements. Protein quality of FM by-product is also low, as it is comprised of a large proportion of connective tissue (Aksnes and Mundheim 1997; Caballero et al., 1999; Kim et al., 2018). Kim et al. (2018) reported lower dry matter contents of the first two limiting amino acids, lysine (4.3 vs 5.5%) and methionine (1.8 vs 2.2%) in tuna meal by-product compared to WD brown fish meal. In the current study the lysine (4.09 vs 4.30%) and methionine (1.59 vs 1.70%) levels in the FM byproduct meal were also lower than in the WD fish meal (Table 3.1.3.1.1).

Upon closer examination of results in the current study (Table 3.1.3.1.11), the apparent digestibility for dry matter, protein and energy also tended to be lower for the YTK fed the Diet 3, which contained the highest proportion of FM by-product (21.4% FM by-product). High ash levels have been reported to interfere with nutrient digestion in a range of fish species. Stone et al. (2000) reported a reduction in dry matter, energy and nitrogen apparent digestibility in Silver Perch (*Bidyanus bidyanus*) fed high ash meat by-products. Protein digestibility has also been reported to be negatively correlated with high ash content in high ash meat meals derived from food waste streams for Rainbow Trout (Watanabe and Pongmaneerat, 1991), Gilthead Seabream (*Sparus aurata*) (Nengas et al., 1995) and Olive Flounder (*Paralichthys olivaceus*) (Rahman et al., 2016). Reduced nutrient digestibility may have contributed to the lower performance of the YTK fed the diet containing 21.4% FM by-product. This suggest that FM by-product inclusion may be limited in commercial diets for YTK. Additionally, consideration must always be given to the ash and protein quality of animal ingredients derived from processing waste streams when selecting ingredients for commercial YTK diets.

It is important for all aquaculture producers to reduce their reliance on marine derived dietary ingredients in order to improve the sustainable production of fish. Sustainability is not only an important environmental issue, it is also an important marketing tool, and may be measured by the fish in-fish out ratio (Tacon and Metian, 2008; Jackson, 2009; Terpstra, 2015). The fish in-fish out ratio is related to the level of wild derived marine ingredients required to produce one kg of fish and takes into account the FCR (Tacon and Metian, 2008; Jackson, 2009; Terpstra, 2015). All alternative protein sources used in this study, including the FM by-product, were derived from sustainable sources. The inclusion of the alternative protein sources resulted in improvements in the fish in-fish out ratios of between 4.8 to 17.9% and 25.4 to 35.1%, respectively, for fish fed diets where WD FM was substituted by 33.3% or 66.7% (Table 3.1.3.1.6).

With regard to diet ingredient costs, all of the alternative protein ingredients used in this study were cheaper than WD FM. This resulted in approximate savings in diet ingredient costs ranging from 60 to \$150 tonne^{-1}, depending on the ingredient used and level of WD FM substitution (Table 3.1.3.1.1). Given, there were no significant differences in growth and FCR, actual savings realized by producers may be considerable and would contribute to significant improvements in productivity for the Australian YTK industry. However, cost savings and improvements in sustainability cannot be fully realised until diets containing the alternative protein sources are validated in pilot-scale on-farm trials.

Overall, the results in this study were encouraging. However, the growth performance of the large YTK fed diets containing the tested alternative protein sources may be further improved with enhanced essential amino acid fortification. Diets for large YTK in the current study were formulated using specific nutritional information derived from a range of related and no-related species (Stone and Bellgrove, 2013). The methionine content of the diets for large YTK in the current study were formulated to be 1%, with analysed levels ranging from 1.01-1.13% (Table 3.1.3.1.3). Throughout the K4P project, and after we commenced the current study, new amino acid requirement information for juvenile YTK was developed. Booth et al. (Manuscript 3.1.5.3) investigated the methionine requirements of juvenile YTK and based on growth performance and feed utilisation estimated it to be ~2% of the diet. Similar to other fin fish species (NRC, 2011), there is also new evidence that suggests that cysteine can spare a significant proportion of essential

methionine for juvenile YTK (Booth et al., Manuscript 3.1.5.3). Given the higher nutritional requirements for faster growing smaller fish (NRC, 2011), it is possible that the methionine requirement of large fish in the current study may be lower, and may have been satisfied by a combination of methionine and cysteine. This demonstrates the importance of our ongoing quest to improve our understanding of the nutrient requirements for YTK at all stages of development.

Conclusions and Recommendations

Results from the current study are encouraging and provide valuable commercially relevant information to reduce the dietary WD FM inclusion levels in production diets for large YTK. Reducing dietary WD FM inclusions in current commercial diets with alternative ingredients derived from cheaper sustainable sources may lead to improved diet sustainability and diet cost savings. Sustainability, as measured by the fish in-fish out ratio, was improved by up to ~35% by the incorporation of a combination of PM and FM by-product. This may provide Australian YTK producers with major advantages in terms of market access and improved consumer perception. Diet cost were reduced considerably, which in turn, may lead to reductions in production costs for the industry. In addition, information pertaining to the replacement of WD fish meal with alternative protein sources will improve flexibility for feed manufactures to select raw materials that most economically meet the nutrient criteria in diet formulations for YTK. This is particularly advantageous, as availability and prices for fish feed ingredients vary greatly, especially in periods of drought. Based on results from the current study, we may recommend that when using SPC that diets contain no less than 20% WD FM. When using PM, we may recommend that diets contain 20% FM (derived from a combination of FM from wild stocks and seafood by-products). When using FM by-product, we may recommend that diets contain a total of 30% FM, where 10% is derived from wild stocks, and 20% is derived from seafood by-products. These results are for large YTK of the size range investigated in the current study and these recommendations are dependent on the changing cost of raw materials. We recommend that WD FM substitution with SPC, PM and FM by-product in diets be followed up with further pilot scale commercial trials before full diet formulation flexibility is realised.

Findings

- Reducing dietary WD FM inclusion levels with PM, FM by-product and SPC may lead to improved diet sustainability and diet cost savings, compared to current commercial diets.
- With regard to diet ingredient costs, all of the alternative protein ingredients used in this study were cheaper than WD FM. This resulted in approximate savings in diet ingredient costs ranging from 60 to \$150 tonne^{-1}, depending on the ingredient used and level of WD FM substitution (Table 3.1.3.1.1).
- With regard to improved sustainability, the inclusion of the alternative protein sources resulted in improvements in the fish in-fish out ratios of between 4.8 to 17.9% and 25.4 to 35.1%, respectively, for fish fed diets where WD FM was substituted by 33.3% or 66.7% (Table 3.1.3.1.6).
- Based on results from the current study, we may recommend that when using SPC that diets contain no less than 20% WD FM. When using PM, we may recommend that diets contain 20% FM (derived from a combination of FM from wild stocks and seafood by-products).
- When using FM by-product, we may recommend that diets contain a total of 30% FM, where 10% is derived from wild stocks, and 20% is derived from seafood by-products.
- An improvement in FCR based on the information provided within this Manuscript, will assist feed manufacturers in formulating commercial diets that achieve one of the overarching

goals of the K4P project, which was to provide information to assist producers to achieve FCRs of < 2.2 for large YTK between 1.5-3.5 kg.

- In addition, this information improves flexibility for feed manufactures to select raw materials that most economically meet the nutrient criteria for commercial diet formulations. Ultimately, the extent of WD FM substitution is a commercial decision for YTK producers and Australian feed manufacturers.

Publications

No publications have resulted from this R&D to date.

Acknowledgements

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The authors would also like to acknowledge the support of the South Australian Research and Development Institute (SARDI) and Marine Innovation Southern Australia for their financial support of Assoc. Prof. David Stone and the provision of the SARDI SAASC experimental facilities at West Beach, South Australia. Thanks to Dr Trent D'Antignana of Nutrisea for input into experimental design. Dr Nicole Ruff of Skretting Australia for input into experimental design, diet formulation and diet manufacture. We would also thank Dr Richard Smullen of Ridley for input into experimental design and diet formulation. Thanks to Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd.) and Dr Marty Deveney (SARDI SAASC) for the diagnosis and prescription of skin and gill fluke treatments. We also thank Leigh Kuerschner, Ben Crowe, Jackson Doherty, Filipa Duarte, Krishna-Lee Currie, Mark Purvis and Jessica Buss for their technical assistance during the experiment.

References

Aksnes, A., Mundheim, H., 1997. The impact of raw material freshness and processing temperature for fish meal on growth, feed efficiency and chemical composition of Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 149, 87-106.

Bansemer, M.S., Forder, R.E.A., Howarth, G.S., Suitor, G.M., Bowyer, J., Stone, D.A.J., 2015. The effect of dietary soybean meal and soy protein concentrate on the intestinal mucus layer and development of subacute enteritis in Yellowtail Kingfish (*Seriola lalandi*) at suboptimal water temperature. Aquaculture Nutrition 21, 300-310.

Baeverfjord, G., Krogdahl, A., 1996. Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: a comparison with the intestines of fasted fish. Journal of Fish Diseases 19, 375-387.

Barrows, F.T., Stone D.A.J., Hardy R.W., 2007. The effects of extrusion conditions on the nutritional value of soybean meal for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 265, 244-252.

Bowyer, J.N., Qin, J.G., Smullen, R.P., Adams, L.R., Thomson, M.J.S., Stone, D.A.J., 2013a. The use of a soy product in juvenile yellowtail kingfish (*Seriola lalandi*) feeds at different water temperatures: 1. Solvent extracted soybean meal. Aquaculture 384-387, 35-45.

Bowyer, J.N., Qin, J.G., Smullen, R.P., Adams, L.R., Thomson, M.J.S., Stone, D.A.J., 2013b. The use of a soy product in juvenile yellowtail kingfish (*Seriola lalandi*) feeds at different water temperatures: 2. Soy protein concentrate. Aquaculture 410-411, 1-10.

Caballero, M.J., López-Calero, G., Socorro, J., Roo, F.J., Izquierdo, M.S., Férnandez, A.J., 1999. Combined effect of lipid level and fish meal quality on liver histology of gilthead seabream (*Sparus aurata*). Aquaculture 179, 277-90.

Chown, S., 2015. Impact of diet retention on gastric evacuation time, growth and the development of gastric dilation in Yellowtail Kingfish (*Seriola lalandi*). Honours thesis, Flinders University, Adelaide, Australia. 85 pp.

Crowe, B.H, McWhorter, T.J., Harris, J.O., Bansemer, M.S., Stone, D.A.J., 2018. Effect of partial wild derived fish meal replacement on bile acid production and liver structure in yellowtail kingfish, *Seriola lalandi*. (Poster). The 18th International Symposium on Fish Nutrition and Feeding, 3rd - 7th June, Las Palmas de Gran Canaria, Canary Island, Spain.

Davidson, J., Barrows, F.T., Kenney, P.B., Good, C., Schroyer, K., Summerfelt, S.T., 2016. Effects of feeding a fishmeal-free versus a fishmeal-based diet on post-smolt Atlantic salmon *Salmo salar* performance, water quality, and waste production in recirculation aquaculture systems. Aquacultural Engineering 74, 38-51.

De Santis, C., Ruohonen, K., Tocher, D.R., Martin, S.A.M., Król, E., Secombes, C.J., Bell, J.G., El-Mowafi, A., Crampton, V.O., 2016. Atlantic salmon (*Salmo salar*) parr as a model to predict the optimum inclusion of air classified faba bean protein concentrate in feeds for seawater salmon. Aquaculture 444, 70-78.

Gatlin, D.M. III, Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, K., Sealey, W., Skonberg, D., Souza, E.J., Stone, D., Wilson, R., Wurtelel, E., 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquaculture Research 38, 551-579.

Hernández, C., Hardy, R.W., Contreras-Rojas, D., López-Molina, B., González-Rodríguez, B., Domínguez-Jimenez, P., 2014. Evaluation of tuna by-product meal as a protein source in feeds for juvenile spotted rose snapper *Lutjanus guttatus*. Aquaculture Nutrition 20, 574-582.

Higgs, D.A., Balfry, S.K., Oakes, J.D., Rowshandeli, M., Skura, B.J., Deacon, G., 2006. Efficacy of an equal blend of canola oil and poultry fat as an alternative dietary lipid sources for Atlantic Salmon (*Salmo salar* L.) in sea water. I: effects on growth performance, and whole body and fillet proximate and lipid composition. Aquaculture Research 37, 180-191.

Jackson, A., 2009. Fish in-fish out, ratios explained. Aquaculture Europe 34, 1-6.

Kim, K.D., Jang, J.W., Kim, K.W., Lee, B.J., Hur, S.W., Han, H.S., 2018. Tuna by-product meal as a dietary protein source replacing fishmeal in juvenile Korean rockfish *Sebastes schlegeli*. Fisheries and Aquatic Sciences 21, 21-29.

Nengas, I., Alexis, M.N., Davies, S.J., Petichakis, G., 1995. Investigation to determine digestibility coefficients of various raw materials in diets for gilthead sea bream, *Sparus aurata*, L. Aquaculture Research 26, 185-194.

National Research Council (NRC), 2011. Nutrient Requirements of Fish and Shrimp, National Academy Press, Washington, DC, pp. 1-376.

Ouraji, H., Zaretabar, A., Rahmani, H., 2013. Performance of rainbow trout (*Oncorhynchus mykiss*) fingerlings fed diets containing different levels of faba bean (*Vicia faba*) meal. Aquaculture 416-417, 161-165.

Rahman, M.M., Han, H.S., Kim, K.W., Kim, K.D., Lee, B.J., Lee, S.M., 2016. Apparent digestibility coefficients of the extruded pellet diets containing various fish meals for olive flounder, *Paralichthys olivaceus*. Fisheries and Aquatic Sciences 19, 19-27.

Sealey, W.M., Hardy, R.W., Barrows, F.T., Pan, Q., Stone, D.A.J., 2011. Evaluation of 100% fish meal substitution with chicken concentrate, protein poultry by-product blend, and chicken and egg Concentrate on growth and disease resistance of juvenile Rainbow Trout, *Oncorhynchus mykiss*. Journal of the World Aquaculture Society 42, 46-55.

Skretting Australia, 2015. Skretting Australia Annual sustainability report 2015. Global G.A.P.

Stone, D.A.J., Bellgrove E., 2013. A literature review: the current status of knowledge of the nutritional requirements of yellowtail kingfish. In: Stone, D.A.J. and Bowyer, J.N. (Eds). Final Report. Sustainable Feeds and Feed Management for Yellowtail Kingfish (*Seriola lalandi*). South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2013/000200-1. SARDI Research Report Series No. 751. pp. 92-121.

Stone, D.A.J., Bowyer, J.N., 2013. Final Report. Sustainable Feeds and Feed Management for Yellowtail Kingfish (*Seriola lalandi*). South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2013/000200-1. SARDI Research Report Series No. 751. 220 pp.

Stone, D.A.J., D'Antignana, T., Bansemer, M.S., 2016. Final Report. Refining Yellowtail Kingfish feeds and feed management. Prepared by the South Australian Research and Development Institute (Aquatic Sciences), Adelaide. AS-CRC Project No. 2013/730. 144 pp.

Stone, D.A.J., Bellgrove, E.J., Forder, R.E.A., Howarth, G.S., Bansemer, M.S., 2018. Inducing sub-acute enteritis in yellowtail kingfish (*Seriola lalandi*): the effect of dietary inclusion of soybean meal and grape seed extract on hindgut morphology and myeloperoxidase activity. North American Journal of Aquaculture 80, 59-68.

Tacon, A.G.J., Metian, M., 2008. Global overview on the used of fish meal and fish oil in industrially compounded aquafeed: trends and future prospects. Aquaculture 285, 148-158.

Terpstra, A.H.M., 2015. The use of fish meal and fish oil in aquaculture and calculation of the fish-in-fish-out (FIFO) ratio. PhD. Universitate Vadensi, The Netherlands. pp.19.

Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquaculture Research 41, 717-732.

Van den Ingh, T.S.G.A.M., Krogdahl, Å., Olli, J.J., Hendriks, H.G.C.J.M.J., Koninkx, G.J.F., 1991. Effects of soybean-containing diets on the proximal and distal intestine in Atlantic salmon (*Salmo salar*): a morphological study. Aquaculture 94, 297-305.

Watanabe, T., Pongmaneerat, J., 1991. Quality evaluation of some animal protein sources for rainbow trout *Oncorhynchus mykiss*. Nippon Suisan Gakkaishi 57, 495-501.

Zhou, Q.C., Zhao, J., Li, P., Wang, H.L. and Wang, L.G., 2011. Evaluation of poultry by-product meal in commercial diets for juvenile cobia (*Rachycentron canadum*). Aquaculture 322, 122-127.

Table 3.1.3.1.1. The proximate, amino acid and mineral composition of four protein test ingredients.

Item (as fed)	Wild derived fish meal	Fish meal by-product	Poultry meal	Soy protein concentrate
Analysed proximate composition (g 100 g^{-1})				
Moisture	7.9	5.1	5.6	7.9
Crude protein	64.4	60.2	65.0	59.4
Crude lipid	7.8	11.0	11.3	2.2
Ash	17.0	20.2	14.1	6.4
Carbohydrate[1]	3.0	4.0	4.0	24.0
Gross energy (MJ kg^{-1})	14.30	15.00	15.90	15.00
Analysed amino acids (g 100 g^{-1})				
Alanine	3.22	3.16	3.18	2.05
Arginine	3.62	3.60	4.08	4.02
Aspartic Acid	5.12	5.06	4.26	5.99
Glutamic Acid	7.35	6.75	7.00	9.75
Glycine	3.19	3.22	4.58	1.95
Histidine	1.47	1.79	1.12	1.33
Hydroxyproline	0.54	0.70	1.53	<0.04
Isoleucine	2.47	2.50	2.22	2.53
Leucine	4.23	4.18	4.06	4.09
Lysine	4.30	4.09	3.13	3.22
Methionine	1.70	1.59	1.01	0.54
Phenylalanine	2.44	2.37	2.37	2.84
Proline	2.36	2.42	3.80	2.57
Serine	2.13	2.07	2.91	2.56
Threonine	2.38	2.40	2.24	2.05
Tyrosine	1.86	1.71	1.51	1.58
Valine	3.01	2.99	3.07	2.71
Total amino acids	51.41	50.59	52.08	49.77
Analysed minerals (mg kg^{-1})				
Calcium	45000	68000	44000	3700
Copper	5.2	4.9	5.2	6.5
Iodine	1.800	1.100	1.100	0.025
Iron	540	350	470	130
Magnesium	2900	2300	1400	3800
Manganese	15.00	4.80	11.00	36.00
Phosphorus	31000	39000	26000	7400
Potassium	8400	2900	6400	22000
Selenium	1.8	7.20	0.86	0.022
Sodium (mg 100 g^{-1})	1100	660	390	<1
Zinc	83	170	95	44

[1] Carbohydrate = 100 - (moisture + lipid + protein + ash).

Table 3.1.3.1.2. The fatty acid composition of four protein test ingredients.

Item (as fed)	Wild derived fish meal	Fish meal by-product	Poultry meal	Soy protein concentrate
Analysed fatty acids (mg 100 g^{-1})				
Saturated Fatty Acids				
C4:0 Butyric	<10	<10	<10	<10
C6:0 Caproic	<10	<10	<10	<10
C8:0 Caprylic	<10	<10	<10	<10
C10:0 Capric	<10	<10	<10	<10
C12:0 Lauric	<10	<10	<10	<10
C14:0 Myristic	476	352	90	<10
C15:0 Pentadecanoic	47	143	23	<10
C16:0 Palmitic	1771	2497	2610	295
C17:0 Margaric	55	176	34	2
C18:0 Stearic	460	924	881	84
C20:0 Arachidic	23	55	23	9
C22:0 Behenic	16	44	<10	11
C24:0 Lignoceric	<10	<10	<10	<10
Mono-unsaturated Fatty Acids				
C14:1 Myristoleic	<10	<10	23	<10
C16:1 Palmitoleic	507	462	667	<10
C17:1 Heptadecenoic	<10	<10	<10	<10
C18:1 Oleic	811	1452	4622	411
C20:1 Eicosenic	101	132	45	4
C22:1 Docosenoic	16	22	<10	<10
C24:1 Nervonic	<10	<10	<10	<10
Poly-unsaturated Fatty Acids				
C18:2n6 Linoleic	133	143	1435	1188
C18:3n6 gamma-Linolenic	16	11	11	<10
C18:3n3 alpha-Linolenic	55	33	147	143
C20:2n6 Eicosadienoic	16	33	23	<10
C20:3n6 Eicosatrienoic	16	11	23	<10
C20:3n3 Eicosatrienoic	8	22	<10	<10
C20:4n6 Arachidonic	109	275	124	<10
C20:5n3 Eicosapentaenoic	1037	550	11	<10
C22:2n6 Docosadienoic	<10	<10	<10	<10
C22:4n6 Docosatetraenoic	16	33	23	<10
C22:5n3 Docosapentaenoic	172	132	23	<10
C22:6n3 Docosahexaenoic	1537	2992	34	<10
∑LC n-3 PUFA	2746	3674	68	0
Total Saturated	2863	4279	3684	407
Total Mono-unsaturated	1435	2178	5368	418
Total Poly-unsaturated	3104	4224	1865	1333
Omega 6 Fatty Acids	289	506	1639	1190
Omega 3 Fatty Acids	2816	3718	226	143
Total Mono Trans Fatty Acids	78	22	45	<10
Total Poly Trans Fatty Acids	39	22	34	7
P:M:S Ratio	1.1:0.5:1	1:0.5:1	0.5:1.5:1	3.3:1:1

Table 3.1.3.1.3. The proximate composition, rancidity values and amino acid composition and estimated cost savings compared to Diet 1 (control diet) of the six test diets.

Diet[1]	Diet 1	Diet 2	Diet 3	Diet 4	Diet 6	Diet 7
Analysed proximate composition (g 100 g^{-1})						
Moisture	8.7	7.5	7.4	7.7	7.2	7.8
Crude protein	45.4	45.7	46.0	44.9	46.1	46.1
Crude lipid	24.1	24.8	23.9	24.7	25.0	24.3
Ash	8.9	9.0	9.8	8.4	8.8	7.8
Carbohydrate[2]	13.0	13.0	13.0	14.0	13.0	14.0
Gross energy (MJ kg^{-1})	18.80	19.10	18.90	19.10	19.30	19.20
Rancidity test						
p-Anisidine Value	5.3	5.2	3.7	4.5	5.9	5.4
Peroxide Value (mEqO2 kg^{-1})	6.3	5.9	6.5	7.5	8.3	9.1
Analysed amino acids (g 100 g^{-1})						
Alanine	1.93	1.90	1.93	1.95	1.98	1.87
Arginine	2.26	2.26	2.31	2.31	2.32	2.30
Aspartic Acid	2.92	2.87	2.92	2.91	2.95	3.04
Glutamic Acid	6.63	6.60	6.64	6.60	6.75	6.99
Glycine	2.01	2.03	2.04	2.15	2.18	1.92
Histidine	1.28	1.24	1.28	1.31	1.27	1.31
Hydroxyproline	0.34	0.38	0.38	0.45	0.45	0.29
Isoleucine	1.41	1.39	1.42	1.39	1.40	1.44
Leucine	3.06	2.99	3.09	3.07	3.11	3.12
Lysine	2.41	2.34	2.38	2.34	2.40	2.35
Methionine	1.08	1.04	1.05	1.01	1.13	1.05
Phenylalanine	1.86	1.82	1.89	1.86	1.88	1.95
Proline	2.27	2.29	2.33	2.40	2.48	2.35
Serine	1.61	1.58	1.62	1.67	1.68	1.72
Threonine	1.47	1.44	1.48	1.46	1.48	1.47
Tyrosine	1.13	1.11	1.16	1.13	1.16	1.14
Total Amino Acids	35.60	35.20	35.90	36.00	36.60	36.30
Taurine	1.02	0.99	1.03	1.08	0.98	0.98
Approx. diet cost saving ($ tonne^{-1})[3]	-	70	150	120	150	60

[1] Diet 1, Control (30%WD FM); Diet 2, 20%WD FM +10.70% FM by-product; Diet 3, 10% WD FM + 21.40% FM by-product; Diet 4, 20% WD FM + 11.32% PM; Diet 6, 10% WD FM +10.70% FM by-product dietary +11.32% PM; Diet 7, 20% WD FM +10.88% SPC (where WD = wild derived; FM = fish meal; PM = poultry meal and SPC = soy protein concentrate)

[2] Carbohydrate = 100 - (moisture + lipid + protein + ash).

[3] Approximate diet cost savings data provided by Skretting Australia (Dr Leo Nankervis, Aug 2017).

Table 3.1.3.1.4. The fatty acid composition of the six test diets.

Diet[1]	Diet 1	Diet 2	Diet 3	Diet 4	Diet 6	Diet 7
Analysed fatty acids (mg 100 g^{-1})						
Saturated Fatty Acids						
C4:0 Butyric	<10	<10	<10	<10	<10	<10
C6:0 Caproic	<10	<10	<10	<10	<10	<10
C8:0 Caprylic	<10	<10	<10	<10	<10	<10
C10:0 Capric	<10	<10	<10	<10	<10	<10
C12:0 Lauric	<10	<10	<10	<10	<10	<10
C14:0 Myristic	795	769	693	716	775	753
C15:0 Pentadecanoic	72	74	72	74	75	73
C16:0 Palmitic	4989	5208	5043	5014	5075	4909
C17:0 Margaric	96	99	120	99	100	97
C18:0 Stearic	1542	1612	1601	1655	1675	1555
C20:0 Arachidic	48	50	48	49	50	49
C22:0 Behenic	48	25	24	25	50	24
C24:0 Lignoceric	24	25	48	49	50	49
Mono-unsaturated Fatty Acids						
C14:1 Myristoleic	24	25	24	25	25	24
C16:1 Palmitoleic	1326	1364	1291	1309	1325	1337
C17:1 Heptadecenoic	<10	<10	<10	<10	<10	<10
C18:1 Oleic	6965	7266	7098	7459	7150	6926
C20:1 Eicosenic	193	174	167	173	200	194
C22:1 Docosenoic	24	<10	<10	<10	<10	<10
C24:1 Nervonic	72	74	48	49	75	73
Poly-unsaturated Fatty Acids						
C18:2n6 Linoleic	2531	2678	2677	2841	2725	2722
C18:3n6 gamma-Linolenic	24	25	24	25	50	49
C18:3n3 alpha-Linolenic	458	446	454	469	450	462
C20:2n6 Eicosadienoic	24	25	24	25	50	24
C20:3n6 Eicosatrienoic	24	25	24	49	50	24
C20:3n3 Eicosatrienoic	<10	<10	<10	<10	<10	<10
C20:4n6 Arachidonic	193	198	215	222	250	194
C20:5n3 Eicosapentaenoic	1542	1463	1267	1433	1600	1652
C22:2n6 Docosadienoic	<10	<10	<10	<10	<10	<10
C22:4n6 Docosatetraenoic	<10	<10	<10	<10	<10	<10
C22:5n3 Docosapentaenoic	241	223	191	222	250	243
C22:6n3 Docosahexaenoic	1615	1637	1577	1482	1700	1604
∑LC n-3 PUFA	3398	3323	3035	3137	3550	3499
Total Saturated	7664	7936	7696	7706	7875	7582
Total Mono-unsaturated	8628	8928	8676	9065	8800	8554
Total Poly-unsaturated	6724	6820	6501	6842	7150	7023
Omega 6 Fatty Acids	2844	3026	3011	3211	3150	3062
Omega 3 Fatty Acids	3880	3794	3489	3606	4000	3985
Total Mono Trans Fatty Acids	217	223	215	222	250	219
Total Poly Trans Fatty Acids	241	248	215	247	250	267
P:M:S Ratio[2]	0.9:1.1:1	0.9:1.1:1	0.8:1.1:1	0.9:1.2:1	0.9:1.1:1	0.9:1.1:1

[1] Diet 1, Control (30%WD FM); Diet 2, 20%WD FM +10.70% FM by-product; Diet 3, 10% WD FM + 21.40% FM by-product; Diet 4, 20% WD FM + 11.32% PM; Diet 6, 10% WD FM +10.70% FM by-product dietary +11.32% PM; Diet 7, 20% WD FM +10.88% SPC (where WD = wild derived; FM = fish meal; PM = poultry meal and SPC = soy protein concentrate)

[2] Ratio of poly-unsaturated fatty acids to mono-unsaturated fatty acids to saturated fatty acids.

Table 3.1.3.1.5. Summary of water quality parameters from the 36 week experiment.

Item[1]	Temperature (°C)	Dissolved oxygen (mg L^{-1})	Dissolved oxygen (% saturation)	pH	Salinity (mg L^{-1})	Ammonia (ppm)	CO_2 (mg L^{-1})
Mean	16.7 ± 2.8	8.1 ± 0.6	102.9 ± 5.3	7.80 ± 0.16	38 ± 0	0.07 ± 0.11	1 ± 0
Range	13.0 - 23.5	5.9 - 10.7	79.0 - 131.0	7.40 - 8.28	36 - 38	0.00 - 0.25	0 - 3

[1] Values means ± standard deviation.

Table 3.1.3.1.6. Growth performance, feed utilisation, proximate body composition, nutrient retention and the fish in-fish out ration of Yellowtail Kingfish fed different wild derived fish meal replacement diets for 36 weeks.

Diet[1,2]	1	2	3	4	6	7	ANOVA[3]
Growth performance							
Initial weight (kg)	2.52±0.01	2.52±0.02	2.53±0.02	2.52±0.01	2.53±0.01	2.52±0.01	$P = 0.981$
Final weight (kg)	4.31±0.04	4.29±0.05	4.28±0.07	4.31±0.07	4.33±0.01	4.44±0.04	$P = 0.321$
Biomass gain (kg tank^{-1})[4]	30.45±0.52	30.23±0.75	29.76±0.77	30.40±1.10	30.71±0.29	32.66±0.65	$P = 0.157$
SGR (% d^{-1})	0.21±0.00	0.21±0.00	0.21±0.00	0.21±0.01	0.21±0.00	0.22±0.00	$P = 0.120$
Initial fork length (mm)	544±2	545±1	545±1	549±1	547±2	546±1	$P = 0.165$
Final fork length (mm)	630±4	630±2	629±3	635±3	637±5	636±1	$P = 0.368$
Length growth rate (mm d^{-1})	0.17±0.01	0.17±0.01	0.16±0.00	0.16±0.01	0.18±0.00	0.17±0.00	$P = 0.163$
Final Condition factor	1.72±0.02	1.72±0.01	1.72±0.00	1.68±0.02	1.68±0.04	1.73±0.01	$P = 0.272$
Feed utilisation (as fed)							
Apparent feed consumption (kg tank$^{-1)}$	68.90±0.93	69.99±2.25	72.62±0.73	70.84±2.10	71.69±0.73	74.02±1.18	$P = 0.235$
Apparent feed intake (% BW d^{-1})	0.50±0.01	0.51±0.01	0.53±0.00	0.52±0.01	0.52±0.01	0.53±0.01	$P = 0.409$
Apparent FCR	2.26±0.04	2.32±0.04	2.44±0.05	2.33±0.05	2.33±0.01	2.27±0.08	$P = 0.193$
Proximate composition (wet basis)[4]							
Moisture (%)	63.9±0.8	63.6±0.4	63.4±0.4	62.4±1.1	64.5±0.7	63.8±0.7	$P = 0.494$
Protein (%)	19.8±0.2	19.1±0.7	19.8±0.1	19.5±0.3	19.3±0.3	19.7±0.1	$P = 0.647$
Lipid (%)	15.5±0.2	15.8±0.3	15.4±0.7	15.8±0.8	15.4±0.7	15.2±0.5	$P = 0.958$
Ash (%)	2.0±0.2	2.0±0.1	2.3±0.3	2.5±0.3	2.4±0.1	1.9±0.3	$P = 0.378$
Carbohydrate (%)	<1	<1	<1	<1	<1	<1	$P = 1.000$
Energy (MJ kg^{-1})	9.10±0.10	9.07±0.03	9.10±0.25	9.20±0.31	8.97±0.27	8.97±0.18	$P = 0.967$
Nutrient retention[5]							
Apparent PD	22.74±0.17	20.58±1.79	21.07±0.54	21.72±0.35	20.66±0.68	21.95±0.96	$P = 0.546$
Apparent ED	23.04±0.35	22.02±0.49	21.31±1.53	22.56±1.23	21.02±1.38	21.71±0.76	$P = 0.758$
Fish in-fish out ratio[6]	2.30	1.89	1.49	2.19	1.71	2.13	*NA*
Difference to Diet 1 (%)	-	-17.9	-35.1	-4.8	-25.4	-7.1	*NA*

[1] Values are mean ± SE; $n = 3$.
[2] Diet 1, Control (30%WD FM); Diet 2, 20%WD FM +10.70% FM by-product; Diet 3, 10% WD FM + 21.40% FM by-product; Diet 4, 20% WD FM + 11.32% PM; Diet 6, 10% WD FM +10.70% FM by-product dietary +11.32% PM; Diet 7, 20% WD FM +10.88% SPC (where WD = wild derived; FM = fish meal; PM = poultry meal and SPC = soy protein concentrate)
[3] A significance level of $P < 0.05$ was used for all statistical tests.
[4] Initial fish proximate composition (wet basis): Moisture 65.1%, protein 17.2%, lipid 15.4%, ash 1.9%, carbohydrate < 1%, energy 8.60 MJ kg^{-1}.
[5] ED = energy deposition; PD = protein deposition.
[6] Fish in-fish out ratio (FI-FO) = FCR × 0.75 × 0.5 × [(% fish meal in feed / 22.5) + ((% fish oil in feed - 0.08 × % fish meal in feed) / 5)] (Terpstra, 2015).

Table 3.1.3.1.7. Fatty acid composition (mg 100 g-1) of whole Yellowtail Kingfish fed different wild derived fish meal replacement diets for 36 weeks.

		Diet[1,2,3]						
	Initial	1	2	3	4	6	7	ANOVA[4]
Saturated Fatty Acids								
C14:0 Myristic	462	408.2±8.4	405.6±9.8	369.7±8.6	395.6±18.7	410.8±21.5	414.0±12.2	*P* = 0.310
C15:0 Pentadecanoic	77	46.5±0.6	47.4±0.8	46.3±2.1	47.5±2.3	46.2±2.2	45.5±1.6	*P* = 0.958
C16:0 Palmitic	3295.6	2661.6±87.1	2711.7±61.1	2567.5±119.9	2692.2±136.7	2652.7±171.6	2631.4±70.5	*P* = 0.958
C17:0 Margaric	77	51.7±5.4	63.2±1.0	61.7±2.8	47.5±2.3	61.6±2.9	55.6±5.6	*P* = 0.059
C18:0 Stearic	954.8	827.0±30.3	853.1±21.3	816.5±20.5	849.2±36.1	842.8±50.6	812.5±15.1	*P* = 0.901
C20:0 Arachidic	46.2	15.5±0.2	15.8±0.3	15.4±0.7	15.8±0.8	21.0±6.3	15.2±0.5	*P* = 0.614
C24:0 Tetracosanoic[2]	15.4	20.7±5.3	26.4±5.4	20.3±4.5	20.7±4.4	25.8±5.5	25.3±5.2	*P* = 0.887
Saturated Fat	5005	4072.5±131.6	4149.4±101.3	3929.0±160.7	4100.5±195.6	4086.9±262.5	4034.3±98.6	*P* = 0.957
Mono-unsaturated Fatty Acids								
C16:1 Palmitoleic	693	857.7±13.7	864.0±25.5	817.9±36.5	855.1±49.1	852.3±44.4	843.2±24.6	*P* = 0.944
C18:1 Oleic	5590.2	5199.3±138.6	5376.1±99.0	5303.3±229.9	5411.3±280.0	5168.6±358.7	5076.6±126.3	*P* = 0.885
C18:1 Vaccenic	NA	408.2±8.4	416.2±14.5	390.7±14.8	417.2±25.1	401.2±28.1	398.8±11.9	*P* = 0.895
C20:1 Eicosenoic	308	175.7±6.2	184.4±7.0	169.8±7.6	179.3±8.9	175.0±13.5	172.2±10.8	*P* = 0.896
C22:1 Cetoleic	46.2	82.7±5.5	89.6±6.0	77.2±3.4	89.2±1.9	82.0±5.6	81.2±7.8	*P* = 0.561
C22:1 Docosenoic	NA	15.5±0.2	26.4±5.4	15.4±0.7	21.0±5.1	15.4±0.7	15.2±0.5	*P* = 0.112
C24:1 Tetracosenoic	61.6	31.0±0.4	42.2±5.5	30.9±1.4	31.7±1.5	30.8±1.5	35.7±6.3	*P* = 0.229
Mono Unsaturated Fat	6699	6790.9±172.3	7014.5±143.3	6846.5±298.2	7026.4±361.4	6756.2±449.3	6652.8±175.5	*P* = 0.926
Poly-unsaturated Fatty Acids								
C18:2n-6 Linoleic	1386	1834.1±24.5	1859.0±32.2	1872.5±83.5	1904.0±79.7	1830.7±68.3	1804.2±53.2	*P* = 0.883
C18:3n-3 Alpha Linolenic	154	211.8±5.3	216.0±7.8	216.1±9.6	221.7±10.8	210.0±5.3	202.2±8.2	*P* = 0.651
C18:3n-6 Gamma Linolenic	15.4	15.5±0.2	15.8±0.3	15.4±0.7	15.8±0.8	15.4±0.7	15.2±0.5	*P* = 0.958
C20:2n-6 Eicosadienoic	46.2	31.0±0.4	36.8±4.9	35.7±4.2	36.6±3.9	36.4±7.0	40.1±4.0	*P* = 0.823
C20:3n-6 Dihomo-gamma-linoleic	30.8	31.0±0.4	31.6±0.5	30.9±1.4	31.7±1.5	30.8±1.5	25.6±5.7	*P* = 0.551
C20:4n-6 Arachidonic	107.8	113.5±3.7	121.0±9.8	123.5±5.5	120.9±1.9	117.6±1.8	111.8±13.2	*P* = 0.843
C20:5n-3 Eicosapentaenoic	261.8	583.0±44.2	542.8±47.9	519.9±26.6	553.9±25.8	551.3±26.5	548.3±59.7	*P* = 0.931
C22:4n-6 Docosatetraenoic	30.8	20.7±5.3	21.0±5.1	30.9±1.4	21.0±5.1	20.4±4.9	15.2±0.5	*P* = 0.268
C22:5n-3 Docosapentaenoic	154.0	232.3±7.1	231.9±15.3	231.5±10.3	237.5±11.5	224.8±8.5	218.2±23.3	*P* = 0.936
C22:6n-3 Docosahexaenoic	770.0	980.2±66.6	1006.3±89.1	1060.7±58.0	986.5±47.0	948.9±62.1	910.3±133.6	*P* = 0.843
∑LC n3 PUFA	1185.8	1795.5±117.7	1781.0±151.9	1812.0±94.7	1777.9±79.3	1725.0±96.8	1676.8±216.4	*P* = 0.979
Poly Unsaturated Fat	2987.6	4512.7±143.3	4498.5±205.8	4532.7±208.5	4567.5±189.4	4430.7±105.0	4351.7±311.4	*P* = 0.977
Total Mono Trans Fat Acids	46.2	41.5±5.6	42.2±5.5	30.9±1.4	47.5±2.3	36.4±7.0	35.1±3.9	*P* = 0.240
Total Poly Trans Fatty Acids	215.6	82.5±25.7	95.4±32.2	83.7±27.5	97.3±34.2	89.6±28.1	87.7±30.3	*P* = 0.999
Total Omega 3	1355.2	2410.0±141.6	2360.5±177.7	2377.6±116.2	2373.5±99.7	2322.8±107.3	2279.2±253.0	*P* = 0.993
Total Omega 6	1617.0	2056.1±23.4	2085.4±36.2	2108.9±90.3	2135.8±85.6	2066.7±77.7	2027.2±69.3	*P* = 0.892

[1] Values are mean ± SE; *n* = 3.

[2] Diet 1, Control (30%WD FM); Diet 2, 20%WD FM +10.70% FM by-product; Diet 3, 10% WD FM + 21.40% FM by-product; Diet 4, 20% WD FM + 11.32% PM; Diet 6, 10% WD FM +10.70% FM by-product dietary +11.32% PM; Diet 7, 20% WD FM +10.88% SPC (where WD = wild derived; FM = fish meal; PM = poultry meal and SPC = soy protein concentrate)

[3] Values for the following fatty acids < 10 mg 100 g^{-1} and were excluded from the table: C4:0 Butyric, C6:0 Caproic, C8:0 Caprylic, C10:0 Capric, C12:0 Lauric, C14:1 Myristoleic, C17:1 Heptadecenoic, C20:3n-3 Eicosatrienoic

[4] A significance level of $P < 0.05$ was used for all statistical tests, NA = not statistically analysed due to < 10 values.

Table 3.1.3.1.8. Essential and non-essential amino acid composition (g 100 g^{-1}) of Yellowtail Kingfish fed different wild derived fish meal replacement diets for 36 weeks.

		Diet[1,2]						
	Initial	1	2	3	4	6	7	ANOVA[3]
Essential								
Arginine	0.820	0.803±0.123	1.000±0.053	0.910±0.071	0.963±0.032	0.933±0.041	0.977±0.003	*P* = 0.388
Histidine	0.690	0.997±0.052	1.153±0.130	0.987±0.061	0.983±0.058	1.037±0.063	0.977±0.062	*P* = 0.574
Isoleucine	0.670	1.087±0.127	1.063±0.123	1.133±0.167	1.143±0.157	1.003±0.149	0.937±0.141	*P* = 0.903
Leucine	1.200	1.300±0.058	1.300±0.100	1.200±0.000	1.200±0.000	1.267±0.067	1.200±0.058	*P* = 0.875
Lysine	1.600	1.580±0.340	1.430±0.276	1.123±0.189	1.280±0.223	1.350±0.275	1.367±0.219	*P* = 0.624
Methionine	0.440	0.587±0.047	0.587±0.044	0.537±0.007	0.533±0.003	0.560±0.021	0.540±0.021	*P* = 0.623
Phenylalanine	0.590	0.697±0.043	0.760±0.078	0.650±0.064	0.693±0.039	0.720±0.053	0.690±0.056	*P* = 0.832
Threonine	0.690	0.750±0.010	0.797±0.041	0.700±0.050	0.733±0.007	0.747±0.012	0.683±0.044	*P* = 0.259
Valine	0.710	1.163±0.179	1.120±0.133	0.983±0.060	0.947±0.077	1.053±0.101	0.960±0.120	*P* = 0.719
Non-essential								
Alanine	0.820	1.433±0.233	1.333±0.088	1.200±0.115	1.233±0.033	1.267±0.067	1.133±0.067	*P* = 0.583
Aspartic acid	1.400	1.300±0.513	2.033±0.233	1.600±0.289	1.733±0.133	1.900±0.153	1.700±0.200	*P* = 0.571
Glutamic acid	1.900	1.900±0.458	2.400±0.153	2.233±0.176	2.400±0.115	2.333±0.088	2.333±0.033	*P* = 0.599
Glycine	0.590	1.133±0.033	1.267±0.176	1.220±0.194	1.167±0.120	1.257±0.184	1.067±0.067	*P* = 0.907
Proline	0.530	0.576±0.274	0.963±0.120	0.843±0.208	0.930±0.085	0.943±0.080	0.897±0.019	*P* = 0.537
Hydroxy proline	0.065	0.113±0.061	0.337±0.103	0.293±0.120	0.257±0.087	0.290±0.118	0.210±0.021	*P* = 0.608
Serine	0.620	0.513±0.203	0.810±0.117	0.600±0.173	0.673±0.088	0.767±0.088	0.607±0.122	*P* = 0.671
Tyrosine	0.490	0.603±0.029	0.617±0.041	0.553±0.018	0.563±0.013	0.583±0.035	0.563±0.033	*P* = 0.626
Taurine	0.140	0.220±0.020	0.217±0.009	0.210±0.010	0.207±0.007	0.213±0.009	0.247±0.042	*P* = 0.764

[1] Values are mean ± SE; $n = 3$.
[2] Diet 1, Control (30%WD FM); Diet 2, 20%WD FM +10.70% FM by-product; Diet 3, 10% WD FM + 21.40% FM by-product; Diet 4, 20% WD FM + 11.32% PM; Diet 6, 10% WD FM +10.70% FM by-product dietary +11.32% PM; Diet 7, 20% WD FM +10.88% SPC (where WD = wild derived; FM = fish meal; PM = poultry meal and SPC = soy protein concentrate)
[3] A significance level of $P < 0.05$ was used for all statistical tests.

Table 3.1.3.1.9. Mineral composition (mg kg-1) of Yellowtail Kingfish fed different wild derived fish meal replacement diets for 36 weeks.

		Diet[1,2]						
	Initial	1	2	3	4	6	7	ANOVA[3]
Calcium	5000	3700±321	4000±1222	4033±845	3800±289	4167±338	3567±669	$P = 0.989$
Copper	0.38	0.59±0.01	0.55±0.02	0.57±0.03	0.58±0.03	0.60±0.03	0.62±0.04	$P = 0.634$
Iron	18	18±1	20±1	24±7	20±1	18±1	19±0	$P = 0.694$
Magnesium	310	273±9	283±28	273±20	267±9	280±6	270±12	$P = 0.976$
Manganese	0.39	0.35±0.02	0.41±0.05	0.38±0.07	0.39±0.03	0.37±0.01	0.35±0.07	$P = 0.919$
Phosphorus	4200	3500±58	3700±800	3600±624	3400±231	3733±186	3467±463	$P = 0.994$
Potassium	3000	3200±0[a]	3167±33[a]	2967±33[b]	3100±58[ab]	3100±58[ab]	3167±33[a]	$P = 0.019$
Selenium	0.46	0.49±0.02	0.51±0.01	0.50±0.02	0.46±0.01	0.49±0.00	0.46±0.00	$P = 0.129$
Sodium	NA	827±30	837±13	813±46	863±7	843±9	833±19	$P = 0.798$
Zinc	9.7	10.9±1.2	10.1±0.4	9.7±0.8	10.2±0.4	9.8±0.2	10.4±0.6	$P = 0.822$

[1] Values are mean ± SE; $n = 3$.

[2] Diet 1, Control (30%WD FM); Diet 2, 20%WD FM +10.70% FM by-product; Diet 3, 10% WD FM + 21.40% FM by-product; Diet 4, 20% WD FM + 11.32% PM; Diet 6, 10% WD FM +10.70% FM by-product dietary +11.32% PM; Diet 7, 20% WD FM +10.88% SPC (where WD = wild derived; FM = fish meal; PM = poultry meal and SPC = soy protein concentrate)

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values within each row without a common superscript are significantly different ([a] indicates the highest value; $P < 0.05$).

Table 3.1.3.1.10. Blood biochemistry and haematology of Yellowtail Kingfish fed different wild derived fish meal replacement diets for 36 weeks.

Diet[1,2,]	1	2	3	4	6	7	ANOVA[3]
Biochemistry[4]							
Sodium (mmol L^{-1})	203.68±2.70	202.96±0.65	203.67±0.43	203.40±0.42	201.88±1.23	202.30±1.67	$P = 0.920$
Potassium (mmol L^{-1})	2.07±0.06	3.16±0.70	2.36±0.30	3.41±0.63	2.20±0.17	2.65±0.44	$P = 0.273$
Urea (mmol L^{-1})	3.06±0.16	2.91±0.19	3.24±0.28	3.60±0.35	3.13±0.30	2.97±0.23	$P = 0.494$
Creatinine (mmol L^{-1})	0.023±0.003	0.024±0.005	0.019±0.001	0.024±0.002	0.020±0.001	0.023±0.003	$P = 0.682$
Calcium (mmol L^{-1})	3.12±0.02	3.20±0.15	3.06±0.05	3.13±0.04	3.03±0.03	3.22±0.14	$P = 0.635$
Protein (g L^{-1})	38±0	39±2	39±1	40±1	37±1	38±1	$P = 0.658$
Albumin (g L^{-1})	11±0	12±1	12±0	12±0	11±0	11±0	$P = 0.571$
Globulin (g L^{-1})	27±0	28±2	28±1	28±1	26±1	27±1	$P = 0.614$
Total Bilirubin (mmol L^{-1})	1±0	2±0	2±0	1±0	1±0	1±0	$P = 0.234$
ALT (IU L^{-1})	8±1	8±1	9±1	9±1	7±1	9±0	$P = 0.519$
ALP (IU L^{-1})	28±2	29±4	31±0	32±3	28±2	26±3	$P = 0.585$
Magnesium (mmol L^{-1})	2±0	2±0	1±0	1±0	1±0	2±0	$P = 0.253$
Cholesterol (mmol L^{-1})	5±0	6±0	5±0	5±0	5±0	5±0	$P = 0.495$
Triglyceride (mmol L^{-1})	2±0	2±0	3±0	2±0	2±0	2±0	$P = 0.329$
Bile Acids (mmol L^{-1})	22±16	12±5	18±12	14±11	16±11	26±23	$P = 0.983$
Haematology[5]							
RBC ($\times 10^{12}$)	2.51±0.09	2.03±0.26	2.30±0.16	2.49±0.03	2.11±0.06	2.30±0.04	$P = 0.149$
HGB (g L^{-1})	107±6	113±3	103±2	111±3	106±2	106±2	$P = 0.286$
PCV (L L^{-1})	0.56±0.01	0.57±0.02	0.57±0.02	0.57±0.01	0.57±0.01	0.58±0.00	$P = 0.933$
MCV (fl)	184.2±1.9	187.2±2.1	184.5±3.0	184.0±1.9	173.7±11.5	186.4±1.5	$P = 0.499$
MCH (pg)	42.8±1.0	68.5±15.1	44.8±6.1	45.8±1.6	53.3±0.2	47.8±1.0	$P = 0.148$
MCHC (g L^{-1})	233±8	380±96	256±29	248±10	290±3	255±7	$P = 0.212$
WBC ($\times 10^{9}$)	7.0±0.1	6.6±0.5	7.0±0.2	6.9±0.1	6.7±0.1	6.9±0.1	$P = 0.720$
Granulocytes (%)	5±1	5±0	5±1	5±1	5±0	4±1	$P = 0.936$
Lymph (%)	95±1	95±0	95±1	95±1	95±0	96±1	$P = 0.979$
Mono (%)	0±0	0±0	0±0	0±0	0±0	0±0	NA
Eosin (%)	0±0	0±0	0±0	0±0	0±0	0±0	NA
Baso (%)	0±0	0±0	0±0	3±3	0±0	0±0	$P = 0.458$
Platelets ($\times 10^{9}$)	19±6	13±2	16±3	15±1	12±2	15±1	$P = 0.674$

[1] Values are mean ± SE; $n = 3$, SE less than 0.01 are reported as "0.00".

[2] Diet 1, Control (30%WD FM); Diet 2, 20%WD FM +10.70% FM by-product; Diet 3, 10% WD FM + 21.40% FM by-product; Diet 4, 20% WD FM + 11.32% PM; Diet 6, 10% WD FM +10.70% FM by-product dietary +11.32% PM; Diet 7, 20% WD FM +10.88% SPC (where WD = wild derived; FM = fish meal; PM = poultry meal and SPC = soy protein concentrate).

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$). NA = not statistically analysed due to zero values.

[4] ALT = alanine aminotransferase; ALP = alkaline phosphatase.

[5] Baso = basophil; Eosin = eosinophil; HGB = haemoglobin; Lymph = lymphocytes; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; MCV = mean corpuscular volume; Mono = monocytes; PCV = packed cell volume; RBC = red blood cell count; WBC = white blood cell count.

Table 3.1.3.1.11. Visceral somatic parameters, gastrointestinal tract morphology and apparent digestibility coefficients for Yellowtail Kingfish fed different wild derived fish meal replacement diets for 36 weeks.

Diet[1,2]	1	2	3	4	6	7	ANOVA[3]
Visceral somatic parameters							
Intraperitoneal fat (%)	1.68±0.20	1.52±0.25	1.78±0.09	1.62±0.13	1.77±0.16	1.71±0.31	*P* = 0.944
Visceral index (VSI; %)	5.94±0.20	5.86±0.28	6.67±0.25	6.20±0.18	6.36±0.13	6.18±0.38	*P* = 0.303
Hepatosomatic index (HSI; %)	0.85±0.06	0.85±0.04	0.91±0.07	0.90±0.05	0.83±0.03	0.89±0.08	*P* = 0.890
Stomach morphology							
Gastric dilation score[4]	0.11±0.11	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	*P* = 0.458
Hindgut morphology							
Muscularis thickness (µm)	998±81	1131±114	1109±37	1040±37	1050±45	1012±71	*P* = 0.717
Submucosa thickness (µm)	50±18	33±2	42±8	56±14	46±11	29±3	*P* = 0.536
Villi length (µm)	1419±45	1302±77	1249±141	1363±68	1384±42	1220±32	*P* = 0.427
Villi thickness (µm)	106±10	104±1	120±13	108±3	102±3	114±9	*P* = 0.598
Lamina propria thickness (µm)	17±3	14±2	21±4	15±3	13±3	18±6	*P* = 0.691
Lamina propria/villi thickness (%)	14.84±1.42	13.46±1.41	16.53±1.55	13.75±2.21	12.17±2.63	14.53±3.53	*P* = 0.862
Mucus cells per 100µm	4.39±1.02	3.05±0.65	4.73±1.30	3.61±0.53	3.18±0.27	3.11±0.69	*P* = 0.625
Eosinophilic droplets in epithelial cells	2±0	2±0	2±0	2±0	3±0	2±0	*P* = 0.656
Melanomacrophage centres	3±1	2±1	3±1	3±0	2±0	2±0	*P* = 0.162
Apparent digestibility coefficient (ADC; %)							
Dry matter	44.4±9.8^{ab}	56.5±0.8^{a}	33.3±0.0^{b}	40.4±4.2^{ab}	59.2±2.4^{a}	49.0±5.0^{ab}	*P* = 0.023
Protein	73.7±5.4^{ab}	84.5±2.4^{a}	68.4±3.4^{b}	80.7±1.4^{ab}	86.2±0.3^{a}	81.3±3.7^{ab}	*P* = 0.016
Energy	61.4±6.6	65.5±2.5	49.0±2.1	53.5±3.1	67.3±4.3	64.6±3.2	*P* = 0.055

[1] Values are mean ± SE; *n* = 3.
[2] Diet 1, Control (30%WD FM); Diet 2, 20%WD FM +10.70% FM by-product; Diet 3, 10% WD FM + 21.40% FM by-product; Diet 4, 20% WD FM + 11.32% PM; Diet 6, 10% WD FM +10.70% FM by-product dietary +11.32% PM; Diet 7, 20% WD FM +10.88% SPC (where WD = wild derived; FM = fish meal; PM = poultry meal and SPC = soy protein concentrate).
[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).
[4] Gastric dilation score based on Chown (2015).

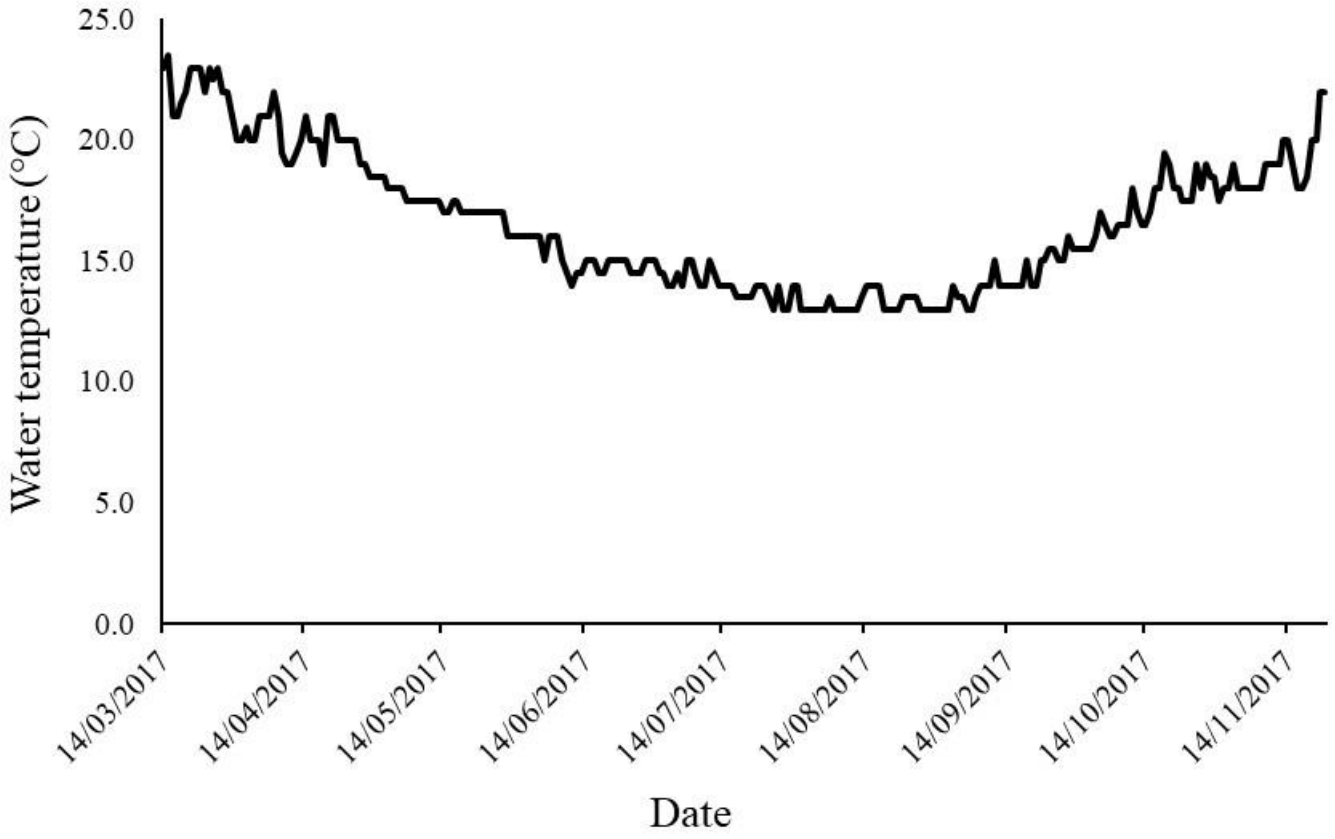


Figure 3.1.3.1.1. Water temperature profile between stocking and final weight check at harvest of the 36 week experiment (average 16.6 °C [range 23.5-13.0 °C]).

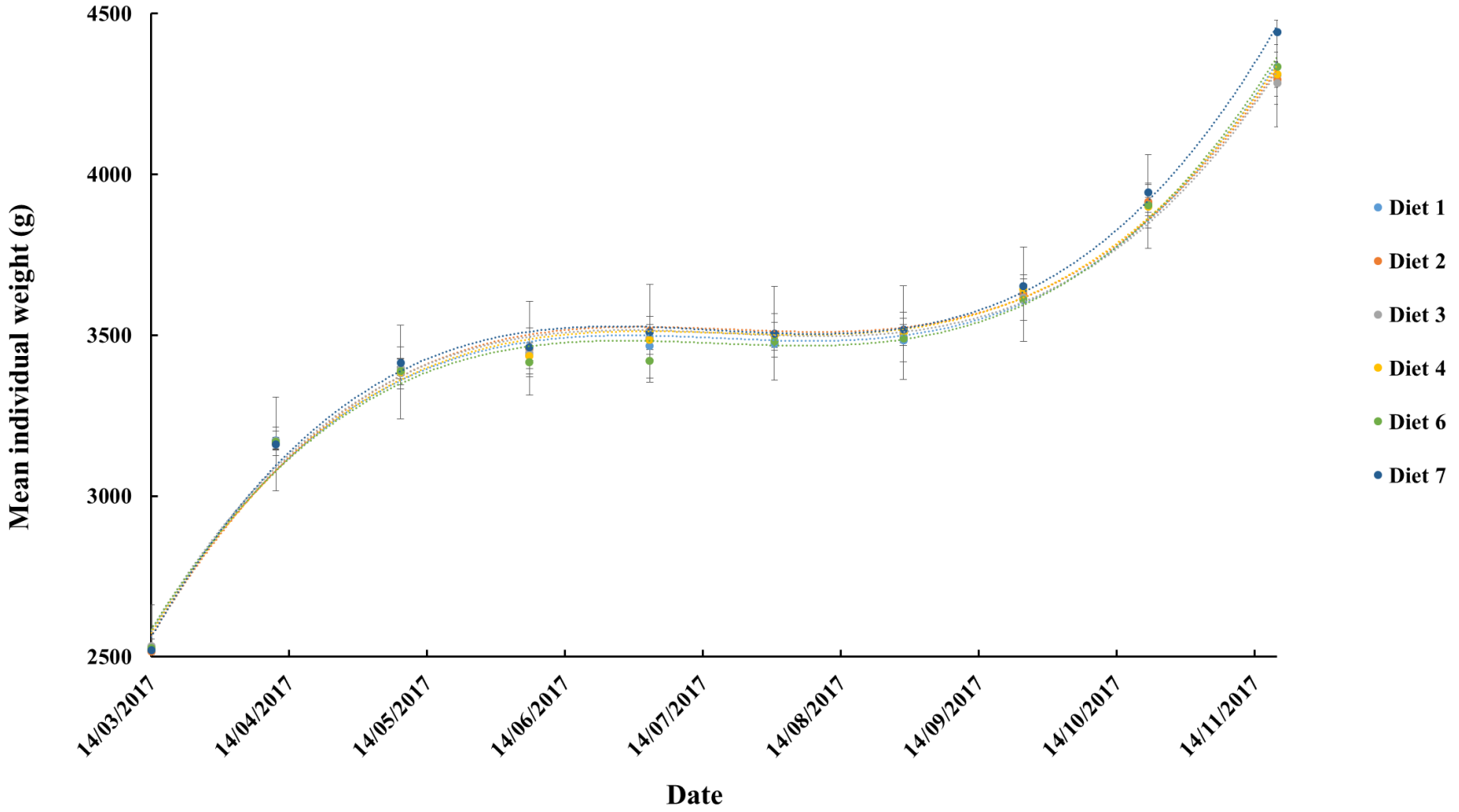


Figure 3.1.3.1.2. Mean individual weight of Yellowtail Kingfish fed different wild derived fish meal replacement diets at week 0, 4, 8, 12, 16, 20, 24, 28, 32 and 36.

Values are mean ± SE; *n* = 3.

Diet 1, Control (30%WD FM); Diet 2, 20%WD FM +10.70% FM by-product; Diet 3, 10% WD FM + 21.40% FM by-product; Diet 4, 20% WD FM + 11.32% PM; Diet 6, 10% WD FM +10.70% FM by-product dietary +11.32% PM; Diet 7, 20% WD FM +10.88% SPC (where WD = wild derived; FM = fish meal; PM = poultry meal and SPC = soy protein concentrate).

3.1.4. Chapter - Digestibility of raw materials by sub-adult Yellowtail Kingfish.

3.1.4.1. Manuscript - Apparent digestibility of common raw materials by Yellowtail Kingfish (Seriola lalandi).

Chinh T.M. Dam[a], Abigail Elizur[a], Tomer Ventura[a], Michael Salini[b], Richard Smullen[b], Igor Pirozzi[c,d] and Mark Booth[c]

[a] University of Sunshine Coast, Maroochydore, Queensland 4558, Australia

[b] Ridley, 4/31 Robart Court, Narangba, Queensland 4504, Australia

[c] New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Locked Bag 1, Nelson Bay, New South Wales 2315, Australia.

[d] College of Science and Engineering and Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville 4801, QLD, Australia

This manuscript may be referenced as: Dam, C.T.M., Elizur, A., Ventura, T., Salini, M., Smullen, R., Pirozzi, I. and Booth, M. (2019). 3.1.4.1. Manuscript - Apparent digestibility of common raw materials by Yellowtail Kingfish (*Seriola lalandi*). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.132-159.

Abstract

The apparent digestibility of 14 raw materials by Yellowtail Kingfish (*Seriola lalandi*; YTK) was examined using the diet substitution method and yttrium oxide as the inert marker. Each raw material was examined in triplicate and faecal material was collected from fish using manual stripping techniques. All raw materials were tested at 30% inclusion, except for blood meal (BLM), which was tested at 15% inclusion. The reference diet was primarily composed of fishmeal (FM). The raw materials examined included: two sources of FM (FM-1, prime quality and FM-2, recycled tuna trimmings); poultry by-product meal (PBM-1 and PBM-2); lupin kernel meal (LKM-1 and LKM-2) and soy protein concentrate (SPC-1 and SPC-2) and a single source of krill meal (KRM), meat meal (MM), BLM, faba beans (FBM), corn gluten meal (CGM) and wheat (WH). With the exception of FM-2 and BLM, marine and land animal protein sources were well digested, recording protein ADCs between 66.5-79.2%. The energy from marine and land animal protein sources was also well digested, ranging from 67.0-83.5%, with the exception of BLM, which recorded a very low energy ADC of 43.0%. Digestibility of protein from plant sources was highest in WH (97.7%), LKM-2 (95.0%), FBM (94.7%) and LKM-1 (86.3%). The energy from LKM-1, LKM-2 and FBM was also well digested (67.4-76.9%); however, energy digestibility was poor in SPC-1 (35.5%), SPC-2 (31.7%), WH (34.0%) and CGM (19.4%). Generally, the ADCs recorded from plant protein sources were more variable than the ADCs recorded from marine and land animal protein sources. Apparent digestibility of amino acids (AAs) from marine and land animal protein sources was fairly consistent and reflected the crude protein ADCs of these raw materials. The recorded ADCs of AAs from plant protein sources was more erratic and the error variance among replicates was higher than observed among replicated marine and land animal protein sources. Mean ADCs of many AAs were > 100% in FBM, LKM-2 and WH whereas the mean ADCs of AAs recorded from YTK fed CGM were close to zero and in some cases negative. The results from this study indicate that YTK are generally efficient at digesting nutrients and energy from marine and land animal protein sources. Plant protein sources such FBM, LKM-1 and LKM-2 appear to have relatively high protein and energy digestibility in YTK and may prove useful as secondary protein and energy sources in aquafeeds. The poor digestibility of the BLM and CGM used in this study suggests these products interfere with digestibility in YTK or there was some form of interaction between these raw materials and other raw materials in the reference diet. The ADCs derived for the raw materials examined in this study will assist in the formulation of research and commercial aquafeeds for this developing aquaculture species.

Introduction

One of the major objectives of the global aquaculture feed sector is to reduce its dependency on fishmeal (FM) and fish oil (FO). This is particularly important for feed producers and farmers of carnivorous fish species which have historically relied on FM and FO to fulfil the protein and energy demands of these valuable aquatic animals (Carter and Hauler, 2000; Wang et al., 2006; Hardy, 2010; Jirsa et al., 2015; Biswas et al., 2017; Moutinho et al., 2017). There are many alternative, commercially available raw materials in the global feed market. These include terrestrial animal by-product meals (e.g. poultry by-product meal (PBM), meat meal (MM), meat and bone meal (MBM), feather meal (FeM), blood meal (BLM) and numerous plant derived feedstuffs (e.g. soybean meal (SBM), canola meal (CM), lupin kernel meal (LKM) and corn gluten meal (CGM). Globally, and in Australia, there are large volumes of the aforementioned commodities produced. Therefore it seems logical they should be the primary focus of research and development by manufacturers supplying the aquafeed sector.

Formulation of a nutritionally adequate diet for any species depends critically on knowledge of the basic nutrient and energy requirements of the target animal and the judicious use of nutrient and energy digestibility data derived from the raw materials used to formulate their diets (Glencross et al., 2007). Only by having this information can the feed formulator limit the risk of formulating an inadequate feed that fails to promote or maximize growth rate and feed efficiency. Feed formulations based on highly digestible raw materials also have obvious benefits for the environment by reducing the waste generated from the undigested feeds.

Many studies have reported apparent digestibility coefficients for commercially available raw materials fed to different fish species (Storebakken et al., 1998; Bureau et al., 1999; Allan et al., 2000; Booth et al., 2001; Cheng and Hardy, 2002; Zhou et al., 2004; Tibbetts et al., 2006; Martins et al., 2009; Wang et al., 2012; Hernández et al., 2015; de Carvalho et al., 2016; Che et al., 2017; Chi et al., 2017). The majority of these studies conclude that the digestibility of raw materials are, and can be, significantly influenced by the origin of the raw material as well as the processing methods applied to it during production (e.g. grinding, cooking, rendering, extrusion, dehulling, protein concentration, fractionation etc.). Moreover, different species have different capacities to digest and utilise nutrient and energy from raw materials, generally dependent on their natural trophic feeding habits (i.e. herbivore, omnivore or carnivore).

Yellowtail Kingfish (*Seriola lalandi*; YTK) is an emerging marine aquaculture species in Australia and other parts of the world (Nakada, 1999; Kolkovski and Sakakura, 2004; Symonds et al., 2014; Martínez-Montaño et al., 2016). It is an economically profitable species (e.g. farm gate value of ≈AUD$15 kg^{-1}), which is well suited to sea-cage farming due to its rapid growth and ravenous feeding behaviour. In warmer conditions YTK can reach a marketable size of > 3-4 kg in as little as 12-15 months. In Australia, YTK have been farmed in South Australia (SA) since 2000 and this state remains the dominant producer. Annual production of YTK in SA was estimated to be approximately 2650-2850 metric tonnes in 2017 and is expected to increase steadily in the next five years (David Head; Clean Seas Seafood, *personal communication*). Offshore farming of YTK is being trialled in New South Wales (NSW) and Western Australia (WA), where small pilot farms are evaluating the potential of this species.

Despite the potential growth of the YTK industry in Australia and the increased demand for high quality sustainable aquafeeds there remains a dearth of knowledge with regard to the digestibility of raw materials by this species. Significant gains in knowledge have been made in previous years with regard to understanding the basic nutritional requirements of YTK (Booth et al., 2010a; Booth et al., 2013b; Stone and Bellgrove, 2013), but a comprehensive data-base on the digestibility of common raw materials is yet to be collated. This is most likely due to the inherent difficulty in determining the digestibility of raw materials from problematic species like YTK (see Booth and Pirozzi, 2017). Despite the well documented challenges involved in conducting digestibility experiments with YTK, the growth of the YTK industry in Australia will be constrained unless more information on the digestibility of raw materials becomes available. Only then can new commercial aquafeeds be formulated on a digestible nutrient and energy basis. Formulating aquafeeds on this basis will ensure there is less risk of undersupplying the nutrient and energy requirements of farmed YTK which in turn should lead to increased levels of production and greater economic benefit for farmers. It will also be difficult to reliably decrease the use of FM and FO in commercial feeds for YTK unless data on other raw materials becomes available.

The aim of this study was to determine the digestibility of 14 common raw materials for YTK and to provide the Australian YTK industry with a raw material data-base that documents the apparent digestibility coefficients (ADCs) of dry matter, crude protein, fat, gross energy and amino acids for each product. The raw materials examined included: two sources of FM (FM-1, prime quality and FM-2, recycled tuna trimmings); two PBM (PBM-1 and PBM-2); two LKM (LKM-1 and LKM-2) and two soybean meal concentrates (SPC-1 and SPC-2) and a single source of krill meal (KRM), MM, BLM, faba beans (FBM), CGM and wheat (WH). All raw materials examined in this study are commonly used in Australian aquafeeds and were obtained from the one source (Ridley, Narangba, QLD).

Materials and Methods

Animal ethics statement

Experiments were done under the authority granted by the NSW Department of Primary Industries (NSW DPI) Fisheries Animal Care and Ethics Committee (Aquaculture Nutrition ACEC Authority 93/5-Port Stephens) and the Animal Ethics Committee of the University of the Sunshine Coast (AN/S/16/46).

Overview of experimental approach

Two 4-week digestibility experiments were conducted. Experiment 1 determined the digestibility of FM-1, FM-2, PBM-1, MM, LKM-1, FBM and CGM. Experiment 2 determined the digestibility of KRM, PBM-2, BLM, LKM-2, SPC-1, SPC-2 and WH. The same reference (REF) diet was used in both experiments. With the exception of BLM (15% inclusion), the dietary inclusion content of all raw materials examined was set at 30%. The digestibility of the diets and ingredients were determined applying indirect methodology and yttrium oxide (Y_2O_3; Merck, Technipur™, Darmstadt, Germany) was employed as the non-digestible marker. The YTK used in both experiments were from the same cohort and were obtained from the NSW DPI Port Stephens Marine Finfish Hatchery. The average water temperature, pH and dissolved oxygen concentration of both experiments were 20.3 ± 0.1 ^oC; 7.7 ± 0.1 and 9.33 ± 0.3 mg L^{-1} respectively. Faecal material was collected from sedated YTK on a weekly basis using manual stripping methods similar to that described by Booth and Pirozzi (2017). Three replicate cages of YTK (n = 3) were assigned to each of the test diets examined in Experiment 1 and Experiment 2, respectively.

Raw materials and diet preparation

The common reference mash was composed predominantly of prime FM (68%), wheat flour (27%) and FO (3.5%) as well as a vitamin / mineral premix (0.66%). Yttrium oxide was added into the reference mixture at a rate of 20 g yttrium oxide kg^{-1} diet. The formula and nutrient composition of the reference diet is presented in Table 3.1.4.1.1.

The measured proximate and energy composition of raw materials is presented in Table 3.1.4.1.2 and the amino acid composition of raw materials is presented in Table 3.1.4.1.3.

In preparation for diet manufacture the raw materials were milled through a high speed Retsch rotor-mill fitted with a 750 um screen prior to incorporation in test diets. Raw materials were then mixed with the reference mash at the appropriate ratio (Table 3.1.4.1.4 and Table 3.1.4.1.5) on a dry matter basis before being manufactured into extruded pellets using a twin-screw extruder with intermeshing, co-rotating screws (MPF24:25, Baker Perkins, Peterborough, United Kingdom). All diets were extruded operationally through an 8 mm pellet die at the same operating parameters for consistency. Newly manufactured diets were oven dried at 45 ^oC to lower moisture content to < 12%, cooled and sealed in plastic bags and stored frozen (-20 ^oC) until used. Diets were manufactured at the CSIRO Bribie Island Research Centre (Woorim, 4507, QLD, Australia) and shipped to PSFI prior to use in experiments. All diets were stored at -17 °C prior to use in experiments.

Experimental systems, allocation of replicates and faecal collection

Yellowtail Kingfish were progeny of wild broodstock held at the NSW DPI Port Stephen Fisheries Institute (PSFI). Prior to trials YTK were reared in large holding tanks (10 kL) and fed a commercial marine finfish diet (Ridley, Narangba, QLD, Australia.

Each digestibility experiment was done in two 20 kL recirculating aquaculture systems (RAS). Each RAS consisted of two large circular 10 kL tanks (height 1.1 m; diameter 3.5 m) connected to ancillary equipment including a 2 kw pool pump, large sand filter and a 1kL rotating biological contactor (i.e. a bio-filter). Tanks were aerated with compressed air as well as oxygen using fine bubble diffusers. Water was circulated through the tanks at a flow rate of 600 L h^{-1} and approximately 40% was directed to waste to allow a similar amount of fresh top-up water to be continuously added to each system. The 10 kL tanks were also vacuum siphoned on a daily basis to remove settled solids and ensure water quality was maintained at suitable levels. Water temperature in each RAS was controlled to ± 2 °C using a reverse cycle heat exchanger.

Six 200 L floating cylindrical cages made from 10 mm oyster mesh were placed in each of the 10 kL tanks and secured in place to the rim of the tank. All cages were fitted with lids to prevent the escape of fish. This arrangement provided n = 3 replicate cages for each of the 8 dietary treatments evaluated in Experiment 1 and Experiment 2 (i.e. the reference diet and 7 test diets per experiment). Dietary treatments were assigned to cages such that no replicate cage from the same dietary treatment occurred in the same 10 kL tank more than once. In addition, replicate cages were re-allocated to a different tank after each faecal stripping event. These procedures were employed to avoid confounding effects on treatments due to the pseudo-replication of cages.

Six sub-adult YTK weighing 573.9 ± 17.6 g (mean ± S.D) were placed into each replicate cage at the beginning of the experiment. Fish were hand fed their respective test diets twice daily (i.e. 09:00 h and 14:00 h) to excess at a fixed rate of 2.5% of the total biomass per cage. All fish were allowed to acclimatise to their dietary treatment for one week prior to handling and collection of faecal material.

Faecal samples were collected from each fish within each cage using stripping techniques similar to those described by Booth and Pirozzi (2017). Fish were netted directly from their respective cage and placed immediately into a 200 L aerated tank containing anaesthetic (AQUI-S®) until they lost equilibrium. Fish were removed from the sedation tank and the ventral surface was wiped clean before a small amount of pressure was applied to the abdomen to expel urinary products. The ventral area was again wiped clean before faecal material was expelled from the distal intestine into a clean 70 mL container using gentle abdominal pressure. Hands were rinsed between the handling of fish and care was taken to ensure that the faecal samples were not contaminated by urine or mucous. Faecal samples were immediately stored in a freezer at -17 °C. After stripping fish were returned to their respective experiment cages to recover. Faecal samples were generally collected about 6 h after the last meal and fish were never stripped on consecutive days. Faecal samples from the same cage were pooled over time and kept frozen at -17 °C before being freeze-dried in preparation for analysis.

Chemical analysis

Raw materials, diets and faecal samples were analysed for dry matter, nitrogen, crude lipid, ash, gross energy and amino acids. In addition, all diets and faecal samples were analysed for yttrium. Where presented carbohydrate content (NFE) was calculated by difference (NFE = 1000 – crude protein – crude lipid – ash). Sample dry matter was calculated by gravimetric analysis following oven drying at 105 ºC for 24 h. Crude protein (N × 6.25) of samples was based on the determination of total-nitrogen by Leco auto-analyser. Amino acid composition of samples was determined using high-pressure liquid chromatography (HPLC) following acid hydrolysis; therefore amino acids destroyed by acid hydrolysis are not reported. Crude lipid content was determined gravimetrically following extraction of lipids by chloroform and methanol (2:1 v/v) according to the method of Folch et al. (1957). Ash content was determined gravimetrically following loss of mass after combustion in a muffle furnace at 550 °C for 12 h. Gross energy was measured by adiabatic bomb calorimetry. Yttrium was determined based on the method of McQuaker et al. (1979) using inductively coupled plasma mass-spectrophotometry following mixed-acid digestion (ICP-MS: ELAN DRC II, Perkin Elmer).

Calculation of apparent digestibility coefficients of diets and raw materials

The apparent digestibility of nutrients and energy in the reference and test diets (i.e. $ADC_{test\ diet}$) was calculated using the following formula:

$$ADC_{diet} = \left(1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}}\right)\right) \times 100$$

where Y_{diet} and Y_{faeces} represent the yttrium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of interest (i.e. dry matter, crude protein, lipid, gross energy or amino acid) in the diet and faeces, respectively (Maynard et al., 1979). The apparent digestibility of nutrients and energy in raw materials was calculated according to the formulae:

$$Nutr.AD_{ingredient} = \frac{\left(AD_{test} \times Nutr_{test} - \left(AD_{ref} \times Nutr_{ref} \times P_{ref}\right)\right.}{\left(P_{ingredient} \times Nutr_{ingredient}\right)}$$

where $Nutr.AD_{ingredient}$ is the apparent digestibility of a given nutrient or energy in the raw material; AD_{test} is the apparent digestibility of the test diet; AD_{ref} is the apparent digestibility of the reference diet; $Nutr_{Ingredient}$, $Nutr_{test}$ and $Nutr_{ref}$ are the level of the nutrient or energy of interest in the raw material, test diet and reference diet, respectively (Sugiura et al., 1998). $P_{Ingredient}$ and P_{ref} are the proportional amount of test ingredient and reference diet respectively. The nutrient and energy content of all test diets was corrected for minor discrepancies in analytical results to ensure the dry matter ratio of the reference diet and raw material summed to 100% (Bureau et al., 1999).

Statistical analysis

Differences in the apparent digestibility of diets were examined using one-way ANOVA after ensuring data satisfied the assumptions of normality and homogeneity of variances. Where ANOVA proved significant a Dunnett's two-sided multiple comparison test with control was used to compare the apparent digestibility coefficients of the test diets to the reference diet (i.e. the control) in each experiment in order to simplify dietary comparisons. Raw material ADCs were compared based on their origin (i.e. marine and land animal protein sources or plant based protein sources) using ANOVA and if ANOVA proved significant the Tukey-Kramer multiple comparison test was used to separate treatment means. Statistical tests were undertaken using SPSS version 22.0 (SPSS, Michigan Avenue, Chicago, IL, USA). Limits for all critical ranges were set at $P < 0.05$.

The overall digestibility of raw materials was explored using hierarchical clustering. This was done by jointly examining the dry matter, protein and lipid ADCs of all raw materials in a single analysis in order to form a broader view of the similarity / dissimilarity among raw materials. Energy was not considered as it is derived from these macronutrients. The analysis was done on unscaled data using Euclidian distances and a group average (unweighted pair-group) clustering method. The cluster cut-off value was set at 20. The fit of the model was confirmed with a high value for the *Cophenetic* correlation coefficient of 0.793 (NCSS).

Results

Apparent digestibility of the reference diet in Experiment 1 and Experiment 2

The same reference diet was used in Experiment 1 and Experiment 2, however the experiments were done at different times using different animals. Therefore, ANOVA was used to examine whether there was any difference between the respective ADCs of the reference diet between experiments. ANOVA on each parameter indicated there was no significant difference in the dry matter ($F_{1,4} = 0.38$; $P = 0.57$), protein ($F_{1,4} = 2.21$; $P = 0.21$), lipid ($F_{1,4} = 1.13$; $P = 0.36$), gross energy ($F_{1,4} = 0.46$; $P = 0.53$) or average amino acid ADCs ($F_{1,4} = 0.07$; $P = 0.81$) of the reference diet from either experiment. The mean ± SE dry matter, protein, lipid and gross energy ADCs of the reference diet in Experiment 1 were 42.1 ± 3.8, 65.1 ± 3.0, 84.7 ± 2.6 and 56.8 ± 4.0, respectively (Table 3.1.4.1.6). The mean ± SE dry matter, protein, lipid and gross energy ADCs of the reference diet in Experiment 2 were 39.2 ± 2.6, 58.7 ± 3.1, 81.6 ± 1.5 and 53.9 ± 1.6, respectively (Table 3.1.4.1.8). In addition there was close agreement between the average amino acid digestibility recorded for the reference diet in either experiment (Exp.1 = 65.8% vs Exp.2 = 64.7%; Table 3.1.4.1.7 and Table 3.1.4.1.9).

Apparent digestibility of test diets in Experiment 1

The ADCs of dry matter, protein, lipid and gross energy of test diets from Experiment 1 are tabulated in Table 3.1.4.1.6 and the apparent ADCs of dietary amino acids is presented in Table 3.1.4.1.7. There were highly significant differences among the dry matter ADCs of test diets in Experiment 1 ($F_{7,16} = 7.45$; $P = 0.0005$), however according to the Dunnett's comparison only the dry matter ADC of the CGM diet was different to the dry matter ADC of the reference diet (i.e. control diet). There was a significant difference among the protein ADCs of test diets ($F_{7,16} = 6.54$; $P = 0.0009$). According to the Dunnett's comparison the protein ADC of CGM diet was significantly lower than that of the reference diet. Similarly, while there were significant differences among the average AA ADCs of test diets ($F_{7,16} = 15.16$; $P < 0.0001$), only the average AA ADC of the CGM diet (lower) and the FBM diet (higher) were different to the reference control diet (Dunnett's comparison). There was a significant difference among the lipid ADCs of diets ($F_{7,16} = 19.07$; $P < 0.0001$). In this case the Dunnett's comparison indicated the lipid ADC of the CGM and MM diets were significantly lower than the lipid ADC of the reference diet. The gross energy ADC of test diets was also different ($F_{7,16} = 10.05$; $P < 0.0001$). The gross energy ADC of the CGM diet was significantly lower than the gross energy ADC of the reference control diet (Dunnett's comparison).

Apparent digestibility of test diets in Experiment 2

The ADCs of dry matter, protein, lipid and gross energy of test diets from Experiment 2 are tabulated in Table 3.1.4.1.8 and the apparent ADCs of dietary amino acids is presented in Table 3.1.4.1.9. There were significant differences among the dry matter ADCs of test diets in Experiment 2 ($F_{7,16} = 6.5$; $P = 0.0009$), however Dunnett's comparison test found no significant difference between the dry matter ADC of the reference diet and the DM ADC of the other seven diets. There was a significant difference among the protein ADCs of test diets in Experiment 2 ($F_{7,16} = 4.94$; $P = 0.004$). According to the Dunnett's comparison the protein ADC of LKM-2 diet was significantly higher than that of the reference diet. Similarly, while there were significant differences among the average AA ADCs of test diets ($F_{7,16} = 8.96$; $P = 0.0002$), only the average AA ADC of the LKM-2 diet was significantly higher than the average AA ADC of reference diet (Dunnett's comparison). There was a significant difference among the lipid ADCs of diets ($F_{7,16} = 3.0$; $P = 0.032$), but no difference between the lipid ADC of the reference diet and that of any other diet (Dunnett's comparison). The gross energy ADC of test diets was also different ($F_{7,16} = 8.09$; $P = 0.0003$), however there was no difference between the gross energy ADC of the reference diet compared to the gross energy ADC of any other diet in Experiment 2 (Dunnett's comparison).

Apparent proximate digestibility of raw materials

The raw materials examined in this study were grouped into two categories based on their origin; marine and land animal based protein and energy sources (Table 3.1.4.1.10) or plant based protein and energy sources (Table 3.1.4.1.11). Generally, there were less error variances associated with the replicate ADCs for marine and land animal protein sources than there was among the plant protein sources. One spurious replicate associated with the dry matter and gross energy ADC of BLM and one protein ADC from the MM group was excluded from interpretation as the calculated coefficients were extremely low compared to their sister replicates. One replicate value associated with the lipid ADC of WH and the lipid ADC of FBM was also excluded from interpretation due to each value being unusually low. Calculated ADCs for CGM were highly variable, recording both positive and negative coefficients with respect to lipid and gross energy ADCs. CGM also recorded one unusually low value for the ADC of crude protein. All data on the digestibility of CGM was subsequently excluded from statistical analysis. All details regarding the inclusion and exclusion of replicate raw material ADCs are included in the footnotes to Table 3.1.4.1.10 and Table 3.1.4.1.11. Lipid ADCs for BLM, SPC-1, SPC-2 and CGM were not determined as the lipid content of these raw materials was negligible.

Examination of the ADCs of the raw materials grouped into the marine and animal meal category (Table 3.1.4.1.10) indicated there was a significant difference ($F_{6,13} = 3.39$; $P = 0.030$) between the dry matter ADC of FM-2 (43.8%) and the dry matter ADC of MM (69.6%). There was a significant difference among the protein ADCs of the raw materials in this category ($F_{6,13} = 8.25$; $P = 0.0008$). Protein ADC was lowest in BLM (50.6%) and highest in MM (92.4%). The lipid ADCs of the raw materials (excluding BLM) were different ($F_{5,12} = 11.58$; $P = 0.0003$), with the lipid ADC of MM (52.2%) being significantly lower than FM-1 (93.8%). Gross energy ADCs of marine and land based protein sources were also different ($F_{6,13} = 3.81$; $P = 0.021$). In this case the energy ADC of BLM (43.0%) was significantly lower than FM-1 (74.9%) and MM (83.5%).

The dry matter ADCs of raw materials grouped into the plant protein source category were different ($F_{5,12} = 6.65$; $P = 0.004$). Dry matter ADCs for SPC-1, SPC-2, CGM, WH and FBM were all < 36%, whereas the dry matter of LKM-2 and LKM-1 were > 53% (Table 3.1.4.1.11). The protein ADCs of plant sources were also different ($F_{5,12} = 6.04$; $P = 0.005$). Protein digestibility was lowest in CGM (31.4%) and SPC-2 (45.3%), intermediate in SPC-1 (62.5%) and LKM-1 (89.9%) and slightly above 100% in LKM-2, FBM and WH (Table 3.1.4.1.11). Fat digestibility was significantly different among the limited number of plant protein sources ($F_{3,6} = 8.95$; $P = 0.012$). Fat digestibility was lowest and most variable in WH (44.0%), intermediate in FBM (70.6%) and LKM-1 (88.9%) and highest in LKM-2 (93.1%). The gross energy ADCs among plant sources were different ($F_{5,12} = 8.60$; $P = 0.0012$). CGM recorded the lowest gross energy ADC (19.4%), whereas the energy ADCs of SPC-2, WH and SPC-1 were tightly grouped between 31.6% and 35.6% (Table 3.1.4.1.11). The energy ADCs of LKM-2, FBM and LKM-1 were highest and ranged from 67.3% to 76.8%.

The AA digestibility of raw materials categorised into marine and land animal based protein and energy sources or plant based protein and energy sources are presented in Table 3.1.4.1.12 and Table 3.1.4.1.13, respectively. Amino acid ADCs were reasonably stable among the marine and animal protein sources and somewhat more erratic among the plant sources. There was a large degree of variation among the digestibility of AAs. Exploration of the relationship between protein ADCs and average AA ADCs of the marine and land animal sources found there was a highly significant linear relationship between the variables ($P < 0.05$). The equation of the straight line relating average AA ADCs and protein ADCs was estimated as: average AA ADC = 1.1321 x protein ADC -15.2199 ($R^2 = 0.80$; $n = 20$) (Figure 3.1.4.1.1a). Exploration of the relationship between protein ADCs and average AA ADCs of the plant sources also found a highly significant linear relationship between the variables ($P < 0.05$). The equation of the straight line relating average AA ADCs and protein ADCs of plants was estimated as: average AA ADC = 1.0022 x protein ADC -4.1565 (($R^2 = 0.88$; $n = 20$) (Figure 3.1.4.1.1b).

The overall digestibility of the raw materials was explored using hierarchical clustering. The dendrogram is presented in Figure 3.1.4.1.2. Based on the aforementioned dendrogram inputs the analysis indicated there were five distinct clusters; one that grouped most of the marine and land animal sources together (i.e. FM-1, FM-2, KRM, PBM-1, PBM-2, BLM); one that grouped the lupin sources together (LKM-1, LKM-2); one that grouped the refined plant protein sources together (i.e. SPC-1, SPC-2 and CGM) and

one that grouped FB and WH. MM appeared to be dissimilar to most other cluster groups (Figure 3.1.4.1.2).

Discussion

Determination of the nutrient and energy digestibility coefficients of common raw materials is critical to appropriately formulate cost-effective and sustainable feeds for high value species such as YTK (Glencross et al., 2007; Booth and Pirozzi, 2017). However, the accuracy and reproducibility of digestibility coefficients is often questionable and is known to be influenced by the collection method (e.g. either active or passive collection). Active collection methods include manual stripping and dissection which potentially underestimate digestibility because of incomplete digestion and potential contamination of faeces with endogenous material. Active collection also estimates an instantaneous measure of digestibility because samples are usually collected once daily at a similar time point. In contrast, faecal material collected by passive techniques such as settlement (Allan et al., 1999) can potentially overestimate the digestibility of diets and raw materials because of nutrient leaching.

The reproducibility of the techniques adopted in this study was examined by including the same reference diet in each experiment. The results indicated the absolute difference between dry matter, protein, lipid and gross energy ADCs, respectively, of the reference diet from either experiment was only 2.9, 6.4, 3.1 and 2.9 percentage units. These minor numerical differences in ADCs resulted in no statistical differences being identified in the respective proximate categories and energy digestibility of the reference diet between experiments. Furthermore, there was also very close agreement between the ADCs of individual amino acids and the average amino acid ADCs of the reference diet from each experiment (i.e. 65.8% vs 64.7%). Considered together these results greatly increased the level of confidence held in the reference diet ADCs and their subsequent use in the calculation and estimation of raw material digestibility coefficients.

Of great interest in the present study was whether the methodology used with YTK was sensitive enough to discriminate between similar raw materials that were perceived to have lower or higher digestibility. For example, the prime quality FM (FM-1) was expected to be more digestible than the FM made from recycled fish trimmings (FM-2). While there was no statistical difference between the dry matter, protein (and amino acid) or gross energy ADCs of the two FM products (due to the high standard errors), there was clearly a decrease of about 10% in the ADCs of the recycled fishmeal compared to the prime FM. Little difference was found between the proximate ADCs of the PBM, but the dry matter and protein ADCs of PBM-2 tended to be higher than PBM-1. Both FM-1 and PBM-2 had higher content of crude protein and lower content of ash than FM-2 and PBM-1, which indicates these products are probably of higher quality. This might partly explain the improvement in ADCs of these raw materials. The dehulled lupin kernel meals were of different origin. LKM-1 was slightly higher in protein and fat content than LKM-2 and importantly lower in NFE content. The elevated NFE of LKM-2 may explain the lower dry matter and gross energy ADCs recorded for it and why the protein and lipid ADCs of these products were almost similar. Finally, there was little discrepancy between the dry matter and energy ADCs of SPC-1 and SPC-2 and although the difference in protein ADC of the products was not significant, the protein ADC of SPC-1 was far higher. Again, SPC-1 was lower in NFE and slightly higher in crude protein than SPC-2. These results indicate that the faecal collection methods employed in this study are robust enough to broadly discriminate between the digestibility of similar raw materials, at least when using apparent digestibility as a measure of raw material quality. Greater confidence in the apparent ADCs will come from increasing the number of replicates assigned to dietary treatments.

Little comparable data has been published on the digestibility of raw materials by YTK, however a recently published study that used similar methodology indicated the dry matter, protein, fat and gross energy ADCs of extruded wheat were approximately 40.2%, 81.5%, 67.1% and 42.1%, respectively when included at 40% of the diet (Booth and Pirozzi, 2017). These values are reasonably similar to the values found for wheat flour included at 30% in this study. Other reports on the digestibility of raw materials by YTK also determined using stripping techniques are available (Booth et al., 2010b). That study indicated the dry matter, protein, energy and fat ADCs for Peruvian fishmeal were 66%, 80.5%, 81.7% and 92.4% respectively. Interestingly, the apparent digestibility of a CGM from the study of Booth et al. (2010b) was very low compared to that of a wheat gluten product. The low ADCs found for CGM in both the present study and that of Booth et al. (2010b) reflects similar results for Japanese

Flounder (*Paralichthys olivaceus*) (Deng et al., 2010), Silver Perch (*Bidyanus bidyanus*) (Allan et al., 2000) and juvenile *Pseudobagrus ussuriensis* (Che et al., 2017). In contrast digestibility of CGM was higher in Cobia (*Rachycentron canadum*) (Zhou et al., 2004), Atlantic Salmon (*Salmo salar*) (Anderson et al., 1992) and Rainbow Trout (*Oncorhynchus mykiss*) (Glencross et al., 2005; Glencross et al., 2003). The individual AA digestibility of CGM was also low and highly variable, which is consistent with the results of Masumoto et al. (1996) who reported low AA availability of CGM (46.8%) in Japanese Yellowtail (*Seriola quinqueradiata*). These authors hypothesized this was due to the low pH of CGM (pH = 3.2) and its interference with amino acid digestibility, which is supposedly better when pH was > 5.0 (Masumoto et al., 1996). In light of the consistently poor digestibility of CGM in this and previous studies, it would appear prudent to restrict the amount of CGM in the diets of YTK, at least until reasons for the low digestibility can be confirmed. In addition, the relatively poor ADCs recorded for the SPC-1 and SPC-2 suggest incorporation of plant proteins having a high level of refinement may overwhelm the digestive system of YTK. Whether this effect is due to the presence of residual anti-nutritional factors in SPC or simply caused by the high level of substitution (i.e. 30%) remains unclear. These specific raw materials were also grouped by the hierarchical cluster analysis, indicating the overall value of these raw materials to YTK was similar when based on their collective dry matter, protein and lipid ADCs, respectively.

Overall, the average proximate ADCs presented in this study tend to be lower than those cited in other published work. However when considered in terms of the 95% upper confidence limits (95% UCL; Table 3.1.4.1.11 and 3.1.4.1.12), they reflect those commonly cited in the literature. For example Masumoto et al. (1996) reported that protein digestibility of brown fishmeal was 88.7% in Japanese Yellowtail. Similarly, the protein digestibility cited for fishmeal products tested on species such as Rose Spotted Snapper (*Lutjanus guttatus*) (Hernández et al., 2015), Cobia (Zhou et al., 2004; Chi et al., 2017), Mulloway (*Argyrosomus japonicas*) (Booth et al., 2013a), Atlantic Salmon (*Salmo salar*) (Storebakken et al., 1998; Sugiura et al., 1998) and Rainbow Trout (*Oncorhynchus mykiss*) (Alexis et al., 1998; Bureau et al., 1999; Cheng and Hardy, 2002) were greater than 80%. The average digestibility of the two most limiting AAs to animals, lysine and methionine, of FM-1 were 70.5% and 80.2%, respectively. These values are somewhat lower than that reported for Japanese Yellowtail fed brown FM (93.1% vs 92.2 %) (Masumoto et al., 1996). The differences in nutrient digestibility of FM-1 and FM from other international studies is likely related to several factors such as the source of FM, processing method, size of fish, environmental conditions or the faecal collection method.

Apart from BLM, the apparent digestibility of the rendered animal proteins in this study was similar to the ADCs recorded for the marine and land animal proteins. The protein ADC of MM was particular high compared to the other rendered meals, but there is no simple explanation for this result. Nonetheless, the results highlight the potential of using rendered animal meals to replace significant levels of fishmeal in diets for YTK and they support the conclusions made by previous studies on marine fish (Booth et al., 2013c; Hatlen et al., 2015; Ji et al., 2017; Moutinho et al., 2017; Dawson et al., 2018). Digestibility of dry mater and protein from BLM was inferior to the other marine and land animal meals, indicating that YTK could not digest this particular batch of BLM effectively. The average amino acid digestibility of BLM was also somewhat lower than that predicted using crude protein (nitrogen × 6.25). This suggests there was possibly a small amount of non-protein nitrogen present in this batch of BLM. In contrast, results reported by Chi et al. (2017) for Cobia and other species including Rainbow Trout (Bureau et al., 1999; Bureau et al., 2000), Red Drum (*Sciaenops ocellatus*) (McGoogan and Reigh, 1996), Silver Perch (Allan et al., 2000) and Mulloway (Booth et al., 2013a) indicate BLM has high digestibility. The low digestibility of BLM can be due to excessive heat during the rendering and drying process which can lead to damaged proteins and induce covalent cross-linking protein (Cho et al., 1982).

In line with most studies, dry matter and gross energy ADCs measured in YTK were lower for plant products having higher carbohydrate (NFE) content (Masumoto et al., 1996; Allan et al., 2000; Zhou et al., 2004; Yuan et al., 2010; Zhang et al., 2013; Chi et al., 2017). The decline in dry matter and energy digestibility appears to be more significant where the NFE is in the form of non-starch polysaccharides (Lupatsch et al., 1997; Glencross et al., 2012). Regardless, the protein and amino acid ADCs of FBM, LKM-1, LKM-2 and WH approached 100%, meaning these raw materials may be used as background protein sources which aid in control of the bulk-density of finished feeds (Table 3.1.4.1.13). The high protein digestibility of legumes has been reported by others. For example, omnivorous silver perch could digest about 90.5% of protein from FBM (Allan et al., 2000) and ADC protein of 97.1% and 95.9%,

respectively of protein from *Lupinus angustifolius* meal and *L. albus* meal (Booth et al., 2001). Glencross et al. (2004) reported that protein digestibility of LKM was 130.4% in Atlantic salmon.

Strong linear relationships were found between crude protein ADCs and the average amino acid digestibility of marine and land animal proteins sources and plant protein sources (Figure 3.1.4.1.1a and Figure 3.1.4.1.1b). These relationships lend some weight to the veracity of the protein and amino acid data-set and allow prediction of the average digestibility of amino acids in raw materials. However, individual amino acid ADCs varied widely among and within different raw materials, indicating they should not be approximated from protein ADCs or the average value of amino acid ADCs. Therefore, where available, data on individual AAs should be used to populate feed formulation software. This will reduce the risk of undersupplying digestible AAs to YTK when combining different raw materials.

Conclusions and Recommendations

The digestibility coefficients determined in this study will be useful in formulating new commercial feeds for YTK as well as designing specific feeds for use in nutrition experiments with YTK. The data presented here will serve as an extremely useful starting point for constructing a larger data base of raw material digestibility coefficients for this species.

Key findings

- The ADCs derived for the raw materials examined in this study will assist in the formulation of research and commercial aquafeeds for this developing aquaculture species.

Publications

Dam, C.T.M., Elizura, A., Ventura, T., Salini, M., Smullen, R., Pirozzi, I., Booth, M. Apparent digestibility of common raw materials by Yellowtail Kingfish (*Seriola lalandi*). Aquaculture (under review).

Acknowledgements

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

We extend our thanks Ridley for providing the raw materials used in experiments and to David Blyth (CSIRO) for extrusion manufacturing of the experimental diets. We would like to acknowledge Basseer Codabaccus, Brendan Findlay, Ian Russell and Steven Gamble (NSW DPI Fisheries) for their valuable technical assistance. We would also like to thank Cedric Simon and Barney Hines (CSIRO) for undertaking the biochemical analysis on feedstuffs, feeds and faecal material.

References

Alexis, M., Filioglou, M., Theochari, V., 1998. Apparent digestibility measurements of feedstuffs having potential for use in rainbow trout diets. Thalassographica 11, 19–26.

Allan, G.L., Rowland, S.J., Parkinson, S., Stone, D.A.J., Jantrarotai, W., 1999. Nutrient digestibility for juvenile silver perch *Bidyanus bidyanus*: development of methods. Aquaculture 170, 131-145.

Allan, G.L., Parkinson, S., Booth, M.A., Stone, D.A., Rowland, S.J., Frances, J., Warner-Smith, R., 2000. Replacement of fish meal in diets for Australian silver perch, *Bidyanus bidyanus*: I. Digestibility of alternative ingredients. Aquaculture 186, 293-310.

Anderson, J.S., Lall, S.P., Anderson, D.M., Chandrasoma, J., 1992. Apparent and true availability of amino acids from common feed ingredients for Atlantic salmon (*Salmo salar*) reared in sea water. Aquaculture 108, 111-124.

Biswas, A., Araki, H., Sakata, T., Nakamori, T., Kato, K., Takii, K., 2017. Fish meal replacement by soy protein from soymilk in the diets of red sea bream (*Pagrus major*). Aquaculture Nutrition 23, 1379-1389.

Booth, M., Pirozzi, I., 2017. Making sense of nonsense: using regression analysis to deal with highly variable data collected from a yellowtail kingfish (*Seriola lalandi*) digestibility experiment. Aquaculture 485, 39-48.

Booth, M., Allan, G., Smullen, R., 2013a. Digestibility of common feed ingredients by juvenile mulloway *Argyrosomus japonicus*. Aquaculture 414, 140-148.

Booth, M.A., Allan, G.L., Pirozzi, I., 2010a. Estimation of digestible protein and energy requirements of yellowtail kingfish *Seriola lalandi* using a factorial approach. Aquaculture 307, 247-259.

Booth, M.A., Moses, M., Allan, G.L., 2013b. Utilisation of carbohydrate by yellowtail kingfish *Seriola lalandi*. Aquaculture 376-379, 151-161.

Booth, M.A., Allan, G.L., Smullen, R.P., 2013c. Digestibility of common feed ingredients by juvenile mulloway *Argyrosomus japonicus*. Aquaculture 414-415, 140-148.

Booth, M.A., Allan, G.L., Frances, J., Parkinson, S., 2001. Replacement of fish meal in diets for Australian silver perch, *Bidyanus bidyanus*: IV. Effects of dehulling and protein concentration on digestibility of grain legumes. Aquaculture 196, 67-85.

Booth, M.A., Pirozzi, I., Allan, G.L., Fielder, D.S., 2010b. Diet Development: temperate species, Aquafin CRC Project 1B5: Feed Technology For Temperate Fish Species; Final Report to Aquafin CRC & FRDC, Project No. 2004/220, Volume 2. Industry & Investment NSW, Cronulla, Sydney, pp. 376.

Bureau, D., Harris, A., Cho, C., 1999. Apparent digestibility of rendered animal protein ingredients for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 180, 345-358.

Bureau, D., Harris, A., Bevan, D., Simmons, L., Azevedo, P., Cho, C., 2000. Feather meals and meat and bone meals from different origins as protein sources in rainbow trout (*Oncorhynchus mykiss*) diets. Aquaculture 181, 281-291.

Carter, C., Hauler, R., 2000. Fish meal replacement by plant meals in extruded feeds for Atlantic salmon, *Salmo salar L*. Aquaculture 185, 299-311.

Che, J., Su, B., Tang, B., Bu, X., Li, J., Lin, Y., Yang, Y., Ge, X., 2017. Apparent digestibility coefficients of animal and plant feed ingredients for juvenile *Pseudobagrus ussuriensis*. Aquaculture Nutrition 23, 1128-1135.

Cheng, Z.J., Hardy, R.W., 2002. Apparent digestibility coefficients and nutritional value of cottonseed meal for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 212, 361-372.

Chi, S., Wang, W., Tan, B., Dong, X., Yang, Q., Liu, H., Zhang, S., 2017. The Apparent Digestibility Coefficients of 13 Selected Animal Feedstuff for Cobia, *Rachycentron canadum*. Journal of the World Aquaculture Society 48, 280-289.

Cho, C., Slinger, S., Bayley, H., 1982. Bioenergetics of salmonid fishes: energy intake, expenditure and productivity. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 73, 25-41.

Dawson, M.R., Alam, M.S., Watanabe, W.O., Carroll, P.M., Seaton, P.J., 2018. Evaluation of Poultry By-Product Meal as an Alternative to Fish Meal in the Diet of Juvenile Black Sea Bass Reared in a Recirculating Aquaculture System. North American Journal of Aquaculture 80, 74-87.

de Carvalho, R.A., Ota, R.H., Kadry, V.O., Tacon, A.G., Lemos, D., 2016. Apparent digestibility of protein, energy and amino acids of six protein sources included at three levels in diets for juvenile white shrimp *Litopenaeus vannamei* reared in high performance conditions. Aquaculture 465, 223-234.

Deng, J., Mai, K., Ai, Q., Zhang, W., Tan, B., Xu, W., Liufu, Z., 2010. Alternative protein sources in diets for Japanese flounder *Paralichthys olivaceus* (Temminck and Schlegel): II. Effects on nutrient digestibility and digestive enzyme activity. Aquaculture Research 41, 861-870.

Folch, J., Lees, M., Sloane-Stanley, G., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem. 226, 497-509.

Glencross, B., Booth, M., Allan, G., 2007. A feed is only as good as its ingredients–a review of ingredient evaluation strategies for aquaculture feeds. Aquaculture Nutrition 13, 17-34.

Glencross, B., Rutherford, N., Bourne, N., 2012. The influence of various starch and non-starch polysaccharides on the digestibility of diets fed to rainbow trout (*Oncorhynchus mykiss*). Aquaculture 356, 141-146.

Glencross, B., Evans, D., Dods, K., McCafferty, P., Hawkins, W., Maas, R., Sipsas, S., 2005. Evaluation of the digestible value of lupin and soybean protein concentrates and isolates when fed to rainbow trout, *Oncorhynchus mykiss*, using either stripping or settlement faecal collection methods. Aquaculture 245, 211-220.

Glencross, B.D., Boujard, T., Kaushik, S.J., 2003. Influence of oligosaccharides on the digestibility of lupin meals when fed to rainbow trout, *Oncorhynchus mykiss*. Aquaculture 219, 703-713.

Glencross, B.D., Carter, C.G., Duijster, N., Evans, D.R., Dods, K., McCafferty, P., Hawkins, W.E., Maas, R., Sipsas, S., 2004. A comparison of the digestibility of a range of lupin and soybean protein products when fed to either Atlantic salmon (*Salmo salar*) or rainbow trout (*Oncorhynchus mykiss*). Aquaculture 237, 333-346.

Hardy, R.W., 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquacult. Res. 41, 770-776.

Hatlen, B., Jakobsen, J.V., Crampton, V., Alm, M., Langmyhr, E., Espe, M., Hevrøy, E., Torstensen, B., Liland, N., Waagbø, R., 2015. Growth, feed utilization and endocrine responses in Atlantic salmon (*Salmo salar*) fed diets added poultry by-product meal and blood meal in combination with poultry oil. Aquaculture Nutrition 21, 714-725.

Hernández, C., Hardy, R., Márquez-Martínez, D., Domínguez-Jimenez, P., González-Rodríguez, B., 2015. Evaluation of apparent digestibility coefficients of individual feed ingredients in spotted rose snapper *Lutjanus guttatus* (Steindachner, 1869). Aquaculture Nutrition 21, 835-842.

Ji, Y., Gu, Y., Liu, H., Yang, Z., Li, C., 2017. The effects of partial replacement of white fish meal by poultry by-product meal and addition of bile acid in feed on growth, digestibility, and serum enzyme activities of the Chinese soft-shelled turtle. Fisheries Science 83, 83-88.

Jirsa, D., Barrows, F., Hardy, R., Drawbridge, M., 2015. Alternative protein blends as a replacement for fish meal in diets for white seabass, *Atractoscion nobilis*. Aquaculture Nutrition 21, 861-867.

Kolkovski, S., Sakakura, Y., 2004. Yellowtail kingfish, from larvae to mature fish–problems and opportunities. Advances in Aquaculture Nutrition.

Lupatsch, I., Kissil, G.W., Sklan, D., Pfeffer, E., 1997. Apparent digestibility coefficients of feed ingredients and their predictability in compound diets for gilthead seabream, *Sparus aurata L*. Aquaculture Nutrition 3, 81-89.

Martínez-Montaño, E., González-Álvarez, K., Lazo, J.P., Audelo-Naranjo, J.M., Vélez-Medel, A., 2016. Morphological development and allometric growth of yellowtail kingfish *Seriola lalandi* V. larvae under culture conditions. Aquaculture Research 47, 1277-1287.

Martins, D.A., Valente, L.M., Lall, S.P., 2009. Apparent digestibility of lipid and fatty acids in fish oil, poultry fat and vegetable oil diets by Atlantic halibut, *Hippoglossus hippoglossus* L. Aquaculture 294, 132-137.

Masumoto, T., Ruchimat, T., Ito, Y., Hosokawa, H., Shimeno, S., 1996. Amino acid availability values for several protein sources for yellowtail (*Seriola quinqueradiata*). Aquaculture. 146, 109-119.

Maynard, L., Loosli, J., Hintz, H., Warner, R., 1979. Digestive processes in different species. Animal Nutrition. McGraw-Hill Inc., New York, NY, 21-46.

McGoogan, B.B., Reigh, R.C., 1996. Apparent digestibility of selected ingredients in red drum (*Sciaenops ocellatus*) diets. Aquaculture 141, 233-244.

McQuaker, N.R., Brown, D.F., Kluckner, P.D., 1979. Digestion of environmental materials for analysis by inductively coupled plasma-atomic emission spectrometry. Analytical Chemistry. 51, 1082-1084.

Moutinho, S., Martínez-Llorens, S., Tomás-Vidal, A., Jover-Cerdá, M., Oliva-Teles, A., Peres, H., 2017. Meat and bone meal as partial replacement for fish meal in diets for gilthead seabream (*Sparus aurata*) juveniles: Growth, feed efficiency, amino acid utilization, and economic efficiency. Aquaculture 468, 271-277.

Nakada, M., 1999. Yellowtail and related species culture. Encyclopedia of aquaculture.

Stone, D.A.J., Bellgrove, E., 2013. A literature review: the current status of knowledge of the nutritional requirements of yellowtail kingfish. in: Stone, D.A.J., Bowyer, J. (Eds.), Sustainable Feeds and Feed Management for Yellowtail Kingfish (*Seriola lalandi*), ASCRC Project No. 2009/728. South Australian Research and Development Institute (Aquatic Sciences), Adelaide, pp. 92-121.

Storebakken, T., Kvien, I., Shearer, K., Grisdale-Helland, B., Helland, S., Berge, G., 1998. The apparent digestibility of diets containing fish meal, soybean meal or bacterial meal fed to Atlantic salmon (*Salmo salar*): evaluation of different faecal collection methods. Aquaculture 169, 195-210.

Sugiura, S.H., Dong, F.M., Rathbone, C.K., Hardy, R.W., 1998. Apparent protein digestibility and mineral availabilities in various feed ingredients for salmonid feeds. Aquaculture 159, 177-202.

Symonds, J., Walker, S., Pether, S., Gublin, Y., McQueen, D., King, A., Irvine, G., Setiawan, A., Forsyth, J., Bruce, M., 2014. Developing yellowtail kingfish (*Seriola lalandi*) and hāpuku (*Polyprion oxygeneios*) for New Zealand aquaculture. New Zealand Journal of Marine and Freshwater Research 48, 371-384.

Tibbetts, S.M., Milley, J.E., Lall, S.P., 2006. Apparent protein and energy digestibility of common and alternative feed ingredients by Atlantic cod, *Gadus morhua* (Linnaeus, 1758). Aquaculture 261, 1314-1327.

Wang, J., Yun, B., Xue, M., Wu, X., Zheng, Y., Li, P., 2012. Apparent digestibility coefficients of several protein sources, and replacement of fishmeal by porcine meal in diets of Japanese seabass, *Lateolabrax japonicus*, are affected by dietary protein levels. Aquacult. Res. 43, 117-127.

Wang, Y., Guo, J.-l., Bureau, D.P., Cui, Z.-h., 2006. Replacement of fish meal by rendered animal protein ingredients in feeds for cuneate drum (*Nibea miichthioides*). Aquaculture 252, 476-483.

Yuan, Y.-c., Gong, S.-y., Yang, H.-j., Lin, Y.-c., Yu, D.-h., Luo, Z., 2010. Apparent digestibility of selected feed ingredients for Chinese sucker, *Myxocyprinus asiaticus*. Aquaculture 306, 238-243.

Zhang, H., Yi, J., Piao, X., Li, P., Zeng, Z., Wang, D., Liu, L., Wang, G., Han, X., 2013. The metabolizable energy value, standardized ileal digestibility of amino acids in soybean meal, soy protein concentrate and fermented soybean meal, and the application of these products in early-weaned piglets. Asian-Australasian Journal of Animal sSciences 26, 691.

Zhou, Q.-C., Tan, B.-P., Mai, K.-S., Liu, Y.-J., 2004. Apparent digestibility of selected feed ingredients for juvenile cobia *Rachycentron canadum*. Aquaculture 241, 441-451.

Table 3.1.4.1.1. Formulation and nutrient composition of the reference diet used in Experiment 1 and Experiment 2 (g kg^{-1} or MJ kg^{-1} dry matter basis; mean ± SEM; *n* = 2).

Raw material inclusion	**Reference diet (g kg^{-1}) or (MJ kg^{-1})**
Fishmeal - prime quality	680.0
Wheat flour	270.0
Fish oil	35.0
Choline chloride (70%)	6.0
Vitamin C (Stay-C 35®)	0.6
Vitamin / mineral premix	6.0
Yttrium oxide	2.3
Nutrient composition	
Dry matter	1000
Nitrogen	91.3±0.3
Crude protein	570.8±2.0
Ash	125.0±3.8
Lipid	83.7±1.4
Gross energy (MJ kg^{-1})	20.8±0.2
Yttrium	1.76±0.01
Amino acid composition	
Alanine	34.7±0.3
Arginine	26.4±0.7
Aspartic acid (+ asparagine)	52.8±3.9
Cysteine	5.8±0.5
Glutamic acid (+ glutamine)	83.3±4.9
Glycine	38.9±0.8
Histidine	9.8±1.4
Isoleucine	19.9±2.9
Leucine	39.7±3.7
Lysine	35.2±5.7
Methionine	13.1±0.4
Phenylalanine	23.9±1.4
Proline	26.6±2.5
Serine	28.1±1.5
Taurine	13.6±0.6
Threonine	24.0±2.5
Tyrosine	18.8±3.0
Valine	26.4±3.3
∑ **reported amino acids + taurine**	**520.9+38.6**

Table 3.1.4.1.2. Nutrient and energy composition of the raw materials used in Experiment 1 and Experiment 2 (g kg^{-1} or MJ kg^{-1} dry matter basis).

Exp.	Raw material[1]	Nitrogen	Crude protein[¥]	Fat	Ash	NFE[‡]	Gross energy (MJ kg^{-1})
1	Fishmeal prime (FM-1)	114.9	718.4	92.6	180.0	9.0	20.6
1	Fishmeal recycled tuna trims (FM-2)	109.2	682.2	115.5	198.0	4.3	20.8
1	Poultry by product meal - 1 (PBM-1)	111.5	696.7	137.8	163.0	2.5	21.5
1	Meat meal (MM)	76.0	475.2	98.3	380.6	45.9	15.8
1	Dehulled lupin kernel meal -1 (LKM-1)	70.7	442.0	122.9	43.7	391.4	21.7
1	Faba bean meal (FBM)	55.2	345.0	32.6	39.5	582.9	19.1
1	Corn gluten meal (CGM)	113.0	706.4	42.1	24.0	227.6	23.3
2	Krill meal (KRM)	95.6	597.8	253.0	105.7	43.6	25.1
2	Blood meal (BLM)	156.7	979.3	12.1	14.0	-	23.2
2	Poultry by product meal - 2 (PBM-2)	135.1	844.6	147.6	41.7	-	25.1
2	Dehulled lupin kernel meal - 2 (LKM-2)	63.1	394.1	87.6	35.5	482.7	21.2
2	Soybean protein concentrate - 1 (SPC-1)	106.9	668.0	5.7	69.6	256.7	19.9
2	Soybean protein concentrate - 2 (SPC-2)	95.2	594.9	18.0	75.6	311.5	20.5
2	Wheat flour (WH)	35.6	222.5	38.9	34.7	703.9	19.2

[¥]Crude protein content = measured nitrogen content × 6.25.
[‡]Nitrogen free extract (NFE) calculated by difference.
[1] All raw materials supplied by Ridley, Narangba, QLD, Australia.

Table 3.1.4.1.3. Measured amino acid composition of the raw materials used in Experiment 1 and Experiment 2 (g kg^{-1} dry matter basis).

		Amino acid																		
Exp.	**Raw material¥**	**ALA**	**ARG**	**ASP***	**CYS**	**GLU‡**	**GLY**	**HIS**	**ISO**	**LEU**	**LYS**	**MET**	**PHE**	**PRO**	**SER**	**TAU**	**THR**	**TYR**	**VAL**	**∑AA**
1	FM-1	45.6	35.4	68.2	5.0	92.3	45.3	15.1	28.3	49.7	52.7	21.0	29.8	29.7	33.6	7.2	32.2	25.8	35.0	**651.7**
1	FM-2	42.0	33.4	64.5	4.9	82.4	42.6	18.2	26.9	49.0	47.1	18.5	28.2	28.8	29.6	3.7	30.5	24.3	33.9	**607.9**
1	PBM-1	40.3	33.9	54.5	5.9	83.2	59.6	9.5	20.9	40.2	34.4	11.4	24.1	39.9	31.8	3.3	26.4	20.9	28.6	**568.7**
1	MM	35.2	31.7	35.1	2.6	55.8	72.7	4.4	10.3	22.9	21.0	5.8	14.0	43.5	18.0	0.6	13.7	10.1	17.1	**414.3**
1	LKM-1	14.1	39.4	47.5	7.8	91.2	18.0	7.9	16.0	28.8	17.7	2.9	15.9	17.2	24.2	0.0	15.9	22.1	16.1	**402.6**
1	FBM	15.8	23.7	36.6	3.5	54.8	15.4	6.0	11.8	24.4	19.8	4.1	14.6	14.7	17.6	0.5	12.6	12.1	15.7	**303.8**
1	CGM	58.7	16.7	44.5	9.4	150.0	19.8	9.4	23.5	100.0	9.9	15.2	41.7	62.3	38.2	0.0	23.8	38.6	29.9	**691.5**
2	KRM	27.2	24.7	58.8	4.3	72.7	24.8	9.1	25.4	40.2	33.1	14.5	25.0	22.8	24.0	2.4	25.2	26.9	27.1	**488.3**
2	BLM	71.1	29.2	94.6	8.8	85.2	38.6	36.5	7.5	103.8	78.4	12.1	66.6	35.9	50.9	0.0	49.0	31.3	72.7	**872.1**
2	PBM-2	50.3	49.2	78.2	12.2	116.3	52.7	16.2	35.6	62.6	59.0	20.6	35.7	43.5	50.6	2.0	39.5	32.8	44.0	**801.0**
2	LKM-2	16.7	49.4	48.9	7.1	103.3	21.0	11.8	16.5	29.2	21.5	3.1	17.7	19.2	23.7	0.1	17.4	19.0	17.5	**443.0**
2	SPC-1	27.8	37.5	78.6	7.9	119.4	28.7	11.9	26.9	46.5	34.7	8.8	32.9	32.6	35.5	0.0	26.1	25.8	29.4	**610.9**
2	SPC-2	28.2	34.9	73.9	8.4	108.5	29.8	11.2	25.6	44.3	28.8	8.1	31.7	31.0	44.9	0.3	28.4	37.0	29.0	**604.2**
2	WH	8.9	8.1	15.8	3.8	53.3	10.5	4.3	6.2	13.5	7.6	3.2	9.4	16.9	10.8	0.4	7.0	7.6	9.1	**196.3**

¥ Raw material codes as per description in table 3.1.4.1.1.
*Aspartic acid + asparagine,
‡Glutamic acid + glutamine,
Σ Amino acid value includes only the amino acids listed in this table, including taurine.

Table 3.1.4.1.4. Formulation and nutrient composition of the diets used in Experiment 1 (g kg^{-1} or MJ kg^{-1} dry matter basis).

	Experimental diet						
	FM-1	**FM-2**	**PBM-1**	**MM**	**LKM-1**	**FBM**	**CGM**
Reference mash	700	700	700	700	700	700	700
Fishmeal prime quality (FM-1)	300						
Fishmeal reclaimed (FM-2)		300					
Poultry by product meal - 1 (PBM-1)			300				
Meat meal (MM)				300			
Dehulled lupin kernel meal -1 (LKM-1)					300		
Faba bean meal (FBM)						300	
Corn gluten meal (CGM)							300
Nutrient composition							
Nitrogen	96.9	95.8	95.8	88.4	85.9	81.2	96.6
Crude protein	605.9	598.6	598.6	552.6	537.2	507.7	603.7
Ash	144.0	147.5	135.2	206.9	99.3	98.9	94.5
Lipid	76.5	86.9	99.7	87.3	90.7	48.9	70.8
NFE	173.6	167.1	166.6	153.2	272.9	344.5	230.9
Gross energy (MJ kg^{-1})	20.6	20.7	21.1	19.0	21.0	20.3	21.7
Amino acid composition (g kg^{-1})							
Alanine	36.8	35.7	35.2	33.6	27.3	27.8	40.7
Arginine	30.5	29.9	30.1	29.4	31.7	27.1	24.9
Aspartic acid (+ asparagine)	58.0	56.9	53.9	48.1	51.8	48.6	50.9
Cysteine	4.5	4.4	4.7	3.7	5.3	4.0	5.8
Glutamic acid (+ glutamine)	86.4	83.4	83.7	75.5	86.1	75.2	103.7
Glycine	38.9	38.0	43.1	47.1	30.7	29.9	31.2
Histidine	10.6	11.5	8.9	7.3	8.4	7.8	8.9
Isoleucine	21.5	21.1	19.3	16.1	17.9	16.6	20.1
Leucine	41.9	41.7	39.1	33.9	35.6	34.3	57.0
Lysine	41.9	40.2	36.4	32.4	31.4	32.0	29.1
Methionine	16.4	15.7	13.5	11.9	11.0	11.3	14.7
Phenylalanine	24.7	24.2	23.0	19.9	20.5	20.1	28.3
Proline	27.4	27.1	30.4	31.5	23.6	22.9	37.1
Serine	30.0	28.8	29.4	25.3	27.2	25.2	31.3
Taurine	14.7	13.6	13.5	12.7	12.5	12.7	12.5
Threonine	26.5	26.0	24.8	21.0	21.6	20.6	24.0
Tyrosine	21.1	20.6	19.6	16.4	20.0	17.0	24.9
Valine	28.5	28.2	26.6	23.2	22.8	22.7	27.0
∑ reported amino acids + taurine	**560.2**	**547.0**	**535.7**	**488.9**	**485.4**	**455.8**	**572.1**

Table 3.1.4.1.5. Formulation and nutrient composition of the diets used in Experiment 2 (g kg^{-1} or MJ kg^{-1} dry matter basis).

	Experimental diet						
	KRM	**BLM**	**PBM-2**	**LKM-2**	**SPC-1**	**SPC-2**	**WH**
Reference mash	700	850	700	700	700	700	
Krill meal (KRM)	300						
Blood meal (BLM)		150					
Poultry by product meal - 2 (PBM-2)			300				
Dehulled lupin kernel meal - 2 (LKM-2)				300			
Soybean protein concentrate - 1 (SPC-1)					300		
Soybean protein concentrate - 2 (SPC-2)						300	
Wheat flour (WH)							300
Nutrient composition							
Nitrogen	90.2	101.6	104.5	87.6	93.6	92.7	73.1
Crude protein	563.8	635.1	653.0	547.7	585.2	579.6	457.2
Ash	118.5	109.9	100.9	97.9	109.8	109.4	98.2
Lipid	135.5	69.1	100.1	81.3	61.2	59.2	69.0
NFE	182.2	185.9	145.9	273.1	243.8	251.7	375.6
Gross energy (MJ kg^{-1})	22.3	21.4	22.0	20.9	20.8	20.9	20.3
Amino acid composition (g kg^{-1})							
Alanine	31.2	38.7	38.2	28.1	31.4	31.5	25.7
Arginine	27.3	28.6	34.7	34.7	31.2	30.4	22.4
Aspartic acid (+ asparagine)	55.2	59.8	61.0	52.2	61.1	59.7	42.3
Cysteine	4.2	4.9	6.7	5.1	5.3	5.5	4.1
Glutamic acid (+ glutamine)	80.6	84.1	93.6	89.7	94.6	91.3	74.7
Glycine	32.7	36.5	41.1	31.6	33.9	34.2	28.4
Histidine	8.8	12.8	10.9	9.6	9.6	9.4	7.3
Isoleucine	20.7	17.0	23.7	18.0	21.1	20.7	14.9
Leucine	39.0	48.3	45.8	35.7	40.9	40.3	31.0
Lysine	36.0	43.4	43.8	32.5	36.5	34.7	28.4
Methionine	14.5	14.1	16.3	11.1	12.8	12.6	11.1
Phenylalanine	23.2	29.1	26.4	21.0	25.6	25.3	18.5
Proline	25.3	27.8	31.5	24.2	28.2	27.8	23.5
Serine	27.1	31.8	35.1	27.0	30.5	33.4	23.1
Taurine	13.3	15.2	13.1	12.5	12.5	12.6	12.6
Threonine	24.4	27.8	28.7	22.1	24.7	25.4	19.0
Tyrosine	21.4	20.9	23.2	19.1	21.1	24.5	15.6
Valine	26.1	32.8	31.2	23.3	26.8	26.7	20.8
∑ reported amino acids + taurine	**511.1**	**573.6**	**605.0**	**497.6**	**547.9**	**545.9**	**423.5**

.

Table 3.1.4.1.6. Apparent proximate digestibility coefficients (ADC) of the test diets used in Experiment 1.

Test diet	n	Coefficient	Mean (%)	SE	95% LCL	95% UCL
REF-1	3	Dry matter	42.1	3.8	25.7	58.5
	3	Protein	65.1	3.0	52.4	77.8
	3	Lipid	84.7	2.6	73.6	95.8
	3	Gross energy	56.8	4.0	39.7	73.9
FM-1	3	Dry matter	46.4	1.7	39.2	53.5
	3	Protein	66.4	2.7	54.9	77.8
	3	Lipid	87.4	2.0	78.9	95.8
	3	Gross energy	62.2	1.9	54.1	70.2
FM-2	3	Dry matter	42.6	1.4	36.4	48.8
	3	Protein	63.1	0.7	60.0	66.2
	3	Lipid	80.2	0.5	77.9	82.5
	3	Gross energy	60.0	1.8	52.2	67.8
PBM-1	3	Dry matter	46.3	0.8	43.1	49.5
	3	Protein	65.6	0.8	62.3	68.8
	3	Lipid	82.1	0.9	78.2	86.0
	3	Gross energy	61.4	2.6	50.1	72.7
MM	3	Dry matter	50.3	0.7	47.5	53.2
	3	Protein	68.8	3.5	53.9	83.6
	3	Lipid	74.7	0.5	72.4	77.0
	3	Gross energy	63.3	1.0	59.2	67.5
LKM-1	3	Dry matter	49.0	2.9	36.5	61.6
	3	Protein	71.3	3.7	55.5	87.0
	3	Lipid	86.2	2.4	75.9	96.5
	3	Gross energy	63.0	3.4	48.5	77.4
FBM	3	Dry matter	40.2	0.6	37.6	42.9
	3	Protein	72.9	2.0	64.5	81.4
	3	Lipid	81.4	1.6	74.6	88.1
	3	Gross energy	61.0	1.0	56.8	65.2
CGM	3	Dry matter	31.7	2.9	19.3	44.2
	3	Protein	50.3	3.6	35.0	65.6
	3	Lipid	63.3	2.4	53.1	73.5
	3	Gross energy	39.7	2.5	28.8	50.5

Table 3.1.4.1.7. Apparent amino acid digestibility coefficients (ADC) of diets in Experiment 1.

			Apparent amino acid digestibility coefficient of diet (%) Experiment 1																	
Diet	**n**	**Parameter**	**ALA**	**ARG**	**ASP**	**CYS**	**GLU**	**GLY**	**HIS**	**ISO**	**LEU**	**LYS**	**MET**	**PHE**	**PRO**	**SER**	**TAU**	**THR**	**TYR**	**VAL**	**Ave.**
REF-1	3	Mean	70.8	76.1	54.8	32.0	74.2	66.0	55.3	68.1	70.8	70.8	73.9	67.9	67.2	65.7	76.5	62.8	68.3	63.3	**65.8**
		SE	2.3	2.3	3.7	5.2	2.6	3.2	1.7	3.2	3.1	2.6	2.6	2.8	2.9	3.3	1.2	3.5	1.9	3.6	
FM-1	3	Mean	73.5	77.6	55.4	28.0	74.4	66.7	51.6	66.1	71.7	70.4	75.9	69.4	66.5	64.8	73.6	63.6	67.1	63.4	**65.5**
		SE	1.7	2.3	2.0	1.0	1.7	1.9	1.3	2.9	2.2	1.6	0.9	1.9	1.5	3.0	3.6	2.2	2.2	2.9	
FM-2	3	Mean	67.7	70.9	54.2	46.3	72.5	61.4	49.8	64.1	67.4	65.4	75.9	65.6	61.6	55.8	72.4	58.6	59.5	61.3	**62.8**
		SE	2.3	1.4	2.8	2.0	0.7	3.5	2.9	2.0	1.6	2.3	1.1	2.1	1.9	10.6	1.2	3.8	3.9	2.3	
PBM-1	3	Mean	68.8	72.3	52.7	28.5	71.0	65.2	43.2	61.9	66.0	65.7	75.3	65.0	60.2	60.7	77.6	57.9	62.1	58.0	**61.8**
		SE	0.6	1.8	1.7	1.6	0.9	1.0	2.3	1.3	1.5	0.4	0.7	0.7	1.1	0.5	0.8	1.1	0.5	1.6	
MM	3	Mean	75.2	79.9	62.9	29.0	77.3	70.3	56.3	71.7	74.2	74.9	81.1	73.9	67.7	71.9	81.6	69.2	74.0	67.9	**69.9**
		SE	1.0	0.9	1.1	1.4	0.7	1.1	2.4	0.6	0.5	1.1	1.3	0.9	1.8	1.6	2.3	1.3	1.3	0.7	
LKM-1	3	Mean	76.1	82.7	63.0	36.5	77.3	70.5	65.1	67.5	73.9	72.4	82.5	73.1	70.6	71.6	73.8	65.4	71.8	65.1	**69.9**
		SE	3.0	2.8	4.3	6.1	2.7	3.8	3.5	4.9	3.8	3.3	2.0	3.5	2.9	3.9	1.0	4.4	4.2	5.0	
FBM	3	Mean	78.5	80.7	65.5	54.6	80.5	73.9	66.9	77.8	78.1	81.7	84.7	76.0	76.5	77.9	75.9	77.1	74.5	74.6	**75.3**
		SE	2.0	2.1	3.2	5.5	1.9	3.0	3.6	2.3	2.0	2.3	1.8	1.8	2.1	2.2	4.7	2.5	1.9	2.6	
CGM	3	Mean	43.3	54.0	41.8	30.2	47.0	48.2	35.9	41.3	43.2	64.4	65.4	43.9	45.7	42.3	65.5	49.3	38.1	40.3	**46.7**
		SE	1.8	2.2	1.8	3.8	2.6	1.3	4.1	2.1	2.7	0.8	1.6	2.2	1.9	1.7	4.1	1.6	2.2	2.2	

Table 3.1.4.1.8. Apparent proximate digestibility coefficients (ADC) of the test diets used in Experiment 2.

Test diet	n	Coefficient	Mean (%)	SE	95% LCL	95% UCL
REF-2	3	Dry matter	39.2	2.6	28.0	50.5
	3	Protein	58.7	3.1	45.2	72.2
	3	Lipid	81.6	1.5	75.3	87.8
	3	Gross energy	53.9	1.6	47.1	60.7
KRM	3	Dry matter	46.2	2.1	37.3	55.1
	3	Protein	62.3	0.5	60.3	64.3
	3	Lipid	80.1	1.9	71.8	88.5
	3	Gross energy	59.3	1.2	54.2	64.3
BLM	3	Dry matter	41.8	1.4	35.7	47.9
	3	Protein	56.8	1.1	52	61.6
	3	Lipid	80.6	0.7	77.6	83.6
	3	Gross energy	50.8	1.4	44.9	56.6
PBM-2	3	Dry matter	46.5	1.3	41	51.9
	3	Protein	63.6	1.1	59	68.2
	3	Lipid	74.7	2.4	64.6	84.8
	3	Gross energy	58.3	1.5	51.7	65
LKM-2	3	Dry matter	43.5	1.1	38.7	48.4
	3	Protein	68.3	2.1	59.4	77.3
	3	Lipid	84.8	1.5	78.5	91.1
	3	Gross energy	58	2.4	47.7	68.2
SPC-1	3	Dry matter	32.2	0.9	28.3	36.1
	3	Protein	60	2.8	48	72
	3	Lipid	83.3	1.8	75.7	91
	3	Gross energy	48.6	0.9	44.5	52.6
SPC-2	3	Dry matter	34.5	4	17.1	51.9
	3	Protein	54.6	2.7	42.8	66.4
	3	Lipid	76.5	4.5	57.2	95.8
	3	Gross energy	47.3	3.1	34	60.6
WH	3	Dry matter	37.8	0.7	35	40.6
	3	Protein	65.2	1.2	60.1	70.3
	3	Lipid	73.9	2.3	64	83.7
	3	Gross energy	48.3	0.2	47.4	49.1

Table 3.1.4.1.9. Apparent amino acid digestibility coefficients (ADC) of diets in Experiment 2.

			Apparent amino acid digestibility coefficient of diet (%) Experiment 2																	
Diet	**n**	**Parameter**	**ALA**	**ARG**	**ASP**	**CYS**	**GLU**	**GLY**	**HIS**	**ISO**	**LEU**	**LYS**	**MET**	**PHE**	**PRO**	**SER**	**TAU**	**THR**	**TYR**	**VAL**	**Ave.**
REF-2	3	Mean	70.7	73.4	52.9	25.4	71.8	68.2	51.2	67.1	68.4	70.0	70.9	66.3	66.4	67.0	77.6	62.9	72.6	62.6	**64.7**
		SE	2.2	2.6	5.5	6.8	3.1	3.1	3.7	2.7	2.9	2.5	3.7	2.7	2.8	2.7	1.5	3.1	0.5	3.3	
KRM	3	Mean	70.1	71.2	58.1	20.2	73.0	66.9	50.1	65.2	70.5	74.5	73.1	68.8	70.8	66.5	74.0	64.1	70.9	63.8	**65.1**
		SE	0.7	2.6	0.9	6.2	1.1	0.2	4.7	0.9	0.6	0.9	2.2	0.7	1.1	0.7	1.7	1.0	0.7	0.7	
BLM	3	Mean	59.1	71.2	49.5	21.0	67.5	63.4	30.5	67.4	57.4	63.4	68.8	55.1	63.4	59.7	74.0	56.7	66.4	49.8	**58.0**
		SE	2.2	1.5	2.2	2.9	1.8	1.8	1.9	1.8	2.5	2.5	1.1	2.4	1.9	1.6	0.2	1.9	0.6	2.9	
PBM-2	3	Mean	70.4	72.2	55.8	24.9	71.3	67.9	64.1	68.5	71.1	74.6	72.6	68.3	66.4	65.5	76.4	65.4	72.5	65.6	**66.3**
		SE	1.5	0.6	2.4	2.2	0.3	0.9	2.5	1.5	1.4	1.0	1.1	2.0	1.1	2.4	0.6	2.2	2.6	1.7	
LKM-2	3	Mean	78.4	84.6	67.8	49.1	81.3	75.9	66.3	75.3	79.0	81.5	83.1	77.1	76.9	76.1	83.0	74.3	81.0	73.8	**75.8**
		SE	1.0	1.9	0.6	1.4	0.4	0.8	1.2	1.2	0.8	1.1	0.6	1.1	1.0	2.6	0.8	1.1	1.0	0.9	
SPC-1	3	Mean	66.9	69.5	50.5	18.2	66.9	57.8	53.6	62.6	68.5	69.8	75.0	67.8	60.8	63.4	74.4	59.3	72.1	59.1	**62.0**
		SE	1.1	2.1	1.8	5.3	1.6	1.1	3.0	1.6	1.1	2.2	1.5	1.3	1.2	1.5	1.0	1.6	1.3	1.4	
SPC-2	3	Mean	65.6	70.3	56.5	25.9	68.2	61.1	51.9	62.4	67.3	64.5	64.5	66.2	64.0	64.9	73.4	58.5	70.6	59.1	**61.9**
		SE	2.9	4.9	3.0	6.3	2.6	2.4	3.1	3.0	2.6	2.4	3.4	2.8	2.7	2.8	2.0	3.0	1.8	2.9	
WH	3	Mean	73.4	77.0	60.4	30.0	79.3	70.9	51.9	72.0	75.0	76.0	77.7	72.7	75.9	69.7	77.7	68.8	77.7	67.7	**69.7**
		SE	0.3	1.2	0.3	0.8	0.3	0.2	5.7	0.4	0.4	0.9	0.9	0.4	0.4	0.9	1.5	0.5	0.7	0.4	

Table 3.1.4.1.10. Apparent proximate digestibility coefficients (ADC) of marine and land animal protein and energy sources (raw materials).

Origin	n	Raw material	Coefficient	Mean (%)	SE	95% LCL	95% UCL
Marine	3	**FM-1**	Dry matter [ab]	56.3	5.5	32.8	79.9
	3		Protein [ab]	68.7	7.6	35.8	101.5
	3		Lipid [c]	93.8	6.6	65.3	122.3
	3		Gross energy [b]	74.9	6.3	47.9	102.0
Marine	3	**FM-2**	Dry matter [a]	43.8	4.8	23.2	64.5
	3		Protein [ab]	59.3	2.1	50.2	68.5
	3		Lipid [ab]	71.6	1.5	65.1	78.2
	3		Gross energy [ab]	67.5	6.1	41.3	93.8
Marine	3	**KRM**	Dry matter [ab]	62.3	6.9	32.7	92.0
	3		Protein [b]	70.4	1.5	63.8	77.0
	3		Lipid [bc]	78.9	3.7	63.1	94.7
	3		Gross energy [ab]	69.8	3.4	55.2	84.4
Animal	3	**PBM-1**	Dry matter [ab]	56.2	2.6	45.0	67.3
	3		Protein [ab]	66.5	2.2	56.9	76.1
	3		Lipid [bc]	77.9	2.4	67.7	88.1
	3		Gross energy [ab]	72.0	8.6	35.0	109.0
Animal	3	**PBM-2**	Dry matter [ab]	63.3	4.2	45.2	81.4
	3		Protein [bc]	71.3	2.8	59.3	83.2
	3		Lipid [ab]	64.4	5.9	38.9	90.0
	3		Gross energy [ab]	67.0	4.5	47.7	86.3
Animal	3	**MM**	Dry matter [b]	69.6	2.2	60.0	79.2
	2		Protein [c]	92.4	2.8	57.4	127.3
	3		Lipid [a]	52.2	1.7	44.8	59.6
	3		Gross energy [b]	83.5	4.0	66.3	100.8
Animal	2	**BLM***	Dry matter [ab]	65.4	2.6	32.4	98.4
	3		Protein [a]	50.6	4.8	29.9	71.3
	0		Lipid	na	na	na	na
	2		Gross energy [a]	43.0	3.3	1.1	84.9

Different superscript letters associated with each coefficient category indicate means are significantly different (ANOVA; Tukeys Test; $P < 0.05$). Lipid ADC for BLM not compared statistically.

Note: one replicate dry matter ADC value of 37.8% and one replicate gross energy ADC value of 18.3% excluded from the BLM group. One replicate protein ADC value of 52.8% excluded from the MM group.

Table 3.1.4.1.11. Apparent proximate digestibility coefficients (ADC) of plant based protein and energy protein sources (raw materials).

Origin	n	Raw material	Coefficient	Mean (%)	SE	95% LCL	95% UCL
Legume	3	**FBM**	Dry matter [abc]	35.9	1.9	27.6	44.1
	3		Protein [b]	103.5	9.7	61.7	145.3
	2		Lipid [ab]	70.6	4.9	8.3	132.9
	3		Gross energy [c]	71.7	3.5	56.6	86.9
Legume	3	**LKM-1**	Dry matter [c]	65.3	9.7	23.5	107.1
	3		Protein [ab]	90.0	14.8	26.2	153.7
	3		Lipid [b]	88.9	6.7	59.9	117.8
	3		Gross energy [c]	76.9	10.9	30.1	123.6
Legume	3	**LKM-2**	Dry matter [bc]	53.4	3.8	37.1	69.8
	3		Protein [b]	101.3	9.2	61.9	140.8
	3		Lipid [b]	93.1	5.1	70.9	115.2
	3		Gross energy [bc]	67.4	7.9	33.5	101.2
Oilseed	3	**SPC-1**	Dry matter [a]	15.8	3.0	2.8	28.8
	3		Protein [ab]	62.5	8.4	26.5	98.5
	0		Lipid	na	na	na	na
	3		Gross energy [ab]	35.5	3.2	21.9	49.1
Oilseed	3	**SPC-2**	Dry matter [ab]	23.3	13.5	-34.7	81.4
	3		Protein [a]	45.3	9.0	6.6	83.9
	0		Lipid	na	na	na	na
	3		Gross energy [a]	31.7	10.5	-13.6	76.9
Gluten	1	**CGM**	Dry matter	26.8	na	na	na
	2		Protein	31.4	7.8	-67.7	130.5
	0		Lipid	na	na	na	na
	1		Gross energy	19.4	na	na	na
Cereal	3	**WH**	Dry matter [abc]	34.4	2.3	24.7	44.1
	3		Protein [b]	104.7	8.3	69.2	140.2
	2		Lipid [a]	44.0	12.1	-109.7	197.7
	3		Gross energy [ab]	34.0	0.8	30.6	37.3

Different superscript letters associated with each coefficient category indicate means are significantly different (ANOVA; Tukeys Test; $P < 0.05$). Lipid ADCs for SPC-1, SPC-2 and CGM not compared statistically.

Note: one replicate lipid ADC value of 34.7% excluded from the FBM group. Two replicate dry matter ADC values (-0.07% and -3.4%), one replicate protein ADC value (3.5%) and two replicate gross energy ADC values (-3.6% and -4.0%) excluded from the CGM group. One replicate lipid ADC value of 3.0% excluded from the WH group. Please note that data on CGM was not analysed statistically.

Table 3.1.4.1.12. Apparent amino acid digestibility coefficients (ADC) of marine and land animal protein and energy sources (raw materials).

			Apparent amino acid digestibility coefficient of raw materials (%)																	
Origin	**Raw mat.**	**Param.**	**ALA**	**ARG**	**ASP**	**CYS**	**GLU**	**GLY**	**HIS**	**ISO**	**LEU**	**LYS**	**MET**	**PHE**	**PRO**	**SER**	**TAU**	**THR**	**TYR**	**VAL**	**Ave.**
Marine	FM-1	Mean	78.4	82.6	58.4	27.2	77.6	65.9	49.7	63.8	75.8	70.5	80.2	73.5	65.9	61.7	63.7	64.9	61.9	64.0	**65.9**
		SE	4.7	6.2	5.9	2.0	5.7	5.9	2.6	7.4	6.6	4.3	2.0	5.3	4.5	8.8	14.0	5.8	5.6	7.9	
		LCL	58.1	56.2	33.1	18.6	53.1	40.7	38.4	32.0	47.5	52.1	71.7	50.8	46.4	23.7	3.5	39.9	37.6	30.0	
		UCL	98.7	109.1	83.6	35.7	102.1	91.1	61.0	95.7	104.0	88.8	88.6	96.2	85.5	99.7	123.9	89.9	86.2	98.0	
Marine	FM-2	Mean	61.5	63.9	55.0	65.6	71.4	48.8	46.5	58.6	63.0	56.3	80.6	62.8	50.9	66.7	52.7	51.1	41.7	58.2	**58.6**
		SE	6.9	3.9	8.5	4.1	2.4	11.2	5.6	5.3	4.8	6.5	2.7	6.1	5.9	0.3	6.2	10.4	10.3	6.5	
		LCL	32.0	47.2	18.3	48.0	60.9	0.5	22.3	35.7	42.5	28.4	69.1	36.7	25.5	65.5	25.9	6.3	-2.6	30.4	
		UCL	91.1	80.6	91.8	83.2	81.9	97.2	70.7	81.4	83.4	84.3	92.0	88.9	76.2	67.8	79.5	95.9	86.0	86.0	
Marine	KRM	Mean	68.0	62.9	67.6	10.1	73.0	66.2	45.1	61.1	72.6	84.7	74.2	72.4	81.0	67.0	58.9	66.7	71.6	65.4	**64.9**
		SE	2.6	8.6	2.8	13.5	4.2	1.0	12.4	2.4	2.0	3.2	5.7	2.2	3.8	2.5	10.2	3.0	1.7	2.2	
		LCL	56.8	25.9	55.6	-48.1	54.8	61.8	-8.1	50.9	63.9	71.1	49.7	62.8	64.7	56.4	15.0	54.0	64.2	55.8	
		UCL	79.2	99.9	79.6	68.2	91.1	70.6	98.3	71.3	81.2	98.3	98.7	82.0	97.3	77.6	102.7	79.4	79.0	75.1	
Animal	PBM-1	Mean	64.6	68.1	50	28.3	65.8	62.2	27.5	50.4	57.5	54.6	81.1	60.4	50.1	49.3	80	48	46.7	47.7	**55.1**
		SE	2	5.1	5.9	3.2	3.2	2.6	6.0	4.0	4.9	1.5	2.2	2.2	2.7	1.5	4.2	3.3	1.5	4.9	
		LCL	56.1	46.2	24.6	14.6	52.1	50.8	1.9	33.3	36.5	48.2	71.8	51.0	38.4	42.8	62.0	34	40.2	26.5	
		UCL	73.1	89.9	75.4	42	79.4	73.5	53.1	67.6	78.5	60.9	90.3	69.8	61.7	55.7	98.0	62.1	53.2	69	
Animal	PBM-2	Mean	69.6	69	59.2	22.7	68.2	69.3	75.4	69.6	73.6	80.7	72.9	70.2	65.8	64.5	72.7	69.1	75.1	69.2	**67.6**
		SE	4.1	1.4	6.5	3.5	0.8	2.4	5.1	3.4	3.6	2.5	2.5	5.0	2.7	5.6	3.6	5.2	5.9	4.1	
		LCL	52.1	63.1	31	7.6	64.8	58.9	53.3	55.1	58.1	69.9	62.2	48.5	54.3	40.3	57.2	46.7	49.7	51.7	
		UCL	87.1	74.9	87.3	37.7	71.6	79.7	97.5	84.1	89.1	91.4	83.7	91.9	77.4	88.6	88.3	91.5	100.6	86.7	
Animal	MM	Mean	85.3	89.6	95.8	29.5	93.7	74.6	64.3	86.1	92.3	91.4	108.3	97	69.1	90.4	114.3	91.4	85.6	83.7	**85.7**
		SE	3.4	2.6	5.1	3.6	3.5	2.5	8.4	2.8	2.6	5.4	5.3	3.9	4.3	6.9	19.0	5.9	5.5	3.0	
		LCL	70.9	78.2	73.8	14.2	78.6	63.9	28.3	74.2	81.1	68.3	85.7	80	50.8	60.7	32.5	66.1	62.1	71	
		UCL	99.8	100.9	117.7	44.8	108.8	85.3	100.2	98	103.4	114.6	131	113.9	87.4	120.2	196.1	116.6	109	96.4	
Animal	BLM	Mean	24.8	53.6	34.3	6.9	33.7	41.5	2.0	64.8	28.8	44.4	52.5	30.1	49.4	38.3	12.1	39.7	53.4	21.6	**35.1**
		SE	8.7	9.1	9.9	8.4	12.6	12	4.2	21.2	8.4	9.4	5.9	7.5	9.8	6.6	3.2	7.2	2.6	9	
		LCL	-12.5	14.6	-8.4	-29.1	-20.5	-10.3	-16.2	-26.5	-7.5	4.1	26.9	-2.2	7.4	9.8	-1.5	8.5	42.4	-17	
		UCL	62	92.7	76.9	42.8	87.9	93.3	20.2	156.2	65.1	84.6	78.1	62.4	91.4	66.8	25.7	70.9	64.4	60.1	

Table 3.1.4.1.13. Apparent amino acid digestibility coefficients (ADC) of plant protein and energy sources (raw materials).

			Apparent amino acid digestibility coefficient of raw materials (%)																	
Origin	**Raw mat.**	**Param.**	**ALA**	**ARG**	**ASP**	**CYS**	**GLU**	**GLY**	**HIS**	**ISO**	**LEU**	**LYS**	**MET**	**PHE**	**PRO**	**SER**	**TAU**	**THR**	**TYR**	**VAL**	**Ave.**
Legume	FBM	Mean	113.9	95.2	106.2	88.4	109.5	110.4	95.8	109.7	109.8	125.7	131.7	105.2	112.2	116.5	67.6	129.7	86.1	114.4	**107.1**
		SE	11.2	7.1	14.1	12.6	9.4	18.7	11.4	9.7	9.2	11.3	8.6	7.7	9.8	9.4	39.6	11.6	7.5	11.3	
		LCL	65.7	64.6	45.5	34.0	69.2	30.0	47.0	68.1	70.3	76.9	94.8	72.2	69.9	75.9	-103	79.6	53.7	65.6	
		UCL	162.1	125.9	166.9	142.7	149.8	190.8	144.7	151.2	149.3	174.4	168.7	138.2	154.5	157	238.1	179.8	118.5	163.1	
	LKM-1	Mean	102.8	95.2	88	42.9	87.7	86.6	86.3	67.2	87.9	80.9	127.7	91.3	82.8	85.1	47.1	73	74.1	72.2	**82.2**
		SE	18.2	7.1	16	11.1	9.3	21.6	9.6	17.1	15.8	17.5	10.7	14.3	12.2	14.1	9.2	17.9	11.7	21.8	
		LCL	24.4	64.8	19	-4.8	47.7	-6.4	44.8	-6.6	19.7	5.8	81.4	29.7	30.3	24.6	7.7	-4.0	23.7	-21.8	
		UCL	181.2	125.7	157	90.5	127.7	179.6	127.8	140.9	156	156	173.9	152.9	135.3	145.6	86.6	150	124.6	166.1	
	LKM-2	Mean	111.6	97	105.1	66.8	98.7	112.4	83.6	94	108.8	121.6	129.5	105.1	106.8	101.6	131	107.1	102.1	108	**105.0**
		SE	5.4	4.2	2.2	2.7	1.1	3.9	2.7	4.3	3.2	5.2	3.4	4.1	3.8	9.6	7.3	4.2	2.9	3.9	
		LCL	88.6	78.9	95.8	55.3	93.9	95.6	71.8	75.6	94.9	99.2	115	87.5	90.2	60.5	99.5	88.8	89.5	91.2	
		UCL	134.7	115.1	114.5	78.2	103.5	129.2	95.4	112.4	122.7	144.1	144	122.7	123.3	142.7	162.6	125.3	114.7	124.9	
Oilseed	SPC-1	Mean	55.7	60.9	44.9	9.6	55.8	28.5	54.1	54.7	66.2	68.4	81.1	68.9	49.6	58	53	52.2	74.6	51.4	**54.9**
		SE	4.2	5.6	4.8	9.6	4.4	4.7	7.0	4.1	3.4	7.5	5.0	3.6	3.5	4.1	9.5	4.9	3.3	4.3	
		LCL	37.7	36.7	24.5	-31.7	36.7	8.2	23.7	36.9	51.7	36.3	59.4	53.5	34.6	40.3	12.1	31	60.6	33	
		UCL	73.8	85	65.4	50.9	74.8	48.7	84.4	72.5	80.6	100.5	102.8	84.2	64.6	75.8	94	73.4	88.6	69.8	
	SPC-2	Mean	51.1	62.6	61.4	23.7	58.6	43.1	50	53.7	62.4	47.9	44.5	64.7	58.4	62.6	45.5	50.5	70.8	51.1	**53.5**
		SE	10.9	13.4	8.4	11.2	7.9	9.6	7.4	8.1	8.2	9.1	11.8	7.4	7.9	7.0	16.9	8.6	3.7	8.7	
		LCL	4.1	4.9	25.2	-24.6	24.7	1.7	18.1	19	27.3	8.7	-6.2	32.8	24.5	32.5	-27.1	13.4	54.7	13.7	
		UCL	98.1	120.2	97.5	71.9	92.5	84.4	81.9	88.4	97.6	87.1	95.2	96.7	92.4	92.8	118.1	87.6	86.9	88.4	
Gluten	CGM*	Mean	3.6	-14.0	6.6	31.3	8.9	-33.4	8.5	-5.9	16.7	24.7	54.3	13.2	24	1.1	-30.8	19.5	3	-4.8	**7.0**
		SE	4.5	9.3	7.0	6.6	6.5	6.9	10.5	6.0	5.4	6.4	4.2	5.2	3.9	4.7	38.3	5.0	4.5	6.5	
		LCL	-15.7	-54.2	-23.7	3.0	-18.9	-63.2	-36.8	-31.7	-6.7	-2.9	36.1	-9.0	7.0	-19.0	-195	-1.9	-16.4	-32.7	
		UCL	23.0	26.2	36.8	59.6	36.6	-3.7	53.8	19.9	40.1	52.2	72.4	35.4	41	21.2	134.1	41	22.5	23.2	
Cereal	WH	Mean	92.3	89.8	107.5	31.7	104.2	97.9	48.4	93.6	108	121.4	100.6	98.3	106	86.3	82.4	101.9	107	92.3	**92.8**
		SE	2.8	8.1	2.7	1.7	1.4	1.3	20.3	2.4	2.5	7.9	4.8	2.1	1.7	5.2	12.5	3.3	3.4	2.6	
		LCL	80.2	55.1	95.8	24.2	98.4	92.1	-39.0	83.2	97.2	87.5	80.1	89.3	98.6	63.8	28.7	87.8	92.4	81	
		UCL	104.3	124.6	119.2	39.2	110.1	103.7	135.8	103.9	118.7	155.3	121.1	107.3	113.4	108.8	136.1	115.9	121.6	103.6	

*Data on CGM was not analysed statistically.

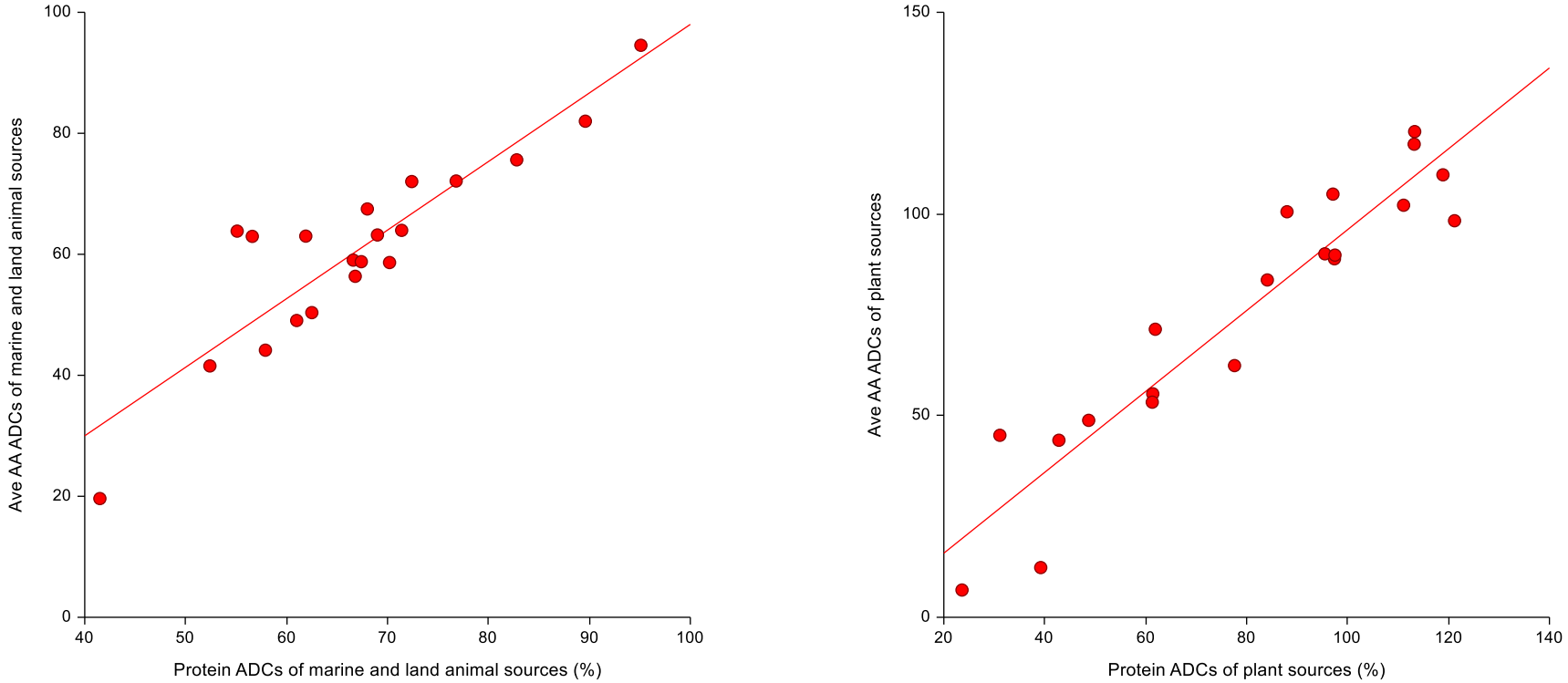


Figure 3.1.4.1.1. Relationships between protein ADCs and average amino acid ADCs of raw materials; left figure describes the linear relationship between marine and land animal sources (average AA ADC = 1.1321 x protein ADC -15.2199; $R^2 = 0.80$; *n* = 20) and right figure describes relationship between plant sources (average AA ADC = 1.0022 x protein ADC -4.1565; $R^2 = 0.88$; *n* = 20).

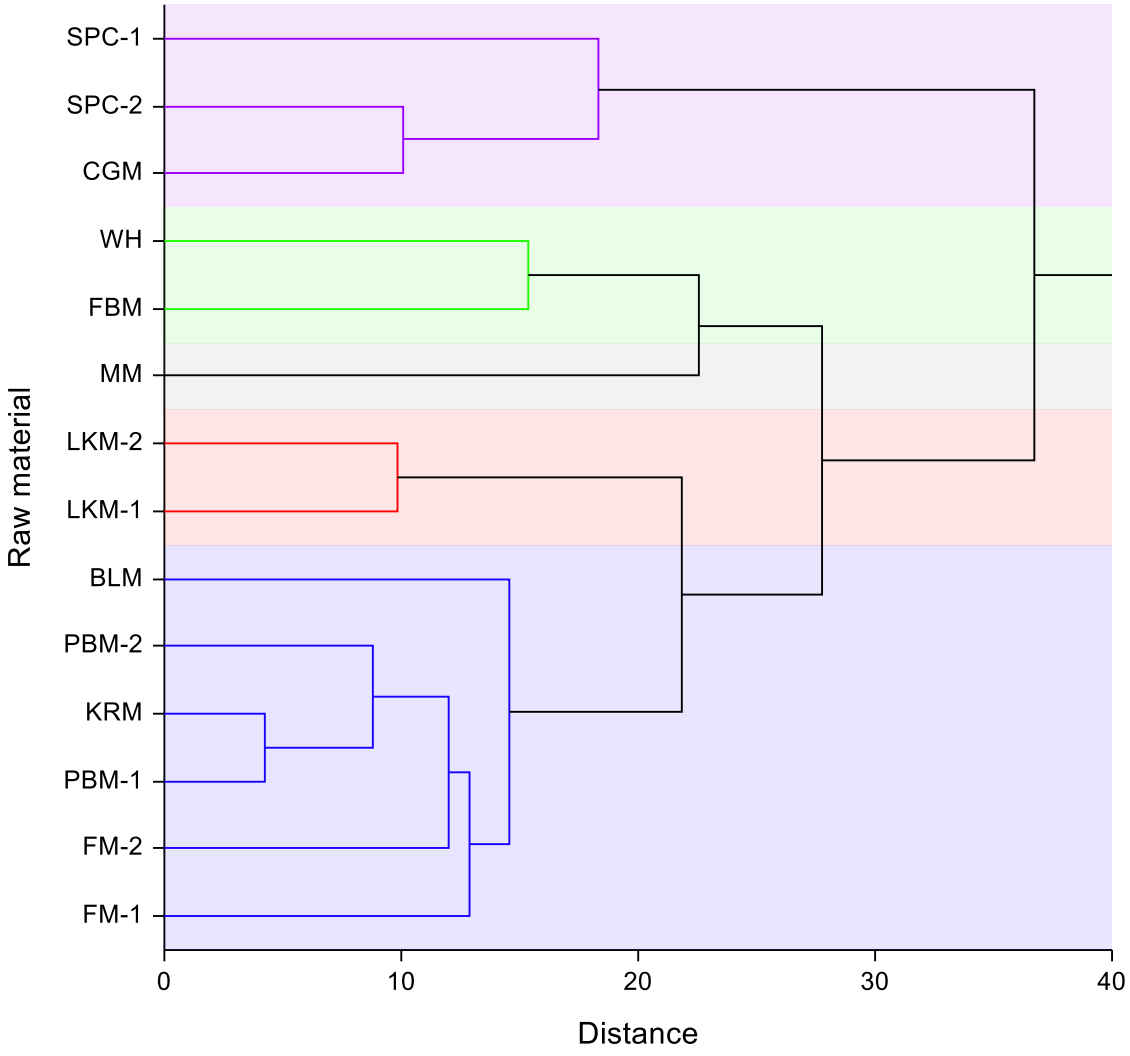


Figure 3.1.4.1.2. Dendrogram indicating the similarity between the overall digestibilities of raw materials as based on three variables; dry matter, protein and lipid ADCs.

3.1.5. Chapter - Requirement studies for juvenile Yellowtail Kingfish.

3.1.5.1. Manuscript - Digestible choline requirement of juvenile Yellowtail Kingfish (Seriola lalandi).

Angela Liu[a], Igor Pirozzi[b, c], Basseer Codabaccus[b, d], Barney Hines[e], Cedric Simon[e], Jesmond Sammut[a] and Mark Booth[b]

[a] School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, NSW, 2052, Australia

[b] New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach, NSW, 2316, Australia

[c] Centre for Sustainable Tropical Fisheries and Aquaculture and College of Science and Engineering, James Cook University, Townsville, Queensland 4811, Australia.

[d] Institute for Marine and Antarctic Studies, University of Tasmania. Private Bag 49, Hobart, TAS, 7001, Australia

[e] CSIRO Agriculture and Food, 306 Carmody Rd, St Lucia, QLD, 4067, Australia

This manuscript may be referenced as: Liu, A., Pirozzi, I., Codabaccus, B., Hines, B., Simon, C., Sammut, J. and Booth, M. (2019). 3.1.5.1. Manuscript - Digestible choline requirement of juvenile Yellowtail Kingfish (*Seriola lalandi*). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027),* Adelaide, June. pp.160-186.

Abstract

There are no published studies quantifying the choline requirements of Yellowtail Kingfish (*Seriola lalandi;* YTK). Therefore, the first aim of this study was to determine the digestible choline requirement of juvenile YTK in the presence of 2-amino-2methyl-1-propanol (AMP), an inhibitor of choline biosynthesis. The second aim was to determine if choline supplementation of a commercial-like diet made from common raw materials is necessary to mitigate the performance of YTK and if the choline supplementation rate is affected by water temperature. Two eight-week experiments were done. The first was a dose-response experiment in which juvenile YTK (mean ± SD = 156.3 ± 15.3 g) were reared at 16 °C and fed five isonitrogenous - isoenergetic diets containing 3 g AMP kg^{-1} diet and either 0.42 (Diet-1), 1.10 (Diet-2), 1.37 (Diet-3), 2.96 (Diet-4) or 6.05 g digestible choline kg^{-1} diet (Diet-5); diets were made from semi-purified ingredients and additional choline was supplied as choline chloride (CC; commercial grade 70%). A sixth diet (Diet-6), comparable to Diet-4, was made without AMP in order to estimate the *de-novo* synthesis of choline by YTK. The second study was a factorial experiment in which juvenile YTK (157.3 ± 11.9 g) were reared at 16 °C or 24 °C and fed a commercial-like diet supplemented with zero, 3.0 or 6.0 g of CC kg^{-1}. This resulted in three diets with an average digestible choline concentration of 1.77, 3.54 and 4.66 g kg^{-1} diet, respectively. Based on a segmental-linear-regression model the results from Experiment 1 indicated the break-point in choline deposition rate occurred when digestible choline intake reached 27.3 mg kg BW^{-1} day^{-1}. The break-point in specific growth rate (SGR) occurred when digestible choline intake reached 26.1 mg kg BW^{-1} day^{-1}. On a dietary basis the breakpoint in choline deposition rate and SGR occurred when diets provided 1.94 and 1.93 g digestible choline kg^{-1} diet, respectively. The *de novo* rate of choline synthesis was estimated to be 4.2 mg choline $kgBW^{-1}$ d^{-1} which is about 15% of the estimated requirement, suggesting juvenile YTK have a limited capacity for *de novo* choline synthesis. Choline retention efficiency proved to be inversely related to the choline content of diets in both experiments. Data from Experiment 2 also indicated there were no significant interactions between water temperature and digestible choline content of diets with

respect to production indices such as SGR, FCR, whole-body composition and protein, lipid and energy retention. Choline deposition rate, SGR and FCR tended to be better in fish fed the commercial-like diet supplemented with 3 g CC kg^{-1}, but there was no additional advantage of raising the content to 6 g CC kg^{-1} diet. The increased performance of YTK fed P-Diet 2 and the fact that the digestible choline content of P-Diet 1 was lower than the estimated requirement established in Experiment 1 confirms the necessity of adding CC to production diets for YTK.

Introduction

Choline is an essential nutrient that is closely related to the B-complex vitamin group. It is a precursor for several metabolites and is required for the structure and function of cells in all animals (Caudill, 2010). Choline is metabolically important as a methyl donor, a constituent of phospholipids (particularly phosphatidylcholine (PC) which is responsible for the structural integrity of cell membranes and contributes to lipid metabolism), and a component of the neurotransmitter acetylcholine (ACh), which plays a vital role in memory and cognitive development (Simon, 1999; Caudill, 2010; NRC, 2011). Most animals can synthesise choline *de novo* through the methylation of phosphatidylethanolamine (PE) catalysed by phosphatidylethanolamine-*N*-methyltransferase (PEMT). However, many animals cannot synthesise sufficient quantities and or the rate of choline synthesis is insufficient to meet their metabolic requirements (Craig and Gatlin, 1996; Duan et al., 2012; Fagone and Jackowski, 2013; Khosravi et al., 2015). Thus, choline must be obtained primarily from the diet.

Identifying the optimal dietary choline requirement in fast-growing fish is important for many reasons. These include preventing deficiencies in fish species that have a limited capacity for *de novo* choline biosynthesis, avoiding potential toxic effects from excess dietary choline (Griffin, 1994; Zeisel and Blusztajn, 1994) and minimising the amount of nutrients entering the waterways. There is some evidence to suggest choline can also ameliorate environmental stressors such as crowding and poor water quality (Yeh et al., 2015), however whether this is due to the direct effect of choline or its interaction with other factors is uncertain. Dietary choline requirement in finfish ranges widely between 0.05 to 4.0 g kg^{-1} diet, depending on the species, size, age, growth rate, digestibility of diet, nutrient interactions, environmental variables and even the response variable and models used to estimate the requirement (NRC, 2011). Since most animals can synthesise choline from methyl donors such as methionine and betaine, studies aimed at quantifying choline requirement have often relied on experimental diets that meet but do not exceed the dietary requirement for methyl donors; i.e. to ensure the sparing of choline does not occur (Mai et al., 2009; Rumsey, 1991). However, this approach is challenging if the requirement for methyl donors in the animal have not been determined. Addition of 2-amino-2-methyl-1-propanol (AMP) to the diet inhibited *de novo* choline biosynthesis in Rainbow Trout (*Oncorhynchus mykiss*) (Rumsey, 1991), Channel Catfish (*Ictalurus punctatus*) (Zhang and Wilson, 1999) and Parrot Fish (*Oplegnathus fasciatus*) (Khosravi et al., 2015), allowing the absolute choline requirement of these fish to be quantified. This approach also provides an indication of the *de novo* choline synthesis capacity of the fish being studied.

Phosphatidylcholine is a major source of cellular choline and a lipotropic factor that prevents excessive lipid accumulation and development of fatty liver disease (e.g. hepatic steatosis) in animals and humans (Luo et al., 2016). Accumulation of fat in the liver is often associated with lower growth and feed efficiency in fish. Such symptoms have been reported in Lake Trout (*Salvelinus nanaycush*) (Ketola, 1976), Hybrid Striped Bass (*Monrone saxatillis* × *M. chrysops*) (Griffin, 1994), Grass Carp (*Ctenopharyngodon idella*) (Wang et al., 1995) and Grouper (*Epinephelus coioides*) (Qin et al., 2017). Many studies have reported that fish fed choline-deficient diets exhibited normal growth, even though they had deleterious liver and organ pathologies. Examples include Channel Catfish (Zhang and Wilson, 1999), Common Carp (*Cyprinus carpio*) (Ogino et al., 1970), White Sturgeon (*Acipenser transmontanus*) (Hung, 1989) and Giant Grouper (*Epinephelus lanceolatus*) (Yeh et al., 2015). In contrast, choline-deficient diets lowered growth but did not alter the liver lipid content of Rainbow Trout (Rumsey, 1991), Yellow Perch (*Perca flavescens*) (Twibell and Brown, 2000), Japanese Yellowtail (*Seriola quinqueradiata*) (Hosokawa et al., 2001) and Cobia (*Rachycentron canadum*) (Mai et al., 2009). Therefore, the literature suggests the lipotropic effects of dietary choline are inconsistent in fish.

Yellowtail Kingfish (*Seriola lalandi;* YTK) is commercially important to many countries including Australia, New Zealand, North and South America, Japan and Taiwan (Sicuro and Luzzana, 2016). It is

a fast growing, carnivorous species with good meat quality and a high market acceptance (Miegel et al., 2010; Abbink et al., 2012). In Australia, choline (normally as feed grade CC) is added to commercial aquafeeds for YTK as a precautionary measure because hitherto there are no published studies quantifying the specific choline requirement of this species. This strategy is aimed at mitigating the risk of inadvertently formulating diets with sub-optimal choline levels as a result of selecting raw materials that have unknown, low or variable amounts of endogenous choline. Supplementation rates are generally based on the estimated choline requirements of the closely related Japanese Yellowtail which were shown to be between 2100 and 2900 mg CC kg^{-1} of diet based on response to weight gain and liver choline concentration, respectively (Hosokawa et al., 2001). However, that study was done using reagent grade CC, used very small fish (25.6 g), lasted only 17 days and was done at very high water temperature (25-29.8 °C).

Australian YTK is farmed in the Spencer Gulf (South Australia), Providence Bay (New South Wales) and Geraldton (Western Australia), where seasonal water temperature can vary between 10-24 °C (Miegel et al., 2010). Being a temperate pelagic species the growth rate of YTK is significantly lower during winter months (Moran et al., 2009; Bowyer et al., 2014; Bansemer et al., 2018). Apart from reduced growth, overwintering of YTK leads to decreased feed efficiency and in some cases nutritional disorders (Pirozzi and Booth, 2009; Abbink et al., 2012). Whether the choline requirement of YTK changes according to water temperature is also unknown. However, the requirements of fish for other methyl donor's such as methionine and betaine are known to vary with temperature which may directly or indirectly influence requirement for choline (Kumar et al., 2012).

The primary aim of this study was to determine the digestible choline requirement of juvenile YTK reared at a constant water temperature of 16 °C. This was done by adopting a dose-response strategy and feeding juvenile YTK semi-purified diets containing feed grade CC (70% CC) and AMP. A secondary aim was to determine if CC supplementation of a commercial-like formula is necessary to mitigate performance of YTK and whether the rate of CC supplementation is temperature-dependent. This was done by formulating a basal diet that resembled the commercial aquafeeds currently being used by the Australian YTK industry and supplementing the formulation with zero (i.e. residual endogenous level of choline), 3.0 g CC kg^{-1} diet and 6.0 g CC kg^{-1} diet. The highest supplementation rate is twice the amount of CC currently used in Australian aquafeeds for YTK. These diets were tested on fish reared at 16 °C and 24 °C.

Materials and Methods

Ethics

All experimental procedures were performed under the NSW Department of Primary Industries (DPI) Fisheries Animal Care and Ethics Research Authority (ACEC93/5). Care, husbandry and termination of fish was done according to 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2015).

Fish stock

All juvenile fish were progeny of wild YTK broodstock held at the DPI Port Stephens Fisheries Institute (PSFI) hatchery (NSW, Australia). Prior to experiments fish were held in 10,000-L tanks and fed a commercially available diet. Prior to any handling, fish were sedated using recommended doses of Aqui-S® (Aqui-S New Zealand Ltd.; 540 g L^{-1} isoeugenol). Fish were fasted for 24 h prior to weighing or termination.

Experiment 1- choline requirement of juvenile YTK at 16 °C

A basal diet was formulated from a mixture of raw materials and semi-purified ingredients. Fishmeal, sodium caseinate and gelatine were used as the major protein sources whilst dextrin and fish oil were used as the major energy sources. Diets were isoproteic and isoenergetic having an average crude protein and gross energy content of 559.5 g kg^{-1} and 23.6 MJ kg^{-1} diet, respectively (Table 3.1.5.1.1). Feed grade

CC (70% CC; equivalent to 0.74 g choline g^{-1}) was added to the basal mixture at 0.0, 1.0, 1.5, 4.1 and 10.0 g kg^{-1} diet resulting in 5 diets (hereafter, Diet-1, Diet-2, Diet-3, Diet-4, Diet-5) with a measured choline content (expressed as total choline throughout this report unless stated otherwise), of 0.59, 1.25, 1.56, 3.11 and 6.22 g kg^{-1}, respectively. The addition of CC to diets was balanced by adjusting the content of diatomaceous earth. Based on the protocol of others 3 g AMP kg^{-1} was also added to these diets to ensure the potential *de novo* synthesis of choline in YTK was restricted (Khosravi et al., 2015). A sixth diet was prepared without AMP (control; Diet-6) to examine the effect of AMP on fish performance and to allow the estimation of *de novo* choline synthesis in YTK. The formula for Diet-6 was the same as Diet-4 and had a measured choline content of 3.22 g kg^{-1} diet (Table 3.1.5.1.1). All experimental diets were top-coated with 20 g kg^{-1} diet of a commercial feed attractant (*commercial in-confidence*) to ensure diets were palatable to the fish.

Experiment 2 – effect of adding choline chloride to a commercial-like diet

Experiment 2 was designed for interpretation using a factorial ANOVA to examine the interaction between CC supplementation (none, 3.0 and 6.0 g CC kg^{-1} diet) and water temperature (16 °C vs 24 °C). In this case a basal diet was formulated with common raw materials used by the Australian aquafeed industry including imported fishmeal and fish oil and locally available rendered animal by-products such as poultry offal meal, meat meal, blood meal and poultry oil; locally available dehulled lupin and wheat flour were also included in the ration. The basal formula was similar to commercial formulations being used in the Australian YTK industry and contained an average crude protein and gross energy content of 526.8 g kg^{-1} and 23.1 MJ kg^{-1} diet, respectively (Table 3.1.5.1.2). Feed grade CC (70% CC; equivalent to 0.74 g choline g^{-1}) was added to the basal mixture at 0.0, 3.0 and 6.0 g kg^{-1} diet resulting in three practical diets; hereafter P-Diet 1, P-Diet 2 and P-Diet 3 with a measured choline content of 2.05, 3.87, 5.44 g kg^{-1}, respectively. The addition of CC to diets was balanced by adjusting the content of diatomaceous earth.

Common procedures used in Experiment 1 and Experiment 2

Dried ingredients were ground to a fine powder (< 300 µm) using a hammer mill then mixed on a dry matter basis according to specific formulations. Fish oil and poultry oil were added as required followed by an appropriate amount of fresh water. The moist dough was cold-pressed into pellets using a meat mincer fitted with an 8 mm die plate. The pellets were then gently oven-dried at 40-50 °C until moisture content was ≤ 10%. Diets were stored at -20 °C until used. To determine the apparent digestibility of experimental diets, 1g yttrium oxide kg^{-1} diet (analytical grade) was added to diets.

Experiments were conducted in an indoor laboratory housing multiple, research-scale, recirculating aquaculture systems (RAS). Each RAS consisted of a series of 200 L translucent, white circular, polyethylene rearing tanks connected to a water management system. Tanks were fitted with a mesh lid to prevent the escape of fish and the outer surface was painted black to prevent the inadvertent disturbance of fish. Each tank was fitted with an air-stone diffuser and additional dissolved oxygen (DO) was provided to the RAS by injecting industrial grade oxygen (BOC) into influent manifolds. Each RAS was comprised of a 1500 L sump, a large sand filter, a twin-cartridge particle filter (30 µm), a foam fractionator and a 750 L fluidised bed bio-filter (B-Cell). The water temperature in each RAS was controlled using reverse cycle refrigeration units capable of maintaining water temperature to ± 2 °C. Photoperiod in both experiments was set at (10 h light and 14 h dark) using dimmed LED lighting.

Water flow to each experiment tank was controlled via the inlet manifold to provide approximately 5-6 L min^{-1}. Effluent water was continuously discharged from each RAS allowing approximately 50% of water to be exchanged daily. All tanks were regularly siphoned to remove organic matter and thoroughly cleaned during weight check procedures.

Eight juvenile YTK were stocked into each 200 L rearing tank in both experiments. All fish were individually weighed and measured (fork length). The average ± SD stocking weight of fish in Experiment 1 was 156.3 ± 15.3 g whereas, the average ± SD stocking weight of fish in Experiment 2 was 157.3 ± 11.9 g. Afterwards, experimental treatments were randomly assigned to n = 3 replicate tanks.

Fish were hand fed to apparent satiation twice daily Monday to Friday and once daily on Saturday and Sunday. Uneaten pellets were collected from individual tanks after each meal in cyclindroconical pellet traps, dried for 24 h at 105 °C and weighed. Actual dry basis feed intake in each tank was calculated by subtracting the amount of dried pellets (adjusted for leaching losses) from the total amount of feed administered to each tank. Both experiments were run for 56 days.

An interim weight check was done in both experiments four weeks after stocking. At this time one fish was randomly selected and removed from each experimental tank for stable isotope analysis (*unpublished data*). At the end of each experiment three fish were randomly selected from each tank and euthanised with an overdose of benzocaine (*ethyl-p-aminobenzoate*) to determine hepatosomatic (HSI) and viscerosomatic indices (VSI). A small sub-section of the liver was taken from the same area in each animal to determine the proximate composition and total choline concentration of liver tissue. The carcasses were then pooled by tank and homogenised in a high-speed mincer-blender (BLIXER-3, Robot-Coupe Australia Pty Ltd., Artarmon, NSW). Homogenised samples were stored at -20 °C freezer until analyses. Homogenates were used to determine the proximate composition and total choline concentration of whole fish.

The apparent digestibility of diets in each experiment was determined in adjunct studies using 2 or 3 replicate tanks of fish per dietary treatment. New groups of fish were of similar size and subjected to the same feeding and husbandry routines as applied during the growth experiments. Faeces were obtained from juvenile fish by manual stripping (Booth and Pirozzi, 2017). Fish were first sedated in tanks before they were captured individually and gently wiped clean using absorbent cloth. Light pressure was then applied to the abdominal region of fish using the thumb and forefinger to expel urinary products. Once urine was expelled the area around the vent was wiped clean. Faecal material was then expelled by applying light pressure to the abdominal region of the fish by running the thumb and forefinger from the pelvic fins towards the vent. Faecal matter from each tank of fish was collected in 50 mL polyethylene sample pots and pooled. The procedure was repeated every three to four days until enough faecal matter was collected for chemical analysis. Samples were stored at -20 °C until chemical analyses.

Water quality was monitored each day prior to feeding from randomly selected tanks using a digital water quality meter (Horiba U-10 and WP-100). Dissolved oxygen (DO) was monitored using a HQ30d (Hach, Loveland, U.S.A) and ammonia concentration was determined using API® ammonia test kits (Mars Fishcare, North America, Inc.). The mean ± SD water temperature in Experiment 1 was 16.0 ± 1.1 °C. The mean ± SD water temperature in each RAS in Experiment 2 was 16.1 ± 1.0 °C and 23.1 ± 1.3 °C, respectively. The recorded salinity (33.0-36.0‰), DO (5.7-9.2 mg L^{-1}), pH (7.3–8.4 units) and total ammonia (< 0.5 mg L^{-1}) was similar in both experiments.

Chemical analyses

Dry matter, protein, lipid, ash and gross energy of raw materials, experimental diets, whole-body and faecal samples (including yttrium) were conducted by CSIRO according to routine methods outlined in AOAC (AOAC, 2006). Liver proximates were done by (Deakin University). Crude protein content was determined by multiplying the nitrogen content of each sample by 6.25. Crude lipid was determined by extraction using chloroform:methanol (2:1) (Folch et al., 1957). The extracts were filtered and washed with a 1% sodium chloride and methanol:1% sodium chloride solution (1:1). Lipids were recovered and quantified gravimetrically.

Amino acid profile analysis of diets was performed according to standard operating procedure SOP QAAA-001 of the Australian Proteome Analysis Facility (APAF; Macquarie University, Sydney Australia). Samples underwent 24 hours liquid hydrolysis in 6M HCl at 110 °C. After hydrolysis, all amino acids were labelled using the Waters AccQTag Ultra chemistry (following supplier's recommendations) and analysed on a Waters Acquity UPLC. The sample was analysed in duplicate and expressed as an average.

The yttrium content of diet and faecal samples was determined by digesting 100 mg of sample in 5 mL of concentrated nitric acid using a microwave digester. The resulting solution was then diluted to 100 mL using 18.2 Ω water. The concentration of yttrium was measured using ICP-MS (Perkin Elmer Sciex Elan DRC II).

Total choline concentration was determined by acid hydrolysis and LC/MS/MS (Model 8030, Shimadzu). Diets, whole-body (freeze-dried) and faecal samples (oven-dried) were desiccated then ground and homogenised into powder with an electric grinder. Each sample was acid-digested at 70 °C for 3 h (Woollard, Indyk, 2000) and pH adjusted. The mixture was then transferred to a 250 mL volumetric flask and diluted to volume with 18.2 Ω water. A 4 mL aliquot of the diluted solution was filtered through a 0.22 µm syringe-filter (Merck Millipore Ltd. Millex®GP). Liver samples were not dried prior to homogenisation due to their small size. Instead liver samples were homogenised by using zirconia/silica beads (1.0 and 0.1 mm diameter) and a bead-beater (Precelly 24, Bertin Technologies). The remaining procedures were the same as for diet, whole carcass and faecal samples. Fourteen levels of solution were prepared for the standard curve, and 250 µM of CC (Sigma-Aldrich, C7017, ≥ 99%) was used as an internal standard. L-α-phosphatidylcholine (Sigma-Aldrich, P3556, from egg yolk, ≥ 99%), lamb liver, soybean meal, fishmeal and skim milk powder (Diploma) were used for validation. A 2.1 × 150mm, pentafluorophenylpropyl analytical column (Sigma-Aldrich, HS F5-3, P/N 567503-U), 3 µm particle size was used for liquid chromatography separation. Choline analyses were performed by CSIRO.

Biometric calculations and apparent digestibility

All biometric indices were calculated using the average value of fish in each replicate tank. The following performance indices were calculated;

- Weight gain (g) = harvest weight of fish (g) – stocking weight of fish (g)
- Condition factor K = (weight of fish (g) / fork length (mm)^3) × 10^5
- Survival (%) = number of fish at harvest / number of fish at stocking × 100
- Specific growth rate (SGR, % d^{-1}) = [ln(final weight (g)) – ln(initial weight (g))] / days × 100
- Feed conversion ratio (FCR) = total feed intake per tank (g) / biomass gain per tank (g)
- Hepatosomatic index (HSI%) = wet liver weight (g) / wet body weight (g) × 100
- Viscerosomatic index (VSI%) = wet visceral weight (g) / wet body weight (g) × 100
- Relative feed intake (g kg BW^{-1} d^{-1}) = individual feed intake (g) / ((ind. Geom. weight (g) / 1000) / days
- Digestible nutrient retention (%) = (nutrient or energy content of fish at harvest – nutrient or energy content at stocking) / digestible nutrient or energy intake × 100.

The apparent digestibility of dry matter, protein, fat, gross energy and total choline in diets was calculated using the following equation;

- Apparent digestibility coefficient (ADC %) = 100 – [100 × ((% nutrient or energy in faeces / % nutrient or energy in diet) × (% Y_{tt} in diet / % Y_{tt} in faeces))]; (Cho et al., 1982).

The digestible nutrient and energy content of diets was calculated by multiplying the appropriate apparent digestibility coefficient of the diet (ADC) by its corresponding nutrient or energy content;

- Apparent digestible nutrient or energy content = ADC (%) × gross nutrient or energy content of diet

Statistical analysis and modelling

The effect of dietary treatment on biometric indices and digestibility of diets was examined using one-way ANOVA in Experiment 1. A segmental-linear-regression model was used to quantify the relationships between digestible choline intake of fish or digestible choline content of diets (x-axis) and SGR or choline deposition rate (y-axis) in YTK from Experiment 1. Two-factor ANOVA was used to assess the interactive effects of dietary treatment (i.e. choline supplementation of none, 3.0 and 6.0 g kg^{-1} diet) and water temperature (16 °C and 24 °C), on the performance of YTK in Experiment 2. One-way and two-way ANOVA were used once normality and homogeneity of variances assumptions (diagnostic plots) were satisfied. A Tukey's Honest Significant Difference *post-hoc* test was used to discriminate among treatment means when ANOVA proved significant ($\alpha = 0.05$). ANOVA and segmental-linear-regression analysis were done in R (R Core Team, 2017) or GraphPad Prism version 7 (GraphPad Software, La Jolla, California USA), respectively.

Results

Digestibility of diets in Experiment 1

The apparent digestibility coefficients (ADC) of dry matter ($F_{5,6}$ = 0.9; P = 0.493), crude protein ($F_{5,6}$ = 0.7; P = 0.628) and gross energy ($F_{5,6}$ = 3.9; P = 0.060) were not significantly affected by dietary treatment. Average ADCs for dry matter, crude protein and gross energy were 70.6%, 92.5% and 82.0%, respectively. In contrast, the ADC of lipid ($F_{5,6}$ = 21.5; P < 0.01) and the ADC of choline ($F_{5,6}$ = 123.6; P < 0.01) were significantly affected by dietary treatment (Table 3.1.5.1.1). Lipid ADCs were significantly higher in Diet-4, Diet-5 and Diet-6 than in Diet-1. Similarly, choline ADCs were significantly higher in Diet-4, Diet-5 and Diet-6 than in Diet-1, Diet-2 and Diet-3 (Table 3.1.5.1.1).

The digestible nutrient content of diets reflected their respective ADCs. Nonetheless there was no difference between the digestible dry matter ($F_{5,6}$ = 0.9; P = 0.49) or digestible protein content of diets ($F_{5,6}$ = 2.6; P = 0.136). There were significant differences among the digestible lipid ($F_{5,6}$ = 16.9; P < 0.01), digestible energy ($F_{5,6}$ = 4.4; P = 0.048) and digestible choline ($F_{5,6}$ = 10748; P < 0.01) content of diets (Table 3.1.5.1.1). Digestible energy content was reasonably stable across diets (17.2 to 21.0 MJ kg^{-1}), whereas digestible lipid content was generally higher in Diet-4, Diet-5 and Diet-6. The estimated digestible choline content of each diet was different, ranging from 0.42 to 6.05 g kg^{-1} diet.

Digestibility of diets in Experiment 2

There were no significant effects (all P > 0.05) of dietary treatment (i.e. choline level), water temperature or the interaction of these factors on ADC's of dry matter (grand mean = 71.7%), crude protein (grand mean = 85.7%), crude lipid (grand mean = 87.8%), gross energy (grand mean = 79.5%) or choline (grand mean = 87.7%) (Table 3.1.5.1.2). Similarly, there were no significant effects of dietary treatment, water temperature or the interaction of factors on the digestible protein (grand mean = 45.6%) or digestible energy content of diets (grand mean = 18.4 MJ kg^{-1}). The effect of dietary treatment on the digestible lipid content of diets approached the level of significance ($F_{2,12}$ = 3.8; P = 0.051), however neither water temperature nor the interaction of factors affected the digestible lipid content of diets (both P > 0.05; grand mean = 16.3%). Digestible lipid content tended to be lower in P-Diet 1 than P-Diet 2 and P-Diet 3. The digestible choline content of diets was significantly affected by dietary treatment ($F_{2,12}$ = 458.3; P < 0.01), but not by water temperature or the interaction of terms (both P > 0.05). Digestible choline content of diets increased systematically from 1.77 to 3.54 and 4.66 g kg^{-1} in diet P-Diet 1, P-Diet 2 and P-Diet 3, respectively (Table 3.1.5.1.2).

Experiment 1 - choline requirement of juvenile YTK at 16 °C

100% survival of YTK was recorded in all dietary treatments with the exception of Diet-1, where a single mortality was recorded (Table 3.1.5.1.3). Dietary treatment significantly affected final weight ($F_{5,12}$ = 124.3; P < 0.01), weight gain ($F_{5,12}$ = 131.6; P < 0.01), SGR, ($F_{5,12}$ = 142.6; P < 0.01), total feed intake ($F_{5,12}$ = 216.6; P < 0.01), relative feed intake ($F_{5,12}$ = 112.6; P < 0.01) and FCR ($F_{5,12}$ = 12.2; P < 0.01). The response of YTK to different diets was consistent, with all variables improving in a step-wise manner as the dietary concentration of choline was increased (Table 3.1.5.1.3). FCR was statistically similar among Diet-2, Diet-3, Diet-4, Diet-5 and Diet-6. However, the FCR of YTK fed Diet-1 was significantly lower (worse) than all other treatments (Table 3.1.5.1.3).

Comparison of Diet-4 (+AMP) and Diet-6 (nil AMP) indicated that total and relative feed intake was significantly higher in Diet-6. The significant increase in intake of Diet-6 explains the significant increase in weight gain of fish fed Diet-6, even though it was ostensibly the same formulation as Diet-4. This indicates that the 0.3% addition of AMP may have negatively affected the palatability of Diet-4 and by extension all diets containing AMP.

Hepatosomatic index (HSI) ranged between 1.2% and 2.1% and viscerosomatic index (VSI) ranged between 6.9% and 8.8%. Both HSI ($F_{5,12}$ = 14.7; P < 0.01) and VSI ($F_{5,12}$ = 8.7; P < 0.01) were significantly affected by dietary treatment; relative liver and viscera weight tended to decline in diets that contained more choline, most notably Diet-5 and Diet-6 (Table 3.1.5.1.3).

The composition of juvenile YTK is presented in Table 3.1.5.1.4. The crude protein content of fish was not affected by dietary treatment ($F_{5,12} = 0.7$; $P = 0.653$), however diet affected the moisture ($F_{5,12} = 30.2$; $P < 0.01$), lipid ($F_{5,12} = 64.1$; $P < 0.01$), ash ($F_{5,12} = 25.5$; $P < 0.01$) and gross energy ($F_{5,12} = 47.0$; $P < 0.01$) content of fish. The gross energy and lipid content of whole fish increased systematically as the dietary level of choline increased. In addition, the lipid content of carcass was highly negatively correlated with moisture content; Pearson's r = -0.98. The choline content of juvenile fish carcass increased with increasing dietary choline ($F_{5,12} = 12.3$; $P < 0.01$) (Table 3.1.5.1.4).

The moisture ($F_{5,12} = 8.4$; $P < 0.01$), crude protein ($F_{5,12} = 4.1$; $P = 0.02$) and crude lipid ($F_{5,12} = 13.1$; $P < 0.01$) content of fish liver was affected by dietary treatment, whereas the ash ($F_{5,12} = 0.6$; $P = 0.695$), NFE ($F_{5,12} = 0.6$; $P = 0.683$) and choline content was not ($F_{5,12} = 2.2$; $P = 0.125$). Fish fed the diet devoid of AMP (Diet-6) had twice as much lipid stored in their liver as fish fed all other test diets (Table 3.1.5.1.4).

The retention of digestible protein ($F_{5,12} = 7.1$; $P < 0.01$) was similar among all diets with the exception of Diet-1, which recorded a very low retention of 16.7%. Lipid retention increased systematically in response to increases in the digestible choline content of diets ($F_{5,12} = 40.0$; $P < 0.01$), being lowest in Diet-1 (8.7%) and highest in Diet-6 (92.0%). Lipid retention of fish fed Diet-4 (+AMP) was significantly lower (49.0%) than fish fed Diet-6 (nil AMP). Digestible energy retention was also significantly affected by dietary treatment ($F_{5,12} = 27.7$; $P < 0.01$), tending to increase in response to increasing digestible choline content of diets. As noted for lipid retention, digestible energy retention was significantly lower in fish fed Diet-4 (33.3%) than in fish fed Diet-6 (44.7%).

The retention of digestible choline was significantly higher in Diet-1, Diet-2 and Diet-3 than Diet-4, Diet-5 and Diet-6 ($F_{5,12} = 26.6$; $P < 0.01$) and was negatively correlated with digestible choline content of diets. There was no difference in the choline retention of fish fed Diet-4 (+AMP; 22.4%) and Diet-6 (nil AMP; 23.6%), indicating the addition of AMP had little influence on choline retention of fish fed these closely related diets (Table 3.1.5.1.4).

Relationships between digestible choline intake and SGR or choline deposition rate are presented in Figures 3.1.5.1.1a and 3.1.5.1.1b, respectively. Best fit values for each model are presented in the table inserts accompanying each figure. Based on the segmental-linear-regression model the results indicated the break-point in choline deposition rate occurred when digestible choline intake reached 27.3 mg kg BW^{-1} day^{-1} ($R^2 = 0.97$; 95% CI = 20.9 to 36.1 mg kg BW^{-1} d^{-1}). The break-point in SGR occurred when digestible choline intake reached 26.1 mg kg BW^{-1} d^{-1} ($R^2 = 0.97$; 95% CI = 21.6 to 31.5 mg kg BW^{-1} d^{-1}).

Relationships between dietary digestible choline content and SGR or choline deposition rate are presented in Figures 3.1.5.1.2a and 3.1.5.1.2b, respectively. On a dietary basis the breakpoint in choline deposition rate occurred when diets provided 1939 mg digestible choline kg^{-1} diet ($R^2 = 0.97$; 95%; CI = 1551 to 2475 mg digestible choline kg^{-1} diet) while the breakpoint in SGR occurred when diets provided 1932 mg digestible choline kg^{-1} diet ($R^2 = 0.97$; 95% CI = 1727 to 2225 mg digestible choline kg^{-1} diet).

The *de novo* rate of choline synthesis in juvenile YTK at 16 °C was estimated by subtracting the choline deposition rate of fish fed Diet-4 (+AMP) from fish fed Diet-6 (nil AMP). This value was calculated to be 4.2 mg choline $kgBW^{-1}$ d^{-1}.

Experiment 2 – effect of adding choline chloride to a commercial-like diet

100% survival of YTK was recorded in all tanks allocated to the cool water RAS. Three fish died from tanks allocated to the warm water RAS; two from P-Diet1 (i.e. one from tank 33 and one from tank 46) and one from P-Diet 2 (i.e. tank 41) (Table 3.1.5.1.5).

Harvest weight, weight gain and SGR were all affected by water temperature, being significantly higher at 24 °C than 16 °C ($P < 0.01$; Table 3.1.5.1.5). However, there was no significant effect of dietary treatment or the interaction of main effects on these response indices (all $P > 0.05$; Table 3.1.5.1.5). Individual total feed intake and relative feed intake in YTK were significantly affected by dietary treatment and water temperature; however, in both cases the effect of water temperature was far more

significant. On average, fish ate significantly more feed at 24 °C than at 16 °C and they consumed more of P-Diet 2 than P-Diet 1.

Food conversion ratio (FCR) was significantly affected by dietary treatment ($F_{2,12}$ = 6.0; P = 0.015) and water temperature ($F_{1,12}$ = 18.0; P < 0.01). On average FCR was slightly better (lower) in YTK fed P-Diet 3 (FCR = 1.4; i.e. the highest level of choline content) and lower in YTK reared at 16 °C (FCR = 1.4 vs 1.5). FCR was worst (highest) in YTK fed P-Diet 1, the diet with the lowest amount of digestible choline (FCR = 1.5; Table 3.1.5.1.5).

Hepatosomatic index (HSI) of YTK in Experiment 2 ranged between 1.1% and 1.3% and viscerosomatic index (VSI) ranged between 6.0% and 7.0%. Neither of these indices was affected by dietary treatment, water temperature or the interaction of the main effects (P > 0.05; Table 3.1.5.1.5).

The moisture, crude protein, lipid and gross energy content of YTK carcass was not significantly affected by dietary treatment, water temperature or the interaction of these factors (P > 0.05; Table 3.1.5.1.6). The ash and choline content of whole fish was also unaffected by dietary treatment and the interaction term; however, both these responses were significantly affected by water temperature; overall choline content of fish was higher and ash content was lower at 16 °C than 24 °C, respectively.

Two-way interactions between dietary treatment and water temperature were absent in all tests on liver composition except for choline concentration (Table 3.1.5.1.6). Dietary treatment had no effect on the moisture, crude protein, crude lipid, ash or NFE content of liver (all P > 0.05; Table 3.1.5.1.6). In contrast, the moisture, crude protein, crude lipid and ash content of liver was significantly affected by water temperature. Moisture and protein content of liver was higher at 16 °C (moisture = 687.8 g kg^{-1}; protein = 157.4 g kg^{-1}; n = 9) than 24 °C (moisture = 655.3 g kg^{-1}; protein = 144.7 g kg^{-1}; n = 9), whereas the lipid and ash content of liver was lower at 16 °C (lipid = 84.2 g kg^{-1}; ash = 13.7 g kg^{-1}; n = 9) than 24 °C (lipid = 131.7 g kg^{-1}; ash = 18.8 g kg^{-1}; n = 9) (Figure 3.1.5.1.3). Dietary treatment and water temperature did not significantly affect liver choline concentration; however, there was a cross-over interaction between the two factors.

Choline deposition rate was significantly affected by diet type ($F_{2,12}$ = 16.9; P < 0.01) and water temperature ($F_{1,12}$ = 34.2; P < 0.01), but not the main effects (Table 3.1.5.1.6). Overall, choline deposition was higher in fish reared at 24 °C than 16 °C (14.6 mg kg BW^{-1} d^{-1} vs 11.6 mg kg BW^{-1} d^{-1}; n = 9) and lowest in fish fed P-Diet 1 (11.1 mg kg BW^{-1} d^{-1}; n = 6) compared to fish fed P-Diet 2 (13.8 mg kg BW^{-1} d^{-1}; n = 6) and P-Diet 3 (14.5 mg kg BW^{-1} d^{-1}; n = 6). The maximum choline deposition rate recorded in Experiment 2 was similar to the highest deposition rates measured in Experiment 1 for fish fed Diet 6 (14.3mg kgBW^{-1} d^{-1}; nil AMP).

The retention of digestible protein, digestible lipid and digestible energy was not affected by dietary treatment, water temperature or the interaction of factors (Table 3.1.5.1.6). Digestible choline retention was significantly affected by dietary treatment ($F_{2,12}$ = 45.6; P < 0.01), and water temperature ($F_{1,12}$ = 16.2; P = 0.001), but there was no interaction between the main effects (Table 3.1.5.1.6). Choline retention efficiency was higher at 16 °C (22.4%; n = 9) than 24 °C (17.1%; n = 9) and higher in fish fed P-Diet 1 (28.7%; n = 6) than P-Diet 2 (16.3%; n = 6) and P-Diet 3 (14.3%; n = 6).

Discussion

Digestible choline intake and dietary choline requirements of juvenile YTK

The digestible choline requirement of juvenile YTK reared at 16 °C was found to be 27.3 mg kg BW^{-1} d^{-1} when using choline deposition rate as the response variable or 26.1 mg kg BW^{-1} d^{-1} when using SGR as the response variable. This represents a 1.2 mg difference between estimates. The 95% CI for each response ranged between 20.9 to 36.1 mg kg BW^{-1} d^{-1} and 21.6 to 31.5 mg kg BW^{-1} d^{-1}, respectively. On a dietary basis, the break-point in choline deposition rate and SGR were reached when diets provided 1.94 g and 1.93 g digestible choline kg^{-1}, respectively. The 95% CI for each response ranged between 1.55 to 2.48 g digestible choline kg^{-1} diet and 1.73 to 2.23 g digestible choline kg^{-1} diet, respectively. It is important to recognise these estimates were made by fitting a segmental linear regression model to the data and that other dose-response models may provide different values. For example fitting a polynomial regression would elevate the requirement. It should also be noted that these requirements

are derived from test diets containing AMP. Nonetheless, we recommend formulating diets for YTK based on the upper limit of the 95% CI's for choline deposition rate and SGR. This conservative approach will ensure all fish within a population receive an adequate amount of digestible choline.

The *de novo rate* of choline synthesis was estimated to be 4.2 mg choline kg BW^{-1} d^{-1} when based on the absolute difference between the choline deposition rate of YTK fed Diet-4 (+AMP) and Diet-6 (nil AMP). The magnitude of the estimate compared to the requirement value suggests juvenile YTK have a limited capacity for *de novo* synthesis of choline. The use of AMP clearly depressed feed intake of fish allocated to Diet-4 compared to those allocated to Diet-6 and by extension, may have depressed feed intake in YTK fed other diets containing AMP. However, this outcome does not affect our estimate of choline requirement in juvenile YTK because the response indices relied on showed an asymptotic response to increasing levels of digestible choline intake. Furthermore, there was also little difference between the choline retention efficiency of fish fed Diet-4 and Diet-6 (i.e. 22.4% to 23.6%), supporting the hypothesis that AMP had little impact on choline metabolism. Based on the *de novo* rate of choline synthesis and the best fit value of choline requirement we can conclude that juvenile YTK held at 16 °C are capable of endogenously synthesising about 16% of their choline requirements under the stated experimental conditions (i.e. 4.2 / 26.1 × 100 = 16%). The *de novo* rate of choline synthesis drops to 11.6% if the upper 95% confidence interval of 36.1 mg kg BW^{-1} $d^{-1}$for is used as the denominator, highlighting the importance of supplementing this compound into diets for YTK.

Experiment 2 revealed that absolute weight gain and FCR tended to improve in YTK fed a commercial-like formulation supplemented with feed grade CC. The basal mash comprised of common raw materials but devoid of CC was determined to have 2.05 g residual choline kg^{-1} diet. Adding 3 g (i.e. standard industry practice) and 6 g CC kg^{-1} (i.e. double industry practice) to the basal diet elevated the dietary choline concentrations to 3.87 and 5.44 g choline kg^{-1}, respectively. However the apparent choline ADCs of these three diets were not greatly affected by water temperature and ranged between 85-91%. The determination of the ADCs allowed estimation of the digestible choline concentration of P-Diet 1, P-Diet 2 and P-diet 3, which were found to be, on average, 1.77, 3.54 and 4.66 g kg^{-1} diet, respectively. Highest weight gain and lowest FCR corresponded with P-diet 2, indicating the amount of digestible choline provided by this diet was more than adequate for juvenile YTK, at least under the conditions imposed in this experiment. In fact, the amount of digestible choline provided by P-Diet 2 was 43% higher than the amount of digestible choline recommended above (i.e. 2.48 g digestible choline kg^{-1} diet; upper 95% CI), and indicates the current industry practice of supplementing 3 g CC kg^{-1} to YTK diets is probably sufficient. Further fortification of the basal mash with CC (i.e. P-Diet 3) had little impact on the SGR and FCR of juvenile YTK held at either water temperature, indicating the addition of an extra 3 g CC kg^{-1} to formulations above the industry standard is unwarranted.

The amount of digestible choline provided by P-Diet 2 was adequate in terms of supporting weight gain (Figure 3.1.5.1.2a), however slightly higher amounts of supplementation may be required to optimise choline deposition rate (Figure 3.1.5.1.2b; Figure 3.1.5.1.3). The trade-off here is that choline retention efficiency in YTK declines rapidly in response to increasing dietary inclusion of CC, suggesting there is little point oversupplying this water soluble nutrient (Aldrich, 2008). Choline toxicity has been reported in Hybrid Striped Bass fed more than 8 g choline bitartrate kg^{-1} diet, but not when provided as CC (Griffin et al., 1994). Excess CC and choline bitartrate have been reported to reduce growth and increase mortality in rodents (Byington, 1978) and cause diarrhoea in humans (Zeisel and Blusztajn, 1994). However, Zeisel and Blusztain (1994) stated that the toxicity was not caused by choline *per se*, but as a result of an ionic imbalance caused by the chloride released from CC.

Production indices of YTK such as SGR and feed intake were enhanced at 24 °C as opposed to 16 °C in Experiment 2. The positive influence of water temperature on production of YTK is well documented (Pirozzi and Booth, 2009; Abbink et al., 2012; Bowyer et al., 2014), however, in this study there was greater interest in the effect of water temperature on choline requirement. Overall choline deposition rate was found to be higher at 24 °C than 16 °C. In contrast, overall choline retention efficiency was found to be lower at 24 °C than at 16 °C. The higher deposition rate at 24 °C might be explained by the higher feed intake of YTK at this temperature, while the poorer retention of choline at 24 °C might be due to increased metabolic rate leading to a higher requirement for choline or increased passage of feed through the gut. Irrespective of the reasons, the implications of this are that farmed YTK will become choline deficient must faster at higher water temperatures than lower water temperatures. Nonetheless, choline retention efficiency was inversely related to digestible choline intake (e.g. choline retention

efficiency = $45.78e^{(-0.244 \times \text{dig. choline intake})}$; $R^2 = 0.89$) in both experiments, demonstrating the digestive system of YTK is easily overwhelmed by excess choline in the form of CC (Table 3.1.5.1.4 and Table 3.1.5.1.6; "the more you put in the more you get out!"). The tissue choline deposition rate of YTK fed Diet-6 (nil AMP) in Experiment 1 (i.e. 14.3 mg kg $BW^{-1}d^{-1}$) compared favourably to the range of choline deposition rates measured in YTK fed diets supplemented with roughly the same amount of CC in Experiment 2 (i.e. P-Diet 2; range 11.9 - 15.7 mg kg $BW^{-1}d^{-1}$), indicating a high degree of similarity in the response to choline intake between studies.

Choline requirement of other fast growing species

As previously noted the choline supplementation rate of YTK thus far has been based on the estimated choline requirements of the closely related Japanese Yellowtail, which were shown to require between 2100 to 2900 mg CC kg^{-1} of diet based on response to weight gain and liver choline concentration, respectively (Hosokawa et al., 2001). The study on Japanese Yellowtail was done using reagent grade CC, used very small fish (25.6 g), lasted only 17 days and was done at very high water temperature (25-29.8 °C). Based on the molecular weight of CC these estimates are equivalent to approximately 1567 and 2163 mg choline kg^{-1} diet. Accounting for differences between experiments and the digestibility of diets the values derived from the study on Japanese Yellowtail are remarkably similar to the upper 95% CI values determined for YTK. In contrast, a study on the choline requirements of Cobia was run for 10 weeks, used reagent grade CC, small fish (4.2 g) and water temperatures between 28.5 °C and 32 °C. These authors also used diets that provided slightly less methionine (1.05% of diet) than required for optimal performance in Cobia (i.e. 1.19% of diet), so endogenous synthesis of choline from methionine would be limited. Based on broken-line regression of weight gain, liver and muscle choline concentration on dietary choline concentration the dietary choline requirement of Cobia was found to be 696, 877 and 950 mg choline kg^{-1} diet, respectively when provided in the form of choline chloride. A comparison of dietary choline requirements of YTK, Cobia and Japanese Yellowtail based on SGR is presented in Figure 3.1.5.1.4. These data have been adjusted to the choline content of diets, not digestible choline content or CC equivalents.

Effects of choline supplementation on tissue composition of YTK

Choline concentration of whole body and liver tissues of juvenile YTK was uniform across both experiments and was mostly unaffected by dietary choline intake The choline content of the whole animals was significantly higher at 16 °C than 24 °C, and although not significant, there was a similar trend in liver choline concentration (Experiment 2). This increase indirectly explains the increased retention efficiency of choline in YTK at lower water temperature. Whole body choline content ranged from 0.6 to 0.9 g kg^{-1} and liver choline content ranged from1.1 to 2.2 g kg^{-1}. The liver response in YTK was different to studies investigating choline requirement of juvenile Japanese Yellowtail (Hosokawa et al., 2001), Cobia (Mai et al., 2009) and Channel Catfish (Zhang and Wilson, 1999), where choline concentration in the liver was found to increase with increasing dietary choline. For this reason the liver and whole-body choline content of YTK from the present study could not be used as suitable indicators of choline status. Liver lipid concentration has been used as an indicator of choline status in mammals; however, as found in this study liver lipid concentration has not been a reliable indicator of choline status in fish (Twibell and Brown 2000).

The lipid concentration of whole fish or livers of YTK fed commercial-like diets in Experiment 2 was not significantly affected by choline content of diets; nor was lipid retention efficiency. In contrast, the lipid content of whole fish and fish liver in Experiment 1 increased in response to increasing choline content of diets; as did lipid retention efficiency. Interestingly the whole body and liver lipid content of fish fed Diet-6 (nil AMP) was nearly double the lipid concentration of the same organs in fish fed Diet 4 (+ AMP) and they resembled the lipid status of fish fed commercial-like diets. The increasing concentration and retention of lipid in the whole body and liver tissue of fish fed the semi-purified diets indicates these fish, especially those fed diets with digestible choline concentrations below the aforementioned requirement level (i.e. Diet-1, Diet-2, Diet-3 and possibly Diet-4) may have been preferentially catabolising lipids for energy or alternatively, the absence of choline may have indirectly restricted or altered lipid synthesis. The significant increase in the lipid content of the tissue and liver of

fish fed Diet-6 (nil AMP) indicates AMP was presumably interfering in lipid metabolism of YTK by antagonism of choline metabolism.

The response of juvenile YTK to increasing choline is in contrast with most other animal models where increasing dietary choline has been shown to either reduce liver lipid or have no effect at all. However, Craig and Gatlin (1996) in conducting a choline requirement study on Red Drum also found that fish fed diets deficient in choline had reduced liver lipid rather than lipid accumulation. Cultured Red Drum are known to store as much as 30% lipid by weight in their liver, which is considered quite high (Craig and Gatlin, 1996). Mai et al., (2009) also found that dietary choline supplementation significantly increased the muscle lipid content of Cobia (not whole body), but in contrast to our study the liver lipid content of Cobia declined from about 56% to 35% as choline content of diets increased. The maximum liver lipid recorded in this study was in fish fed commercial-like diets at 24 °C (15%; P-Diet 1). Like Red Drum and perhaps Cobia, well fed juvenile YTK may have a proclivity for lipid storage in the liver. In addition, like Red Drum, it is likely that the removal of liver lipid in YTK via very-low-density-lipoprotein (VLDL) will not take place prior to storing a certain amount of lipid in the liver required for normal metabolic function of YTK. Without enough choline, the assembly of lipoproteins, uptake and formation of lipid by the liver may be reduced (Minahk et al., 2008; van der Veen et al., 2012).

Interpretation of choline studies

The comparison of choline requirements among species is problematic for several reasons. Firstly, there are diet, abiotic and ontogeny differences to consider (Poston 1991; Rumsey, 1991; Griffin, 1994; Wang et al., 1995; Zhao et al., 2015). Secondly, the data can be difficult to interpret because some author's present requirement data on a choline only basis, some on a choline chloride basis and in some studies it is difficult to determine which basis has been used. In addition the activity of the choline premix or supplement is often not reported and there are often differences in the methodology used to measure the choline concentration of samples and sample types. Synthetic choline sources are commonly added to animal feeds in the form of salts such as choline chloride, choline bitartrate, choline pantothenate and choline xanthate. Free choline is present in many biological materials as acetylcholine or esterified forms including PC, phosphocholine, glycerophosphocholine, lysophosphotidylchoine and sphingomyelin. As some of these compounds are hydrophilic and others are hydrophobic, during analysis extraction of choline from choline-containing compounds and variations in instrumentation used can result in different total choline concentration in samples (Zeisel et al., 2003; Phillips, 2012). A review of various analytical approaches on choline quantification in foods is detailed by Phillips (2012).

Nutritional interactions with other compounds such as betaine and methionine (i.e. both methyl donors like choline), folate and vitamin B_{12} can also influence the determination of choline requirements (da Costa et al., 2006). Methionine has been shown to reduce the requirement for dietary choline in Lake Trout (*Salvelinus namaycush*), Channel Catfish and Rainbow Trout (Ketola, 1976; Rumsey, 1991; Zhang and Wilson, 1999; Kasper et al., 2000). However there are no published studies on the sparing effect of methionine on choline synthesis or choline requirement in YTK or related *Seriola spp*. Choline metabolic pathways have not been closely studied in fish; however, endogenous choline synthesis (via PC) catalysed by PEMT requires methyl donor groups from *S*-adenosyl-methionine (SAM). Moreover, methionine is known to be an important precursor of SAM in terrestrial animals (Zhang, 2018). Previous choline requirement studies on Cobia (Mai et al., 2009) and Red Drum (Craig and Gatlin, 1996) have made test diets marginally lower in either methionine or total sulfur amino acids to limit the potential synthesis of choline from methionine. This is done in order to provide a better estimate of choline requirements in the absence of other methyl donors. All the test diets in this experiment provided about 1% methionine on a crude basis. Based on research presented in Manuscript 3.1.5.3, this amount is now known to be lower than the optimum methionine requirement of juvenile YTK (>1.5% diet). Thus it is also likely the diets in the present work on YTK were marginally deficient in methionine. This, combined with the use of AMP to limit *de novo* synthesis of choline may explain the slightly higher choline requirement of YTK compared to Japanese Yellowtail (Figure 3.1.5.1.4). The interactions between choline, methionine and taurine have been studied in Atlantic salmon and readers interested in developing a greater understanding of the metabolic pathways involved should consult Espe et al. (2015) and Espe et al. (2017).

Practical considerations

In this study we supplemented diets with a feed grade CC reported to be ≈70% CC on a dry weight basis. Therefore, for every gram of CC included in our diets only 0.7 g CC was actually added to the diet mash. Furthermore, CC has a molecular weight of 139.62 g mol^{-1} ($C_5H_{14}ClNO$), and on that basis contains only 74.61% choline. Therefore, using an example from the present study, the addition of 3 g CC kg^{-1} diet elevates the residual choline level of the diet by about 1.56 g choline kg^{-1}. This estimate represents the free choline provided by CC and not the available or digestible fraction of choline. An estimate of that is found by accounting for the digestibility of the dietary choline, which in this study was found to be about 90%. Thus, the addition of 3g CC kg^{-1} mash provides 1.40 g digestible choline kg^{-1} above the choline already provided by the inclusion of other raw materials. The concentration of residual choline provided by the raw materials in the basal mash in Experiment 2 was determined to be 2.05 g choline kg^{-1} diet. Adding this value to the free choline provided by CC in P-Diet 2 and P-Diet 3 sums to 3.61 and 5.18 g choline kg^{-1} diet, respectively. The calculated values for P-Diet 2 and P-Diet 3 are about 6.7% and 4.8% lower than the measured values, however allowing for analytical error, the agreement between the predicted and estimated choline values for the commercial diets is pleasing. A useful example of choline addition based on the raw materials and formulas used in Experiment 2 is provided in Table 3.1.5.1.7.

Yellowtail Kingfish cannot meet the aforementioned choline requirements unless they can physically consume an appropriate amount of feed containing a suitable level of choline. The present work has estimated a 1 kg fish conservatively requires 36.1 mg digestible choline d^{-1} (i.e. upper 95% CI). Using the digestible choline content of P-Diet 2 (3.4 g digestible choline kg^{-1}) as an example this would mean a 1 kg fish would have to consume 10.6 g feed to meet the daily requirement. This equates to a relative feed intake of 1.06% BW d^{-1} which is entirely feasible for YTK of this size. A higher feed intake rate would simply mean a greater amount of choline (and all other nutrients) is consumed.

The relative feed intake of a 1 kg fish in Experiment 2 translates to 1.8% BW d^{-1} at 16 °C and 2.9% BW d^{-1} at 24 °C (Table 3.1.5.1.2). Using P-Diet 2 to feed these fish would result in digestible choline intake of 61.2 mg digestible choline and 98.6 mg digestible choline at 16 °C and 24 °C, respectively. This choline intake is more than adequate in terms of meeting the estimated requirement of YTK at 16 °C and presumably provides a sufficient amount for fish reared at 24 °C. Based on the average DP (45.5%) and DE (18 MJ kg^{-1}) content of the commercial-like formula used in Experiment 2, an intake of 1.8% BW d^{-1} would also provide a 1 kg fish with 8.2 g of DP and 324 kJ DE per day. These values are in close agreement with the daily DP (7.4 g $fish^{-1}$) and DE (272.8 kJ $fish^{-1}$) requirements of 1 kg YTK estimated using bioenergetic models (Booth et al., 2010). Therefore, YTK should be able to meet not only their requirements for choline, but also their requirements for DP and DE consuming commercial diets similar to P-Diet 2.

Key findings

- The digestible choline requirement of juvenile YTK reared at 16 °C was found to be 27.3 mg kg BW^{-1} d^{-1} when using choline deposition rate as the response variable or 26.1 mg kg BW^{-1} d^{-1} when using SGR as the response variable. The 95% CI for these estimates ranged between 20.9 to 36.1 mg kg BW^{-1} d^{-1} when based on choline deposition and 21.6 to 31.5 mg kg BW^{-1} d^{-1} when based on specific growth rate.
- On a dietary basis, the break-point in choline deposition rate and SGR were reached when diets provided 1.94 g and 1.93 g digestible choline kg^{-1}, respectively. The 95% CI for these estimates ranged between 1.55 to 2.48 g digestible choline kg^{-1} diet when based on choline deposition and 1.73 to 2.23 g digestible choline kg^{-1} diet when based on specific growth rate.
- Choline supplementation of commercial-like formulas for YTK will be necessary if the residual amount of choline is less than approximately 2.48 g digestible choline kg^{-1} diet.
- Short term exposure of juvenile YTK to excess digestible choline (up to 4.76-6.05 g kg^{-1} diet) does not appear to be toxic to juvenile YTK at either 16 °C or 24 °C.

- The current industry practice of supplementing 3 g choline kg^{-1} diet provides slightly more digestible choline than required by YTK to meet their digestible choline requirements. However, until further research is done on this vitamin-like compound and its interaction with other nutrients such as methionine, betaine and taurine it would be prudent to continue formulating diets for YTK to ensure they provide at least 3 g digestible choline kg^{-1} diet. This conservative approach should ensure that fish do not experience a choline deficiency under most production conditions.
- Alternative forms of feed grade choline are available. The efficacy of these sources to YTK should be examined.
- The methodology used to quantify total choline is not routinely carried out by many laboratories in Australia and there is a need to remedy this situation, especially if a more thorough understanding of choline synthesis or indeed the metabolism of other methyl donors is in YTK is to be studied.
- The protective nature of choline against environmental stressors is unknown and should be studied. In addition, little is known about the depletion rate of choline in YTK, especially during prolonged fasting or under conditions such as infrequent feeding.

Publications

One publication has resulted from this R&D to date. Liu, A., Pirozzi, I., Codabaccus, B., Hines, B., Simon, C., Sammut, J., Booth, M., Accepted. Digestible choline requirement of juvenile Yellowtail Kingfish (*Seriola lalandi*). Aquaculture.

Acknowledgements

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

This research was also supported by an Australian Government Research Training Program Scholarship. Some of the raw materials used in this experiment were kindly provided by Ridley and as such we extend out thanks to Michael Salini and Simon Tabrett for their assistance. The authors would like to thank Ian Russell, Brendan Findlay, Steve Gamble (NSW DPI) and Susan Cheers (CSIRO) for their technical assistance. We are also grateful to Jay Dent (Clean Seas Seafood) for feedback on some of the data generated in this study.

References

Abbink, W., Blanco Garcia, A., Roques, J.A.C., Partridge, G.J., Kloet, K., Schneider, O., 2012. The effect of temperature and pH on the growth and physiological response of juvenile yellowtail kingfish *Seriola lalandi* in recirculating aquaculture systems. Aquaculture 330-333, 130-135.

ACEC, 2015. A Guide to Acceptable Procedures and Practices For Aquaculture and Fisheries Research - 4th Edition, 4th ed. NSW Department of Primary Industries (Fisheries) for NSW Fisheries Animal Care and Ethics Committee, Nelson Bay, NSW, Australia.

Aldrich, G., 2008. Dealing with choline chloride. https://www.petfoodindustry.com/articles/9-dealing-with-choline-chloride?v=preview. pp. 2.

AOAC, 2006. Official Methods of Analysis. 18th Edition, Association of Official Analytical Chemists, Gaithersburgs, MD.

Bansemer, M.S., Stone, D.A.J., D'Antignana, T., Skordas, P., Kuerschner, L., Currie, K.-L., 2018. Optimizing Feeding Strategies for Yellowtail Jack at Winter Water Temperatures. North American Journal of Aquaculture 80, 128-140.

Booth, M., Pirozzi, I., 2017. Making sense of nonsense: using regression analysis to deal with highly variable data collected from a yellowtail kingfish (*Seriola lalandi*) digestibility experiment. Aquaculture 485, 39-48.

Booth, M.A., Allan, G.L., Pirozzi, I., 2010. Estimation of digestible protein and energy requirements of yellowtail kingfish *Seriola lalandi* using a factorial approach. Aquaculture 307, 247-259.

Bowyer, J.N., Booth, M.A., Qin, J.G., D'Antignana, T., Thomson, M.J.S., Stone, D.A.J., 2014. Temperature and dissolved oxygen influence growth and digestive enzyme activities of yellowtail kingfish *Seriola lalandi* (Valenciennes, 1833). Aquacult. Res. 45, 2010-2020.

Caudill, M.A., 2010. Pre- and Postnatal Health: Evidence of Increased Choline Needs. Journal of the American Dietetic Association 110, 1198-1206.

Cho, C.Y., Slinger, S.J., Bayley, H.S., 1982. Bioenergetics of salmonid fishes: energy intake, expenditure and productivity. Comparative Biochemistry and Physiology 73B, 25-41.

Craig, S.R., Gatlin, D.M., 1996. Dietary choline requirement of juvenile red drum (*Sciaenops ocellatus*). J. Nutr. 126, 1696-1700.

da Costa, K.-A., Niculescu, M.D., Craciunescu, C.N., Fischer, L.M., Zeisel, S.H., 2006. Choline deficiency increases lymphocyte apoptosis and DNA damage in humans. The American Journal of Clinical Nutrition 84, 88-94.

Duan, Y., Zhu, X., Han, D., Yang, Y., Xie, S., 2012. Dietary choline requirement in slight methionine-deficient diet for juvenile gibel carp (*Carassius auratus gibelio*). Aquacult. Nutr. 18, 620-627.

Espe, M., Zerrahn, J.E., Holen, E., Rønnestad, I., Veiseth-Kent, E., Aksnes, A., 2015. Choline supplementation to low methionine diets increase phospholipids in Atlantic salmon, while taurine supplementation had no effects on phospholipid status, but improved taurine status. Aquacult. Nutr., doi: 10.1111/anu.12298

Espe, M., Andersen, S.M., Veiset-Kent, E., Rønnestad, I., Holen, E., Zerrahn, J.E., Aksnes, A., 2017. Choline supplementation increased total body lipid gain, while surplus methionine improved growth and amino acid retention in adult Atlantic salmon (*Salmo salar*). Aquacult. Nutr., doi: 10.1111/anu.12298

Fagone, P., Jackowski, S., 2013. Phosphatidylcholine and the CDP-choline cycle. Biochim Biophys Acta. 1831, 523-532.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. The Journal of Biological Chemistry 226, 497-509.

Griffin, E.M.W., Keith A.; White, M. Randall; Brown, B. Paul, 1994. Dietary choline requirement of juvenile hybrid striped bass. The Journal of Nutrition 124, 1685.

Hosokawa, H., Shimeno, S., Takeda, M., 2001. Dietary choline requirement of fingerling Yellowtail. Suisan Zoshoku 49, 231-235.

Hung, S.S.O., 1989. Choline requirement of hatchery-produced juvenile white sturgeon (*Acipenser transmontanus*). Aquaculture 78, 183-194.

Kasper, C.S., White, M.R., Brown, P.B., 2000. Choline is required by tilapia when methionine is not in excess. J Nutr. 130, 238-242.

Ketola, H.G., 1976. Choline Metabolism and Nutritional Requirement of Lake Trout (*Salvelinus namaycush*). Journal of Animal Science 43, 474-477.

Khosravi, S., Jang, J.-W., Rahimnejad, S., Song, J.-W., Lee, K.-J., 2015. Choline Essentiality and Its Requirement in Diets for Juvenile Parrot Fish (*Oplegnathus fasciatus*). Asian-Australasian Journal of Animal Sciences 28, 647-653.

Kumar, N., Jadhao, S., Jha, A., Kumar, K., Chandan, N., Akhtar, M.S., Aklakur, M., Kumar, S., Rana, R., 2012. Methyl donors potentiates growth, metabolic status and neurotransmitter enzyme in *Labeo rohita* fingerlings exposed to endosulfan and temperature. Fish Physiol. Biochem. 38, 1343-1353.

Luo, Z., Wei, C.-C., Ye, H.-M., Zhao, H.-P., Song, Y.-F., Wu, K., 2016. Effect of dietary choline levels on growth performance, lipid deposition and metabolism in juvenile yellow catfish *Pelteobagrus fulvidraco*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 202, 1-7.

Mai, K., Xiao, L., Ai, Q., Wang, X., Xu, W., Zhang, W., Liufu, Z., Ren, M., 2009. Dietary choline requirement for juvenile cobia, *Rachycentron canadum*. Aquaculture 289, 124-128.

Miegel, R.P., Pain, S.J., van Wettere, W.H.E.J., Howarth, G.S., Stone, D.A.J., 2010. Effect of water temperature on gut transit time, digestive enzyme activity and nutrient digestibility in yellowtail kingfish (*Seriola lalandi*). Aquaculture 308, 145-151.

Minahk, C., Kim, K.-W., Nelson, R., Trigatti, B., Lehner, R., Vance, D., 2008. Conversion of Low Density Lipoprotein-associated Phosphatidylcholine to Triacylglycerol by Primary Hepatocytes. J Biol Chem. 283, 6449-6458.

Moran, D., Pether, S.J., Lee, P.S., 2009. Growth, feed conversion and faecal discharge of yellowtail kingfish (*Seriola lalandi*) fed three commercial diets. New Zealand Journal of Marine and Freshwater Research 43, 917-927.

NRC, 2011. Nutrient Requirements of Fish and Shrimp. National Academy Press Washington, DC. https://doi.org/10.17226/13039. 392 p

Ogino, C., Uki, N., Watanabe, T., Iida, Z., Ando, K., 1970. B vitamin requirements of carp. 4. Requirement for choline. Bulletin of the Japanese Society of Scientific Fisheries 36, 1140-1146.

Phillips, M.M., 2012. Analytical approaches to determination of total choline in foods and dietary supplements. Analytical and Bioanalytical Chemistry 403, 2103-2112.

Pirozzi, I., Booth, M.A., 2009. The routine metabolic rate of mulloway (*Argyrosomus japonicus*: Sciaenidae) and yellowtail kingfish (*Seriola lalandi*: Carangidae) acclimated to six different temperatures. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 152, 586-592.

Poston, H.A., 1991. choline requirement of swim-up rainbow trout fry. The Progressive Fish-Culturist 53, 220-223.

Qin, D.G., Dong, X.H., Tan, B.P., Yang, Q.H., Chi, S.Y., Liu, H.Y., Zhang, S., 2017. Effects of dietary choline on growth performance, lipid deposition and hepatic lipid transport of grouper (*Epinephelus coioides*). Aquaculture Nutrition 23, 453-459.

R Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. 3691 p.

Rumsey, G.L., 1991. Choline-betaine requirements of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 95, 107-116.

Sicuro, B., Luzzana, U., 2016. The State of Seriola spp. Other Than Yellowtail (*S. quinqueradiata*) Farming in the World. Reviews in Fisheries Science & Aquaculture 24, 314-325.

Simon, J., 1999. Choline, betaine and methionine interactions in chickens, pigs and fish (including crustaceans). World's Poultry Science Journal 55, 353-374.

Twibell, R.G., Brown, P.B., 2000. Dietary choline requirement of juvenile yellow perch (*Perca flavescens*). The Journal of Nutrition 130, 95-99.

van der Veen, J.N., Lingrell, S., Vance, D.E., 2012. The Membrane Lipid Phosphatidylcholine Is an Unexpected Source of Triacylglycerol in the Liver. The Journal of Biological Chemistry 287, 23418-23426.

Wang, D., Zhao, L., Tan, Y., 1995. Requirement of the fingerling grass carp (*Ctenopharyngodon taellus*) for choline. Shuichan Xuebao 19, 133-139.

Woollard, D.C., Indyk, H.E., 2000. Determination of choline in milk and infant formulas by enzymatic analysis: collaborative study. Journal of AOAC International 83, 131-138.

Yeh, S.-P., Shiu, P.-J., Guei, W.-C., Lin, Y.-H., Liu, C.-H., 2015. Improvement in lipid metabolism and stress tolerance of juvenile giant grouper, *Epinephelus lanceolatus* (Bloch), fed supplemental choline. Aquaculture Research 46, 1810-1821.

Zeisel, S.H., Blusztajn, J.K., 1994. Choline and human nutrition. Annual Review of Nutrition 14, 269-296.

Zeisel, S.H., Mei-Heng, M., Howe, J.C., Holden, J.M., 2003. Concentrations of choline-containing compounds and betaine in common foods1. The Journal of Nutrition 133, 1302-1307.

Zhang, N., 2018. Role of methionine on epigenetic modification of DNA methylation and gene expression in animals. Animal Nutrition 4, 11-16.

Zhang, Z., Wilson, R.P., 1999. Reevaluation of the choline requirement of fingerling channel catfish (*Ictalurus punctatus*) and determination of the availability of choline in common feed ingredients. Aquaculture 180, 89-98.

Zhao, H.-F., Feng, L., Jiang, W.-D., Liu, Y., Jiang, J., Wu, P., Zhao, J., Kuang, S.-Y., Tang, L., Tang, W.-N., Zhang, Y.-A., Zhou, X.-Q., 2015. Flesh shear force, cooking loss, muscle antioxidant status and relative expression of signaling molecules (Nrf2, Keap1, TOR, and CK2) and their target genes in young grass carp (*Ctenopharyngodon idella*) muscle fed with graded levels of choline. PloS one 10, e0142915.

Table 3.1.5.1.1. Formulation, proximate composition and digestibility of semi-purified diets used in Experiment 1 (g kg^{-1} dry matter basis unless stated otherwise).

Ingredient	Semi-purified diets					
	Diet-1	Diet-2	Diet-3	Diet-4	Diet-5	Diet-6
Fishmeal	100.0	100.0	100.0	100.0	100.0	100.0
Sodium caseinate	400.0	400.0	400.0	400.0	400.0	400.0
Gelatine	80.0	80.0	80.0	80.0	80.0	80.0
Dextrin	175.0	175.0	175.0	175.0	175.0	175.0
Fish oil	170.0	170.0	170.0	170.0	170.0	170.0
Taurine	10.0	10.0	10.0	10.0	10.0	10.0
Vitamin/mineral premix	10.0	10.0	10.0	10.0	10.0	10.0
Vitamin C (Stay-C)	5.0	5.0	5.0	5.0	5.0	5.0
AMP	3.0	3.0	3.0	3.0	3.0	0.0
Choline chloride (70% CC)	0.0	1.0	1.5	4.0	10.0	4.0
Diatomaceous earth[a]	27.0	26.0	25.5	23.0	17.0	29.0
Feed attractant	20.0	20.0	20.0	20.0	20.0	20.0
Total	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0
Analysed composition						
Dry matter	899.0	900.5	891.7	894.0	890.2	888.4
Crude protein	560.0	567.1	550.9	574.4	553.4	551.3
Crude lipid	151.2	135.7	137.1	135.6	147.8	130.5
Ash	88.0	88.4	88.9	87.5	82.2	87.8
Gross energy (MJ kg^{-1})	23.4	23.4	23.7	23.5	24.0	23.5
Total choline	0.59	1.25	1.56	3.11	6.22	3.29
Amino acid profile[b*]						
Hydroxyproline	7.8	8.1	8.3	8.8	9.3	9.1
Histidine	11.3	11.6	11.1	11.6	11.3	11.5
Taurine	8.2	8.5	8.2	8.7	8.4	8.5
Serine	20.9	21.3	20.7	21.3	20.8	21.3
Arginine	20.2	20.8	20.5	21.2	21.1	21.2
Glycine	21.3	22.1	22.2	23.3	24.0	23.7
Aspartic acid	29.5	29.5	28.6	30.0	29.3	29.4
Glutamic acid	83.3	83.9	80.5	84.4	82.8	83.5
Threonine	16.4	16.8	16.3	16.8	16.4	16.8
Alanine	16.5	16.9	16.6	17.3	17.3	17.3
Proline	44.6	45.6	44.2	46.1	45.8	46.3
Lysine	31.7	31.9	30.7	32.1	31.3	31.6
Tyrosine	15.8	16.4	16.3	16.6	16.0	16.5
Methionine	10.9	11.2	11.0	11.3	10.9	11.1
Valine	24.8	25.3	24.3	25.3	24.8	25.2
Isoleucine	20.3	20.8	19.9	20.7	20.3	20.5
Leucine	35.9	36.5	35.2	36.4	35.7	36.3
Phenylalanine	20.3	20.6	19.9	20.6	20.2	20.6
Tryptophan	NA	NA	NA	NA	NA	NA
Apparent digestibility coefficient (%)[c]						
Dry matter	65.4±0.6	71.8±3.4	64.2±6.6	73.8±10.1	75.9±5.9	72.7±9.2
Protein	93.0±0.9	93.5±1.3	90.0±2.3	92.9±3.1	93.4±1.6	92.2±2.9
Lipid	40.8±13.0[c]	71.0±0.1[ab]	63.1±0.6[bc]	84.4±6.7[ab]	89.4±0.2[a]	93.7±1.6[a]
Gross energy	73.3±2.9	83.1±2.4	77.2±2.8	84.6±6.0	87.6±2.8	86.1±5.2
Total choline	70.1±0.3[c]	88.7±1.3[b]	88.0±2.3[b]	95.3±1.6[a]	97.1±0.2[a]	96.9±0.7[a]
Apparent digestible nutrient content (g kg^{-1})						
Dry matter	655.0±7.1	715±35.4	640.0±70.7	740.0±99.0	760.0±56.6	725.0±91.9
Protein	521.0±5.0	530.0±7.4	495.7±12.6	533.4±17.9	517.1±8.8	508.4±16.0
Lipid	61.8±19.7[d]	96.3±0.1[bcd]	86.6±0.8[cd]	114.4±9.1[abc]	132.2±0.3[a]	122.3±2.0[ab]
Gross energy (MJ kg^{-1})	17.2±0.7[b]	19.5±0.6[ab]	18.3±0.6[ab]	19.9±1.4[ab]	21.0±0.7[a]	20.2±1.2[ab]
Total choline	0.42±0.00[a]	1.10±0.02[b]	1.37±0.04[c]	2.96±0.05[d]	6.05±0.01[e]	3.19 ±0.02[f]

*Analysed by APAF Macquarie University.

[a] 1 g of yttrium oxide added at the end of digestibility experiment in exchange of diatomaceous earth.

[b] Calculation based on amino acid residue mass in protein (molecular weight minus water). NA denotes not analysed.

[c] Values with different superscript letters in the same row are significantly different ($P < 0.05$; ANOVA; Tukeys *post-hoc* test).

Table 3.1.5.1.2. Formulation, proximate composition and digestibility of fishmeal-based diets used Experiment 2 (g kg^{-1} dry matter basis unless stated otherwise).

Ingredient	Fishmeal-based practical diets					
	P-Diet 1 (No added CC)		P-Diet 2 (+3 g CC)		P-Diet 3 (+6 g CC)	
Fishmeal	400.0		400.0		400.0	
Poultry meal	70.0		70.0		70.0	
Meat meal	50.0		50.0		50.0	
Blood meal	70.0		70.0		70.0	
Lupins	100.0		100.0		100.0	
Wheat flour	150.0		150.0		150.0	
Fish oil	60.0		60.0		60.0	
Poultry oil	60.0		60.0		60.0	
Vitamin/mineral premix	5.0		5.0		5.0	
Vitamin C (Stay-C)	0.5		0.5		0.5	
Taurine	10.0		10.0		10.0	
Sodium phosphate monobasic	5.0		5.0		5.0	
Choline chloride (70% CC)	0.0		3.0		6.0	
Diatomaceous earth[a]	19.5		16.5		13.5	
Total	1000.0		1000.0		1000.0	
Analysed composition (g kg^{-1})						
Dry matter	958.6		958.4		973.9	
Crude protein	518.5		524.0		538.1	
Crude lipid	179.4		192.7		183.0	
Ash	114.0		105.0		101.3	
Gross energy (MJ kg^{-1})	22.9		23.0		23.3	
Total choline	2.05		3.87		5.44	
Amino acid profile[b*]						
Hydroxyproline	4.0		4.0		4.3	
Histidine	16.6		16.1		16.3	
Taurine	24.0		23.5		24.3	
Serine	17.5		17.2		17.1	
Arginine	26.6		26.0		26.1	
Glycine	21.7		21.4		21.8	
Aspartic acid	37.3		35.9		35.6	
Glutamic acid	57.5		55.5		55.3	
Threonine	18.0		17.5		17.6	
Alanine	22.5		21.9		22.0	
Proline	19.4		19.1		19.2	
Lysine	31.7		30.5		30.5	
Tyrosine	12.0		11.8		11.7	
Methionine	9.6		9.4		9.3	
Valine	23.4		22.8		22.8	
Isoleucine	17.1		16.6		16.5	
Leucine	34.8		33.8		34.0	
Phenylalanine	19.6		19.2		19.2	
Tryptophan	NA		NA		NA	
	Water temperature					
Apparent digestibility coefficient (%)	16°C	24°C	16°C	24°C	16°C	24°C
Dry matter	69.6±7.8	70.7±16.5	68.2±1.5	74.1±7.7	71.1±6.7	76.5±2.6
Protein	84.2±3.2	84.4±12.6	84.6±2.5	86.8±5.3	85.2±4.4	88.9±1.6
Lipid	83.4±5.1	87.4±12.6	86.9±2.4	88.8±1.6	88.6±1.6	91.9±1.8
Gross energy	76.1±5.9	79.4±13.1	75.3±0.5	80.4±5.5	80.7±3.5	85.1±2.3
Total choline	84.6+4.0	87.9±7.7	91.3±1.4	91.6±4.8	83.5±4.2	87.5±3.9
	Water temperature					
Digestible nutrient content (g kg^{-1})	16°C	24°C	16°C	24°C	16°C	24°C
Dry matter	695.6±78.2	707.1±165.0	682.1±14.9	741.1±76.5	710.6±67.4	765.3±25.7
Protein	436.3±16.8	437.6±65.3	455.1±13.6	467.1±28.5	458.3±23.5	478.4±8.7
Lipid	149.6±9.2	156.8±22.7	167.4±4.5	171.1±3.1	162.1±2.9	168.2±3.3
Gross energy (MJ kg^{-1})	17.4±1.3	18.2±3.0	17.3±0.1	18.5±1.3	18.8±0.8	19.9±0.5
Total choline	1.73±0.08	1.80±0.16	3.53±0.05	3.54±0.18	4.55±0.23	4.76±0.22

*Analysed by Macquarie University.

[a] 1 g of yttrium oxide added at the end of digestibility experiment in exchange of diatomaceous earth.

[b] Calculation based on amino acid residue mass in protein (molecular weight minus water). NA denotes not analysed.

Table 3.1.5.1.3. Experiment 1 - Biometric performance of juvenile Yellowtail Kingfish (*Seriola lalandi*) fed semi-purified diets containing graded concentrations of dietary choline after 56 days. Values are mean ± SD of triplicate aquaria.

	Diet-1	Diet-2	Diet-3	Diet-4	Diet-5	Diet-6
Biometric response[a]	+AMP	+AMP	+AMP	+AMP	+AMP	nil AMP
Initial weight (g)	156.1±1.6	155.8±1.8	156.2±1.4	156.0±1.6	156.8±1.0	156.9±0.9
Final weight (g)	193.5±9.8^{a}	256.9±7.0^{b}	273.2±13.4^{b}	337.1±16.1^{c}	351.0±14.9^{c}	412.1±8.6^{d}
Weight gain (g fish^{-1})	37.4±8.2^{a}	101.1±5.2^{b}	117.0±14.6^{b}	181.2±15.7^{c}	194.2±14.0^{c}	255.2±8.7^{d}
SGR (% BW d^{-1})	0.4±0.1^{a}	0.9±0.0^{b}	1.0±0.1^{b}	1.4±0.1^{c}	1.4±0.1^{c}	1.7±0.0^{d}
Survival (%)	95.8±7.2	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
K	1.3±0.1^{a}	1.4±0.0^{ab}	1.4±0.0^{ab}	1.4±0.0^{abc}	1.4±0.1^{bc}	1.5±0.1^{c}
Total feed intake (g fish^{-1})	89.1±9.0^{a}	144.9±7.0^{b}	160.9±7.9^{b}	198.2±6.2^{c}	206.4±8.6^{c}	275.5±5.4^{d}
Relative feed intake (g kg BW^{-1} d^{-1})	9.0±0.9^{a}	12.7±0.7^{b}	13.7±0.4^{b}	15.2±0.3^{c}	15.4±0.4^{c}	19.0±0.4^{d}
FCR	2.5±0.6^{b}	1.4±0.1^{a}	1.4±0.1^{a}	1.1±0.1^{a}	1.1±0.0^{a}	1.1±0.0^{a}
HSI (%)	1.6±0.1^{ab}	2.1±0.4^{a}	1.8±0.0^{a}	1.2±0.0^{bc}	1.2±0.1^{bc}	1.1±0.1^{c}
VSI (%)	7.7±0.3^{bc}	8.8±0.7^{a}	8.0±0.3^{ab}	7.7±0.1^{abc}	6.9±0.5^{c}	7.1±0.0^{bc}

[a] Values with different superscript letters in the same row are significantly different ($P < 0.05$; ANOVA; Tukeys *post-hoc* test).

Table 3.1.5.1.4. Experiment 1 – Whole body and tissue composition of juvenile Yellowtail Kingfish (*Seriola lalandi*) fed semi-purified diets containing graded concentrations of dietary choline after 56 days. Values are mean ± SD of triplicate aquaria.

	Diet-1	Diet-2	Diet-3	Diet-4	Diet-5	Diet-6
Tissue composition[a]	+AMP	+AMP	+AMP	+AMP	+AMP	nil AMP
Whole-body composition (g kg^{-1} wet basis)**[b]						
Moisture	746.8±6.4^{a}	742.4±4.3^{a}	737.3±7.1^{a}	728.9±2.0^{a}	705.8±13.2^{b}	688.8±4.9^{b}
Crude protein	199.5±7.8	206.3±9.2	198.9±15.1	193.3±8.1	201.4±2.1	197.4±7.5
Lipid	18.4±1.5^{a}	26.7±4.6^{a}	31.3±4.1^{ab}	42.2±3.5^{b}	62.6±9.0^{c}	82.8±5.7^{d}
Ash	44.0±2.5^{a}	37.4±0.3^{b}	36.2±0.3^{bc}	32.5±2.1^{bcd}	34.1±0.6^{cd}	31.8±1.6^{d}
Gross energy (MJ kg^{-1})	5.1±0.2^{a}	5.5±0.2^{ab}	5.7±0.2^{ab}	6.2±0.1^{b}	7.0±0.5^{c}	7.9±0.3^{d}
Total choline	0.6±0.0^{a}	0.6±0.1^{a}	0.6±0.0^{a}	0.7±0.0^{ab}	0.7±0.0^{b}	0.7±0.0^{b}
Liver composition (g kg^{-1} wet basis)*[c]						
Moisture	753.9±3.8^{a}	756.4±1.4^{a}	751.2±3.7^{a}	728.2±13.9^{ab}	713.6±30.6^{ab}	681.7±26.2^{b}
Crude protein	146.0±7.1^{a}	150.4±5.1^{ab}	153.4±5.5^{ab}	163.9±0.9^{b}	164.1±11.1^{b}	159.3±3.0^{ab}
Crude lipid	25.8±3.1^{a}	26.9±5.4^{a}	33.7±3.7^{a}	55.5±11.0^{a}	55.6±9.6^{a}	101.0±29.1^{b}
Ash	16.8±3.2	19.9±8.7	20.3±4.5	16.3±0.9	16.1±1.1	16.4±2.0
Nitrogen free extract	57.5±3.3	46.4±15.1	41.4±4.8	36.0±5.8	50.7±32.8	41.5±17.7
Total choline	1.2±0.5	1.4±0.2	1.1±0.2	1.4±0.3	1.5±0.3	1.7±0.0
Tissue choline deposition rate (mg kgBW^{-1} d^{-1})	1.7±0.1^{a}	5.1±1.2^{b}	6.6±0.2^{b}	10.1±0.8^{c}	12.1±0.9^{d}	14.3±0.6^{e}
Digestible nutrient retention (%)						
Protein	16.7±7.3^{a}	29.4±5.8^{ab}	29.1±6.0^{ab}	32.3±1.3^{b}	37.1±1.2^{b}	35.8±3.0^{b}
Lipid	8.7±4.7^{a}	27.8±10.8^{ab}	39.1±8.9^{b}	49.0±4.2^{bc}	69.1±11.6^{c}	92.0±5.1^{d}
Gross energy	14.5±5.4^{a}	23.0±4.0^{ab}	27.0±2.7^{bc}	33.3±2.3^{cd}	39.1±4.5^{de}	44.7±1.1^{e}
Total choline	45.7±.0.7^{a}	36.5±9.5^{a}	35.0±0.4^{a}	22.4±1.6^{b}	13.0±0.8^{b}	23.6±0.9^{b}

*Analysis by DEAKIN; **Analysis by CSIRO

[a] Values with different superscript letters in the same row are significantly different (P < 0.05; ANOVA; Tukeys *post-hoc* test).

[b] Initial moisture, crude protein, lipid, ash, gross energy and choline composition of juvenile YTK carcass was 761.1 g kg^{-1}, 198.7 g kg^{-1}, 19.8 g kg^{-1}, 37.8 g kg^{-1} and 4.9 MJ kg^{-1} and 0.6 g kg^{-1}, respectively. Note Experiment 1 and 2 share the same initial fish for body composition.

[c] Initial moisture, crude protein, lipid, ash, nitrogen free extract and choline composition of juvenile YTK liver was 735.0 g kg^{-1}, 146.3 g kg^{-1}, 33.3 g kg^{-1}, 13.4 g kg^{-1}, 72.0 g kg^{-1} and 1.7 g kg^{-1}, respectively.

Table 3.1.5.1.5. Experiment 2 – Biometric performance of juvenile Yellowtail Kingfish (*Seriola lalandi*) reared at 16 °C or 24 °C and fed a fishmeal-based diet supplemented with different levels of choline chloride. Values are mean ± SD of triplicate aquaria.

							ANOVA		
	Temperature 16 °C			Temperature 24 °C			*P*-value		
Biometric response	P-Diet 1	P-Diet 2	P-Diet 3	P-Diet 1	P-Diet 2	P-Diet 3	Diet	Temp.	Diet × Temp.
Initial weight (g)	156.3±1.3	157.8±2.3	157.6±2.4	157.7±0.9	157.2±2.0	157.0±1.2	0.900	0.968	0.530
Final weight (g)	348.2±22.9	387.8±15.6	376.6±4.0	477.3±46.9	517.9±37.4	490.4±25.9	0.096	<0.001	0.863
Weight gain (g)	191.9±23.5	230.0±13.4	219.0±2.5	319.6±46.1	360.7±36.4	333.4±25.6	0.092	<0.001	0.870
SGR (% BW d^{-1})	1.4±0.1	1.6±0.1	1.5±0.0	2.0±0.2	2.1±0.1	2.0±0.1	0.059	<0.001	0.86
Survival (%)	100.0±0.0	100.0±0.0	100.0±0.0	91.6±7.2	95.2±7.2	100.0±0.0	0.262	0.055	0.262
K	1.6±0.0	1.6±0.1	1.6±0.1	1.6±0.1	1.6±0.0	1.6±0.1	0.478	0.529	0.703
Total feed intake (g $fish^{-1}$)	238.5±21.0	262.9±9.1	244.4±8.4	431.3±15.9	501.6±48.5	434.9±32.3	0.017	<0.001	0.247
Relative feed intake (g kg BW^{-1} d^{-1})	17.9±1.1	18.7±1.1	17.6±0.8	28.1±1.9	31.4±2.1	28.0±1.5	0.046	<0.001	0.306
FCR	1.5±0.1	1.3±0.1	1.3±0.1	1.6±0.1	1.5±0.1	1.4±0.1	0.015	0.001	0.837
HSI (%)	1.2±0.2	1.2±0.3	1.2±0.1	1.3±0.2	1.1±0.1	1.1±0.1	0.722	0.629	0.629
VSI (%)	6.2±0.6	7.0±0.6	6.3±0.5	6.7±0.8	6.4±0.2	6.0±0.1	0.273	0.548	0.211

Table 3.1.5.1.6. Experiment 2 – Whole body and tissue composition of juvenile Yellowtail Kingfish (*Seriola lalandi*) fed a fishmeal-based diet supplemented with different levels of choline chloride at 16 °C and 24 °C. Values are mean ± SD of triplicate aquaria.

							ANOVA		
	Temperature 16 °C			Temperature 24 °C			*P*-value		
	P-Diet 1	P-Diet 2	P-Diet 3	P-Diet 1	P-Diet 2	P-Diet 3	Diet	Temp.	Diet × Temp.
Whole-body composition (g kg^{-1} wet basis)**[a]									
Moisture	686.7±6.2	687.6±4.1	675.9±4.9	676.0±13.6	681.9±21.5	687.3±7.0	0.850	0.768	0.250
Crude protein	207.9±17.0	202.8±2.3	210.0±3.9	204.7±4.5	201.0±10.3	208.4±6.4	0.387	0.614	0.986
Lipid	77.0±3.9	75.7±6.2	85.0±7.3	86.9±12.3	79.4±19.8	71.5±6.0	0.747	0.999	0.187
Ash	32.0±0.7	29.7±2.2	29.9±0.4	34.7±1.3	34.4±3.0	35.9±0.5	0.391	<0.001	0.266
Gross energy (MJ kg^{-1})	7.9±0.1	7.9±0.3	8.4±0.4	8.2±0.5	7.9±1.0	7.5±0.7	0.903	0.466	0.228
Total choline	0.8±0.0	0.8±0.0	0.9±0.0	0.7±0.0	0.7±0.0	0.7±0.0	0.066	<0.001	0.103
Liver composition (g kg^{-1} wet basis)*[b]									
Moisture	684.7±12.2	699.7±8.0	679.1±17.4	640.3±33.2	648.4±50.4	677.2±10.8	0.588	0.023	0.259
Crude protein	161.6±4.9	159.0±11.7	151.5±4.4	142.6±6.3	147.0±14.9	144.4±12.3	0.647	0.018	0.591
Crude lipid	85.1±10.7	72.9±6.0	94.6±9.4	150.7±32.8	136.4±45.4	108.1±2.8	0.465	0.001	0.142
Ash	14.4±0.6	12.7±2.7	14.0±1.7	18.9±7.7	23.0±3.3	14.6±1.5	0.280	0.012	0.114
Nitrogen free extract	54.2±5.6	55.8±3.8	60.9±13.4	47.5±14.6	45.2±21.6	55.6±5.2	0.493	0.222	0.931
Total choline	2.2±0.1	2.0±0.1	2.0±0.5	1.7±0.2	1.9±0.1	2.2±0.2	0.534	0.244	0.029
Tissue choline deposition rate (mg kgBW^{-1} d^{-1})	10.3±0.9	11.9±1.1	12.7±1.1	11.8±0.5	15.7±0.7	16.4±1.8	<0.001	<0.001	0.164
Digestible nutrient retention (%)									
Protein	26.2±1.0	28.5±3.8	29.5±4.8	27.6±8.5	28.1±5.5	34.0±3.7	0.277	0.459	0.703
Lipid	52.1±1.1	48.4±6.0	57.5±2.0	48.8±19.0	40.3±9.0	43.4±0.8	0.427	0.067	0.596
Gross energy	35.2±3.2	39.1±3.5	38.6±1.1	33.3±12.2	32.6±4.7	33.5±1.5	0.833	0.119	0.779
Total choline	33.2±1.7	18.0±2.6	15.9±2.5	24.2±5.0	14.4±1.4	12.6±2.3	<0.001	<0.001	0.190

*Analysis by DEAKIN; **Analysis by CSIRO.

[b] Initial moisture, crude protein, lipid, ash, gross energy and choline composition of juvenile YTK carcass was 761.1 g kg^{-1}, 198.7 g kg^{-1}, 19.8 g kg^{-1}, 37.8 g kg^{-1} and 4.9 MJ kg^{-1} and 0.63 g kg^{-1}, respectively. Note Experiment 1 and 2 share the same initial fish for body composition.

[b]Initial moisture, crude protein, lipid, ash, nitrogen free extract and choline composition of juvenile YTK liver was 729.1 g kg^{-1}, 158.3 g kg^{-1}, 46.1 g kg^{-1}, 13.3 g kg^{-1}, 53.2 g kg^{-1} and 1.2 g kg^{-1}, respectively.

Table 3.1.5.1.7. Hypothetical contribution of raw materials and a 70% choline chloride premix to the digestible choline content of diets for Yellowtail Kingfish (YTK).

		Inclusion level of ingredient (g kg^{-1})			Proportional amount of choline chloride (g kg^{-1})		
Ingredients	Choline chloride equivalents* (g kg^{-1})	P-Diet 1 (no CC)	P-Diet 2 (+3 g CC)	P-Diet 3 (+6 g CC)	P-Diet 1 (no CC)	P-Diet 2 (+3 g CC)	P-Diet 3 (+6 g CC)
Fishmeal	4.126	400	400	400	1.650	1.650	1.650
Poultry meal	1.832	70	70	70	0.128	0.128	0.128
Meat meal	1.903	50	50	50	0.095	0.095	0.095
Blood meal	1.201	70	70	70	0.084	0.084	0.084
Lupins	2.132	100	100	100	0.213	0.213	0.213
Wheat flour	0.155	150	150	150	0.023	0.023	0.023
Fish oil	-	60	60	60	0.000	0.000	0.000
Poultry oil	-	60	60	60	0.000	0.000	0.000
Vit/min premix	-	5	5	5	0.000	0.000	0.000
Vitamin C (Stay-C)	-	0.5	0.5	0.5	0.000	0.000	0.000
Taurine	-	10	10	10	0.000	0.000	0.000
Sodium phosphate monobasic	-	5	5	5	0.000	0.000	0.000
Choline chloride¥ (70% CC)	700	0	3	6	0.000	2.100	4.200
Diatomaceous earth	-	19.5	16.5	13.5	0.000	0.000	0.000
TOTALS	Estimated CC equivalent of diets				2.19	4.29	6.39
TOTALS	Estimated choline of diets‡				1.64	3.22	4.80
TOTALS	Estimated digestible choline content of diets‡‡				1.47	2.90	4.32

*As determined by UpScience Vietnam (formerly Invivo Vietnam), otherwise assumed to be negligible.
¥ Choline chloride content of premix assumed to be as stated (70% CC).
‡ Free choline = total estimated choline chloride equivalent (CC) multiplied by relative amount of choline in CC (i.e. 74.61%).
‡‡ Digestible choline = free choline × apparent digestibility coefficient of 90%.
Vitamin mineral premix assumed to contain no choline for this example.

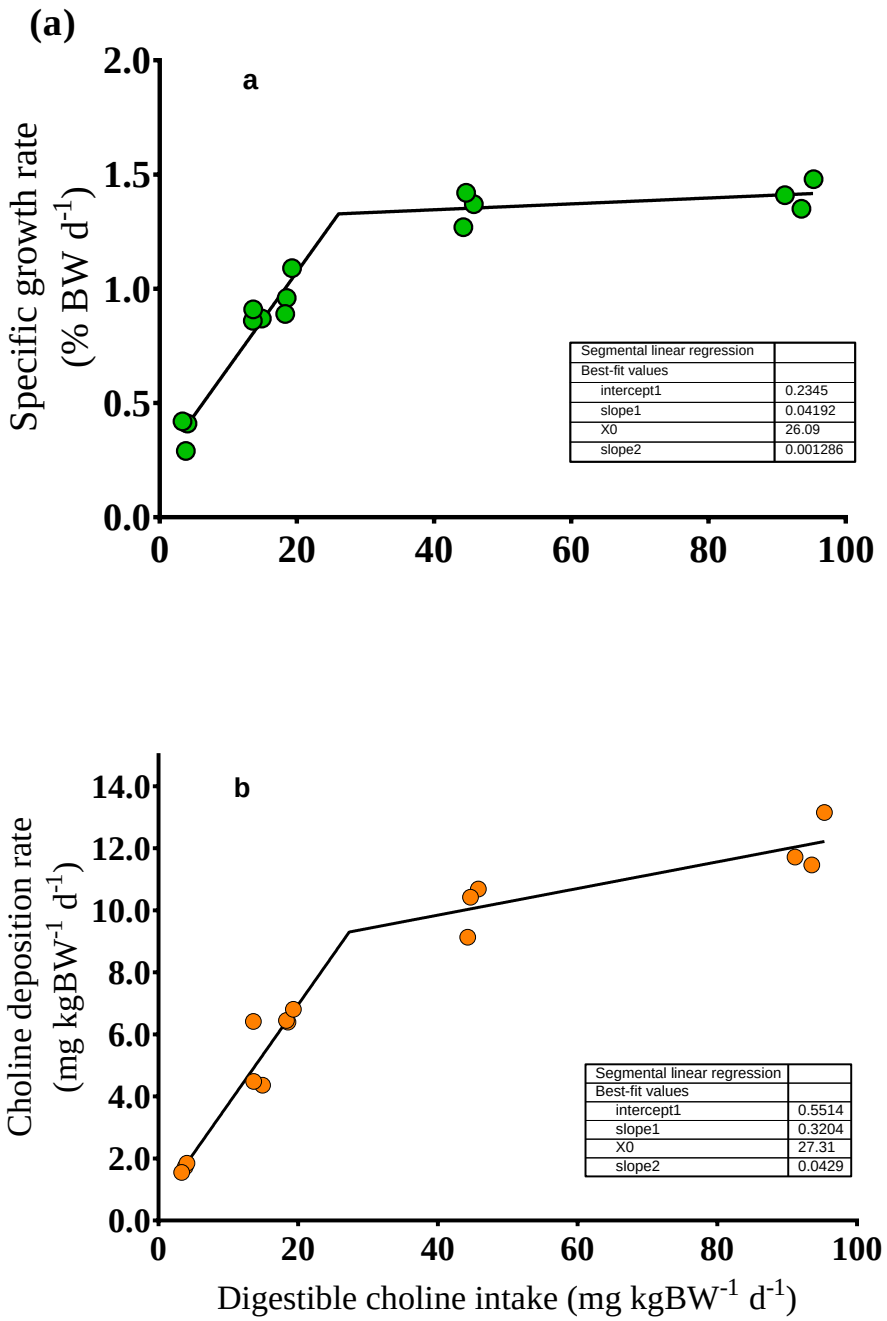


Figure 3.1.5.1.1. Relationships between digestible choline intake and specific growth rate (a) and digestible choline intake and choline deposition rate (b) in juvenile Yellowtail Kingfish (*Seriola lalandi*) from Experiment 1.

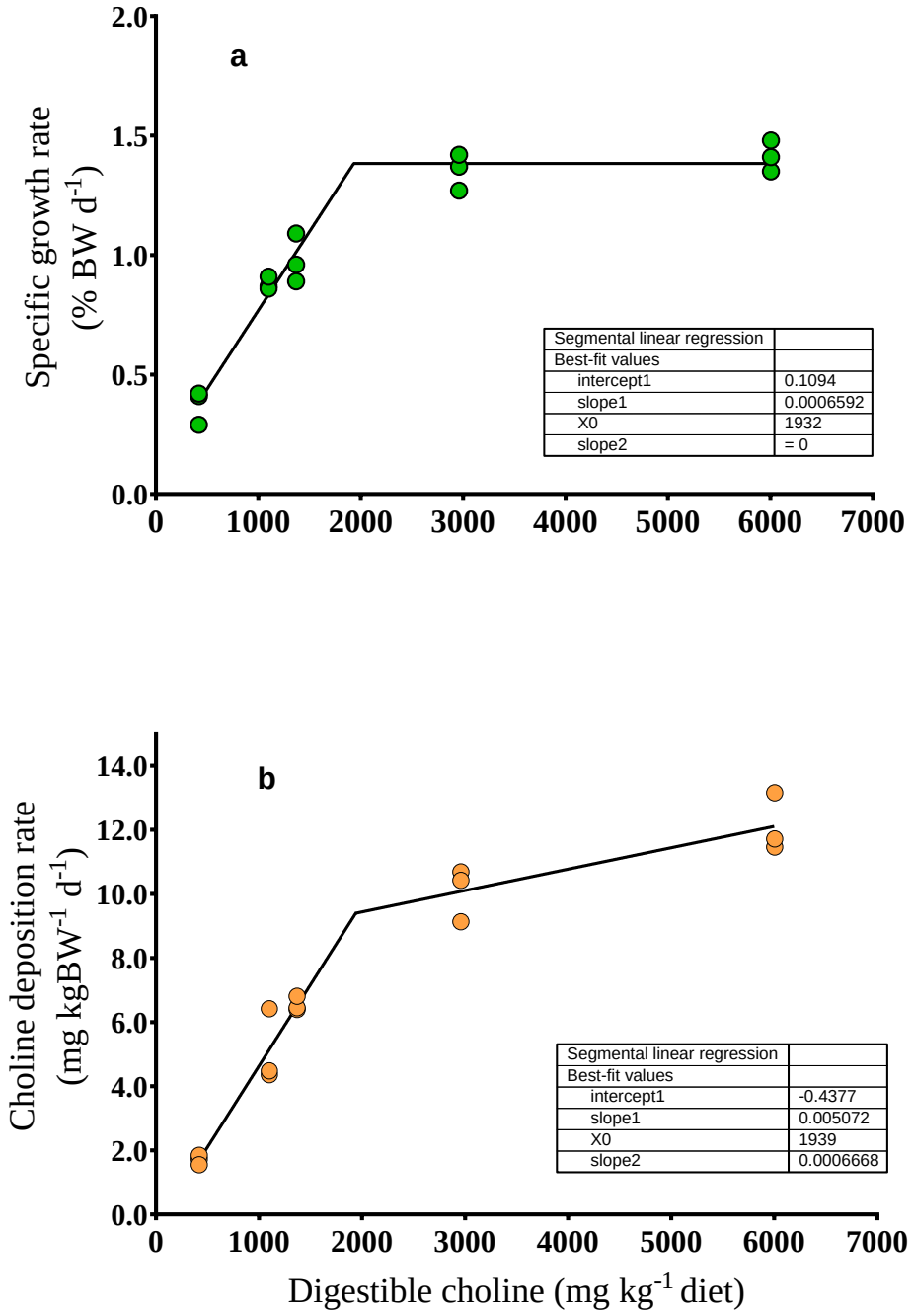


Figure 3.1.5.1.2. Relationships between digestible choline content of diets and specific growth rate (a) and digestible choline content of diets and choline deposition rate (b) in juvenile Yellowtail Kingfish (*Seriola lalandi*) from Experiment 1.

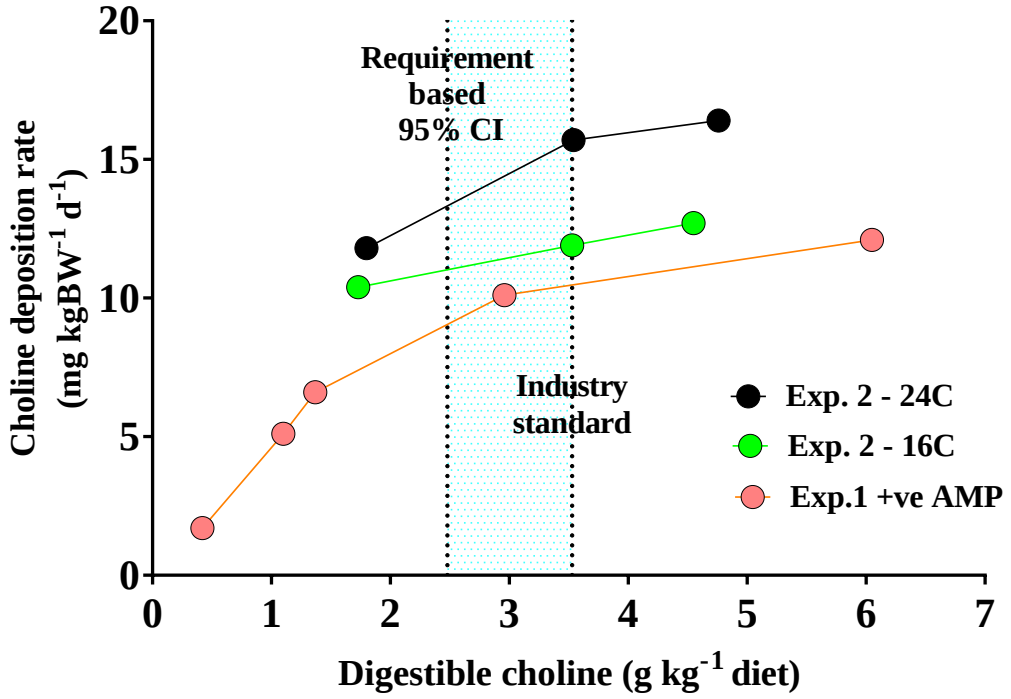


Figure 3.1.5.1.3. Plot of choline deposition rate versus digestible choline intake in juvenile Yellowtail Kingfish (*Seriola lalandi*) by dietary treatment; Experiment 1 and Experiment 2 (all *n* = 3). Treatments from Experiment 2 are also catergorised by temperature. Drop lines indicate the digestible choline requirement of YTK estimated from the upper 95% CI of the broken-line model used in Experiment 1 (2.48 g digestible choline kg^{-1} diet) and the approximate amount of digestible choline provided in commercial diet such as P-Diet 2 using a choline chloride (70% active CC) supplementation rate of 3 g kg^{-1}.

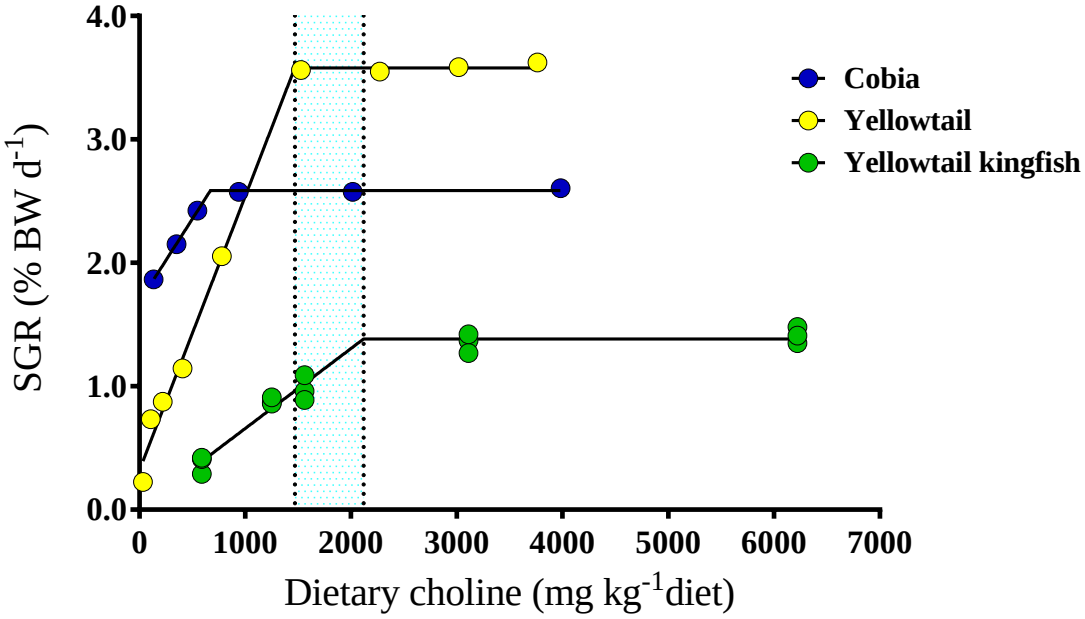


Figure 3.1.5.1.4. Plot of specific growth rate (SGR) versus dietary choline content in juvenile Yellowtail Kingfish (*Seriola lalandi*); this study, juvenile Cobia (*Rachycentron canadum*) (Mai et al., 2009) and juvenile Japanese Yellowtail (*Seriola quinqueradiata*) (Hosokawa et al., 2001). Drop lines indicate the band between dietary choline requirement of Japanese Yellowtail (1469 mg kg $diet^{-1}$) and Yellowtail Kingfish (2120 mg kg $diet^{-1}$) estimated from the broken-line models.

3.1.5.2. Manuscript - Histidine requirement of juvenile Yellowtail Kingfish Seriola lalandi.

Igor Pirozzi[a,b] and Mark Booth[a]

[a] New South Wales Department of Primary Industries, Locked Bag 1, Nelson Bay, NSW 2315
[b] College of Science and Engineering and Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville 4801, QLD, Australia.

This manuscript may be referenced as: Pirozzi, I. and Booth, M. (2019). 3.1.5.2. Manuscript - Histidine requirement of juvenile Yellowtail Kingfish *Seriola lalandi*. In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.187-208.

Abstract

Two separate dose response experiments were designed in an attempt to quantify the dietary requirements of histidine in juvenile Yellowtail Kingfish (*Seriola lalandi*; YTK). In Experiment 1 semi-purified diets where used, while Experiment 2 used practical diets composed predominantly of intact protein sources. In Experiment 1 six test diets were formulated with increasing histidine from 0-2% inclusion representing an expected range from sub and supra optimal histidine requirement. Test diets were formulated using a combination of semi-purified ingredients and crystalline amino acids providing dietary histidine contents from 2.7 to 20.7 g kg^{-1} diet. Experiment 1 was initially carried out at two temperatures; 17 and 22 °C. A control diet treatment was also used for comparative growth. After six weeks YTK fed the test diets had poor and similar relative growth and feed conversion (with respect to temperature) across all diet treatments. Subsequent compositional analyses of the test diets revealed a low tryptophan content. Experiment 1 was rerun at 22 °C using diets fortified with tryptophan, however, results were similar and inconclusive. Subsequent amino acid digestibility analysis indicated that amino acids were digested quite well. YTK may be unable to utilise high levels of crystalline amino acids efficiently; however, this needs to be confirmed. Experiment 2 was subsequently conducted using mainly intact protein sources. No fishmeal was used in these diets. The basal diet was formulated to a low histidine level of 7.45 g kg^{-1} and supplemented at 5.0 g kg^{-1} increments to 28.0 g kg^{-1} diet creating five diets. YTK were stocked at 80 g and grew rapidly to an average of 331 g over seven weeks at 20 °C. No significant differences were found among diet treatments for all variables tested with the exception of histidine intake. The requirement for dietary histidine for YTK is < 7.45 g kg^{-1} diet. Results also suggest there is great potential to produce zero or very low fishmeal diets for juvenile YTK.

Introduction

Histidine is classified as a basic amino acid and plays a role as a coordinating ligand in metalloproteins and as a catalytic site in certain enzymes such as chymotrypsin (Liao et al., 2013). It is abundant in haemoglobin (Olson et al., 1988) and it is the direct precursor of histamine and is a source of carbon atoms for the synthesis of purines. It is thought to mitigate the effects of cataracts in Atlantic salmon (*Samo salar*) in warm water (Waagbo et al., 2010). Histidine and related compounds such as imidazoles may play important roles in osmoregulation and detoxification of reactive carbonyl species (Breck et al., 2005). Histidine and its dipeptides in the white muscle of Japanese Yellowtail (*Seriola quinqueradiata*) appear to function primarily as buffering substances against muscle acidosis brought about by anaerobic metabolism rather than antioxidants (Ogata, 2002).

Quantifying EAA requirements is an important step towards formulating fishmeal replacement diets for aquaculture species. While histidine is considered an important EAA, other EAAs are typically first limiting in diets with high plant based proteins. These include: methionine, lysine, threonine, tryptophan, isoleucine and valine representing the EAAs commonly used by animal feed industries (Nunes et al., 2014). However, rendered animal proteins such as meat meal and feather meal are typically low in

histidine, therefore, it is important to quantify histidine requirement if these ingredients are being considered as protein sources in aquafeeds for YTK.

Most quantitative estimates of EAA are carried out using a dose-response approach where the amino acid of interest is made deficient in the diet while all other known requirements of the animal are met. Response variables such as growth rate, protein deposition or tissue amino acid deposition are then measured and a suitable mathematical model applied to estimate optimum requirement; however other methods including the plasma amino acid concentration and rates of amino acid oxidation have been used and compared to estimates based on whole body amino acid profile. Several studies on histidine requirement of finfish have been conducted. On average the requirement for dietary histidine is approximately 8.0 g kg^{-1} diet; however this can vary from species to species (see Table 3.1.5.2.1) and will also likely be influenced by culture environment and ontogeny.

There are currently no published data on the histidine requirements for YTK. The objective of this study was to determine the dietary histidine requirement of juvenile YTK. This was accomplished in two separate experiments using either 1) semi-purified diets or 2) intact protein sources

Methods

Animal ethics

All experimental procedures were performed under the NSW Department of Primary Industries (DPI) Fisheries Animal Care and Ethics Research Authority (ACEC93/5). Care, husbandry and termination of fish were done according to 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2015).

Fish stock and handling

All juvenile fish were progeny of wild YTK broodstock held at the DPI Port Stephens Fisheries Institute (PSFI) hatchery (NSW, Australia). Prior to experiments fish were held in 10,000-L tanks and fed a commercially available diet. Fish were sedated using recommended doses of Aqui-S® (Aqui-S New Zealand Ltd.; 540 g L^{-1} isoeugenol) prior to any handling. Fish were fasted for 24 h prior to weighing or termination. Fish were initially stocked into experiment systems at an ambient temperature of approximately 20 °C and the temperature adjusted up or down 1 °C per day until the target experiment temperature was achieved. All fish were fed twice daily at approximately 09:00 h and 14:00 h to apparent satiety. Satiation was determined by carefully hand feeding YTK until a loss in feeding activity was observed; any uneaten pellets were removed from each tank, dried and feed intake adjusted accordingly.

Diets

Experiment 1- Semi purified diets

The approach to diet formulation was based on published amino acid requirement studies for other commercially important carnivorous finfish species such as Red Drum (*Sciaenops ocellatus*) (Moon and Gatlin, 1991; Castillo et al., 2015), Rainbow Trout (*Oncorhynchus mykiss*) (Kim et al., 1992), Arctic Char (*Salvelinus alpinus*) (Simmons et al., 1999), Gilthead Seabream (*Sparus aurata*) (Marcouli et al., 2006) and Yellow Croaker (*Pseudosciaena crocea*) (Li et al., 2014); i.e. high (~20% or greater) crystalline amino acid mix in combination with semi-purified ingredients. This approach was necessary particularly for carnivorous species to ensure an adequate supply all essential amino acids, while at the same time restricting the amino acid of interest to elicit a dose response and determine a key breakpoint defining a requirement.

Diet formulations for Experiment 1 are presented in Table 3.1.5.2.2. Six isonitorgenous and isocalorific test diets (Diets 1-6; approx. 47% crude protein, 14.5% crude fat and 21.4 MJ kg^{-1}) were formulated using a combination of intact protein sources and supplemented with essential and nonessential amino

acids. Glycine was substituted with histidine at increments of 0, 0.25, 0.5, 0.75, 1 and 2%. This provided a total dietary histidine content ranging from approximately 0.3 to 2.1%.

The ratio and quantity of the essential amino acid mix (excluding histidine) in this study was designed to supply a similar proportion of essential amino acids to that of the average whole body (dry matter) composition of YTK. These compositional data have been compiled from other feed studies conducted within the K4P project and also published by Booth et al. (2010); Arg 3.7%, Iso 2.5%, Leu 4.1%, Lys 4.6%, Met 1.7%, Phe 2.3%, Thr 2.5%, Val 2.8%, Try 0.5%. These whole body essential amino acid profiles were virtually identical to those reported for Japanese Yellowtail (Watanabe, 2009).

A control diet (Diet 7; 51% crude protein, 16.7% crude fat, 22.6 MJ kg^{-1}) formulated using a range of practical ingredients was also used to provide comparative growth data (Table 3.1.5.2.3). The proximate and amino acid content of protein sources used in this study are presented in Table 3.1.5.2.4.

Amino acid analyses of the diets at the conclusion of Experiment 1 indicated appropriate formulation with respect to substitution of histidine and glycine (Table 3.1.5.2.5). Further, the relative proportion of essential amino acids were similar to that of YTK whole carcass composition (Booth et al., 2010) as intentionally formulated; however, the tryptophan content in Diets 1-6 was quite low. This was likely a consequence of overestimating the tryptophan content of the raw ingredients for formulation. While the amino acid composition of all ingredients was analytically quantified prior to formulation, tryptophan was not initially analysed as this requires additional analysis as it is destroyed during conventional acid hydrolysis (Fountoulakis and Lahm, 1998).

Growth across all treatments was depressed in Experiment 1 and the above indicated a deficiency in tryptophan as a possible explanation for this result. To determine if this was the case the test diets were subsequently reformulated to a dietary tryptophan level of 0.5% by fortifying the crystalline amino acid mix and the Experiment was rerun as outlined below (Experiment Design – Trial 1).

Experiment design - Experiment 1

Ten fish (116 g) per diet treatment (Diet1-7) were stocked into triplicate 200 L tanks in two separate but adjacent RAS's with one dedicated as cool water system (17 ^{o}C) and the other as the warm water system (22 ^{o}C). This experiment was terminated after six weeks due to poor overall growth of fish fed the test Diets 1-6.

Experimental design – Rerun of Experiment 1

The second run of this trial using tryptophan supplemented diets was conducted at one temperature only, 22 °C, and also included two negative tryptophan "control" treatments, equivalent to Diet 4 (1% His) and Diet 6 (2% His) without additional supplemented tryptophan. There was no practical Control diet used in the follow up trial. This trial followed the general protocols as above with the exception that seven fish (186 g) per diet treatment were stocked into triplicate 200 L tanks a single RAS. This experiment was terminated after three weeks as it was clear following a bulk weight check that growth was similarly depressed across all treatments.

Following the termination of this experiment an assessment of Diet 3 was undertaken for proximate and amino acid digestibility. Diet 3 was chosen as an approximate median histidine level of the test diets and representative sub sample to provide some insight into the poor results. Digestibility was determined following protocols below based on n = 2 tanks and seven fish $tank^{-1}$.

Experiment 2- Practical diet - intact protein sources

The results of Experiment 1 were inconclusive and indicated a potential issue with the use of high levels of crystalline amino acids in diets for juvenile YTK, whether that be through physiological overloading issues of crystalline amino acids or issues with the quality of one or more of the amino acid supplements. Therefore, Experiment 2 was conducted using a different approach. The formulation aimed to maintain dietary histidine level in the basal diet as low as possible (~0.8%) while supplying adequate quantities

of dietary protein and other essential nutrients using intact protein sources. The amino acid supplements (Met, Lys, Tau) added to the diets are used in commercial aquafeed production. Protein sources with naturally low histidine levels such as brewer's yeast, feather meal and krill meal were utilised. No fishmeal was used in these diets. The formulations were made on a crude basis as digestibility coefficients for several of the ingredients were unknown; therefore, the overall crude protein content was intentionally slightly higher relative to the diets in Experiment 1 assuming that the digestibility would be somewhat poorer. Five diets were manufactured ranging from zero supplementation of histidine (basal diet; His0.0) through to 2% addition of histidine in 0.5% increments (P.Diet1, P.Diet2, P.Diet3, P.Diet4, P.Diet5) (Table 3.1.5.2.6). The measured proximate and amino acid content of the diets are presented in Tables 3.1.5.2.6 and 3.1.5.2.7 respectively. The reported fat content of Diet 3 was unusually high (24.9%), however, this was likely an analytical error reported by the subcontracted laboratory as the energy, protein and ash content were similar to the other diets which would imply a relatively lower fat content based on the energy equivalent values.

General diet manufacturing protocol

All diets were made at PSFI using laboratory scale equipment. Prior to pellet making all raw materials were ground in a high speed hammer mill (Raymond Laboratory Mill, Transfield Technologies, Rydalmere, NSW, Australia; 1.6 mm screen). Wheat flour was autoclaved for 2 min at 121 °C prior to inclusion in the dry mash. The raw materials and supplements were then dry mixed in a Hobart mixer (Hobart Mixer; Troy Pty Ltd, Ohio, USA) before the addition of oil and fresh water to form a firm dough. The dough was then screw pressed into 6 mm pellets (Dolly, La Monferrina, Castell'Alfero, Italy). The moist pellets were then dried at approximately 60 °C to a moisture content of < 10%.

Experiment aquaculture system

Experiments were conducted in an indoor laboratory housing multiple, research-scale, recirculating aquaculture systems (RAS). Each RAS consisted of a series of 200 L translucent, white circular, polyethylene rearing tanks connected to a water management system. Tanks were fitted with a mesh lid to prevent the escape of fish and the outer surface was painted black to prevent the inadvertent disturbance of fish. Each tank was fitted with an air-stone diffuser and additional dissolved oxygen (DO) was provided to the RAS by injecting industrial grade oxygen (BOC) into influent manifolds. Each RAS was comprised of a 1500 L sump, a large sand filter, a twin-cartridge particle filter (30 µm), a foam fractionator and a 750 L fluidised bed bio-filter (B-Cell). The water temperature in each RAS was controlled using reverse cycle refrigeration units capable of maintaining water temperature to ± 2 °C. Photoperiod in both experiments was set at 10 h light and 14 h dark using dimmed LED lighting. Water quality data for all experiments are presented in Table 3.1.5.2.8.

Experimental design - Experiment 2

Ten fish (80 g) per diet treatment (P.Diet1-5) were stocked into triplicate 200 L tanks in a RAS (as described above) and held at 20 °C. Experiment 2 was run for seven weeks and biometric parameters (Data Analyses section below) assessed. At the conclusion of the experiment a further subsample of fish were taken for compositional analyses. At the conclusion of the experiment YTK were also inspected for cloudy or opaque discoloration of the eyes using a handheld LED illuminated magnifying lens (10×); these symptoms can be indicative of the formation of cataracts.

Compositional analyses

Dry matter, protein, lipid, ash and gross energy of experimental diets and faecal sample (including yttrium) analyses (Experiment 1, Diet 3) were conducted by CSIRO according to routine methods outlined in AOAC (2006). Crude protein content was determined by multiplying the nitrogen content of each sample by 6.25. Crude lipid was determined by extraction using chloroform:methanol (2:1) (Folch et al., 1957). Amino acid profile analysis of diets was performed according to standard operating

procedure SOP QAAA-001 of the Australian Proteome Analysis Facility (APAF; Macquarie University, Sydney Australia).

Digestibility

Digestibility of Diet 3 in Experiment 1 was assessed using stripping techniques. Yttrium oxide (Yttrium III oxide, Sigma-Aldrich) was used as an inert marker. Prior to stripping, fish were anaesthetised using 5-25 mg L^{-1} Aqui-S™. Faeces were collected from the posterior intestine by applying gentle abdominal pressure. Contamination with urine or mucous were minimized and samples were immediately stored at -20 °C. This procedure was repeated twice a week until approximately 3 g dry faecal matter was obtained.

Apparent digestibility of the diet was calculated using the formula:

$$ADC(\%) = 100 \times [1 - \left(\frac{F}{D} \times \frac{D_{marker}}{F_{marker}}\right)]$$

where F = % nutrient in faeces; D = % nutrient in diet; D_{marker} = % marker in diet; F_{marker} = % marker in faeces.

Data Analyses

The following biometric performance variables were used to assess the effects of different feed treatments. The average value of all fish in each tank was used in calculations;

- Weight gain (g $fish^{-1}$) = final weight of fish (g) – initial weight of fish (g)
- Specific growth rate (% d^{-1}) = [Ln(final weight) – Ln(initial weight)] / n days × 100
- Condition factor K = [individual weight of fish (g) / fork length of fish $(mm)^3$] × 10^5
- Feed intake (g $fish^1$ d^{-1}) = individual feed intake (g) / n days
- Growth (g $fish^{-1}$ d^{-1}) = individual weight gain (g) / n days
- Food conversion ratio (FCR) = dry basis feed intake per fish (g) / wet weight gain per fish (g)

Statistical analyses

The effect of different diets on the performance of YTK dietary was examined using one-way ANOVA. ANOVA was performed using NCSS 11 Version 11.0.13 (NCSS 11 Statistical Software (2016). NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss). ANOVA was considered significant when $P < 0.05$. Tukey's multiple comparison procedure was used to discriminate between significantly different treatment means at the 95% confidence interval.

Results

Experiment 1

The experiment was terminated after six weeks as growth of YTK fed the test diets was poor (Table 3.1.5.2.9). There were no significant differences among all test diets for all growth and feed conversion parameters (Table 3.1.5.2.). Survival was also relatively poor for the 22 °C treatment. The average SGR of YTK fed the test diets was 1.1% and 1.4% day^{-1} at 17 °C and 22 °C, respectively. After six weeks the Control group by comparison had approximately double this rate with SGR's of 2.1% and 3.2% day^{-1} at 17 °C and 22 °C respectively. FCR's were also poor among the test diet treatments (Table 3.1.5.2.9). While it would be expected for YTK to perform better on the Control diet, recent work using similar semi-purified diets to determine choline requirement in YTK (Manuscript 3.1.5.1) indicated that the growth response should have been much stronger. This poor and relatively constant response across the test diets in the current study indicated a potentially limiting nutrient in the diets other than histidine.

Following on from this result, diets were fortified with tryptophan and Experiment 1 was rerun as indicated above (see material and methods section). A bulk weight check was performed at week three to determine if the addition of extra tryptophan had enhanced growth. The weight check indicated growth remained depressed and ANOVA found no significant difference across all diet treatments or any trends, indicating a dose-response to increasing levels of histidine intake remained unchanged (Table 3.1.5.2.10). SGR was on average 0.7% day^{-1} and FCR on average was 4.5:1. The adjunct experiment was terminated after three weeks.

Apparent digestibility coefficients for Diet 3 are presented in Table 3.1.5.2.11. Dry matter diet digestibility was poor at 28.7%. There were some anomalies between protein digestibility based on N × 6.25 (79.4%) and protein digestibility calculated as the sum of amino acids (93.5%). Generally amino acid digestibility was good with exception of cysteine which was anomalous at -9.3% digestible.

Experiment 2

At the conclusion of Experiment 2 YTK growth and FCRs were excellent across all diets. YTK more than quadrupled in body weight after 49 days growing from approximately 80 g to 331 g. FCRs were approximately 1.0 across all diets (Table 3.1.5.2.12). However, with the exception of dietary histidine intake, there was no significant difference among any of the variables (Table 3.1.5.2.12). No opaque discoloration of eyes was observed in any individual YTK from any treatment.

Discussion

Trial 1

The results of Experiment 1 were inconclusive and pointed to either a problem with the capacity for YTK to utilise high inclusion levels of crystalline amino acids (CAAs) or the potentially inferior bioavailability of one or more of the supplementary amino acids creating a nutrient deficiency. While the approach in diet formulation using CAAs and semi-purified ingredients to determine amino acid requirements has been implemented successfully for other fish species, and finfish in general have been shown to demonstrate a good capacity to utilise free amino acids (e.g. Espe and Lied, 1994; Rodehutscord et al., 1995), YTK may have a limited capacity to utilise CAAs, particularly at high dietary inclusions.

Histidine amino acid supplements are not typically used in most animal feed industries as it is not limiting in practical feed formulations (Nunes et al., 2014). Eliciting a dose response to determine histidine requirement is particularly challenging, more so than for other amino acids as histidine is reasonably abundant in most intact protein sources. Therefore, the use of higher levels of CAAs are required to meet balanced EAA requirements when formulations are manipulated to provide dietary histidine levels below approximately 0.9%. Studies on turbot (*Scophthalmus maximus*) have shown that dietary protein can be replaced with up to 19% of CAAs; however, higher replacement levels depress growth (Peres and Oliva-Teles, 2005). Red drum grow well on diets supplemented with 22.5% CAAs (Peachey et al., 2018), Japanese Flounder (*Paralichthys olivaceus*) with 25% CAAs (Alam et al., 2001) and Atlantic Salmon grow well with up to 30% dietary CAAs (Espe and Lied, 1994). In this study, CAA were supplemented in Diets 1-6 to 27.5% inclusion which is within the range published for other species; however there are highly variable, species specific responses to the utilisation of dietary CAAs, even among carnivorous species. The use of CAA in diets for YTK has not been studied and thresholds have not been determined. A common response seen in other studies once dietary CAA thresholds are exceeded is depressed growth and poor feed conversion, which was observed in the current study. A better understanding of the implications of using high levels of dietary CAAs is needed, particularly so if considering EAA requirement studies for YTK.

Tyrosine is an aromatic amino acid regarded as a conditionally essential; its dietary content was quite low (3.0 g kg^{-1}) as too was its digestibility (57.5%); equating to a digestible tyrosine content of only 1.61 g kg^{-1}. However the dietary content of digestible phenylalanine was high at 27 g kg^{-1}. Phenylalanine readily converts to tyrosine to such an extent that it can meet the total requirement for aromatic amino acids (TAAAs) if provided in sufficient quantities. The TAAA requirement of most finfish species including YTK are currently unknown; however, TAAAs were provided in test Diets 1-6 at similar

levels to YTK commercial aquafeeds (Table 3.1.5.2.5). Of the few studies that have been conducted, the requirement of TAAAs for Red Drum has been quantified at 21.0 g kg^{-1} (Castillo et al., 2015) and for Grass Carp (*Ctenopharyngodon idella*) at 24.4 g kg^{-1} (Gao et al., 2016). If the TAAA requirements of YTK are similar, then it is unlikely these were limiting as the TAAA content across all test Diets 1-6 was on average 32.2 g kg^{-1} diet. However, this remains to be verified.

Similarly, dietary cysteine content was also relatively low in the test Diets 1-6 being on average 1.0 g kg^{-1} diet. The ADC for cysteine was also anomalous at -9.3%. The ADC value was driven mainly from the result of one replicate sample which had a cysteine digestibility of -26.7%. Irrespective of the cysteine content, the total sulfur amino acid (TSAA) requirement can be met by methionine alone which was supplied in sufficient quantities at 21.0 g kg^{-1} diet on average across Diets 1-6 equating to a TSAA content of approximately 22.0 g kg^{-1} crude basis. The TSAA requirement of juvenile YTK has been quantified at approximately 19.5 g kg^{-1} diet (Manuscript 3.1.5.3), therefore it is unlikely that these nutrients were deficient in the test diets of the current study. This may partly explain the relatively poor cysteine ADC value as methionine was converted to cysteine.

As indicated above, conventional nutritional theory indicates the complete conversion of phenylalanine to tyrosine and methionine to cysteine (Bender, 2012); however, as these processes are enzyme driven there may be species specific variation when considering conversion efficiencies. For example, tyrosine can spare phenylalanine up to 37% in *Catla catla* (Zehra and Khan, 2014) and up to 60% in Red Drum (Castillo et al., 2015). The implication of this is that if TAAAs or TSAAs are supplied at minimum threshold levels, then the ratio of essential to conditionally essential AAs becomes critically important, and this could be exacerbated depending on environmental influences and ontogeny. TSAAs are investigated further in Manuscript 3.1.5.3; however, there is currently no information available on TAAA interactions in YTK.

While apparent digestibility coefficients (ADCs) are not a direct measure of the capacity of an animal to utilise and absorb nutrients, in the absence of utilisation data ADCs may provide an insight to the bioavailability of a nutrient. ADCs for most essential amino acids (Diet 3) were reasonable. The relative difference in protein digestibility (79%) and ∑AA digestibility (93%) is likely due to the influence of non-protein nitrogen.

Experiment 2

Experiment 2 was conducted using diets formulated mainly with intact protein sources and, with the exception of histidine, supplemented only with amino acids commonly used by aquafeed companies (i.e. methionine, lysine, taurine). The histidine level in the basal diet could only be reduced to approximately 7.45 g kg^{-1} diet without compromising the overall balance of the remaining EAAs and without the need to supplement a suite of CAAs. To achieve this however, no fishmeal was used. The results clearly demonstrated that YTK did not benefit from histidine supplementation as growth and FCRs were very good and similar across all diet treatments. Further, histidine has been associated with mitigating cataract formation in salmon held in warm water (Waagbo et al., 2010) and causing cataracts in Red Drum fed histidine deficient diets (Peachey et al., 2018); no cataracts or opaque discoloration of eyes were observed in any YTK in Experiment 2. The results of Experiment 2 indicate that the minimum requirement for histidine in YTK diets is < 7.45 g kg^{-1} diet.

Given the challenge of formulating a practical diet to induce a specific histidine deficiency as outlined in this study, it is unlikely that current commercial YTK aquafeeds are histidine deficient under normal aquaculture practices, not without also being deficient in other EAAs. Histidine levels in commercial YTK diets are almost double the minimum level used in the current study (Table 3.1.5.2.5). The average histidine requirement of aquaculture finfish is 7.8 g kg^{-1} diet (Table 3.1.5.2.1); however, this value is inflated by the histidine requirement for Japanese Flounder which is double, at 15.6 g kg^{-1} diet (Han et al., 2013). If the value for Japanese Flounder is removed from Table 3.1.5.2.1, then the overall average histidine requirement of finfish decreases to 7.2 g kg^{-1} diet.

An encouraging consequence of Experiment 2 was that YTK performed very well on zero fishmeal diets. Feed intake, growth and FCR were all at levels similar to YTK fed a fishmeal based diet and support the results of Manuscript 3.2.5.1 which evaluated the use of low fishmeal and fishery by-product meal in diets for YTK. Predicted growth using the growth model presented in Manuscript 3.2.5.1 estimated a

final body weight of 368 g growing from 80 g after 49 days at 20 °C. This compared well to the average measured final body weight across all diets in Experiment 2 of 331 g. This result is particularly encouraging as the diets were not primarily formulated for maximal growth but rather to reduce the basal level of histidine as low as possible. This highlights the importance of designing a diet that has the correct nutrient specification supplying a balance of all essential nutrients as well as being highly palatable. Brewer's yeast (20% inclusion) and krill meal (20% inclusion) were the two main protein sources, however, these have not been extensively tested in diets for YTK, certainly not at such high levels. YTK can digest krill meal efficiently (Manuscript 3.1.4.1); however, apart from the study done in Manuscript 3.2.5.1 where low fishmeal diets were made with 11% brewer's yeast, no work to date has been conducted on feeds containing this ingredient for YTK. Brewer's yeast has been shown to be an excellent protein source for grouper when included in diets at 30% (Pirozzi et al., 2018) and is generally an underutilised resource. While the results of this experiment indicated good growth performance over seven weeks, longer term trials are necessary to thoroughly evaluate the efficacy of zero fishmeal diets for YTK. Some of the ingredients utilised in this experiment, such as sodium caseinate, may not be commercially viable, however these were included at low levels; therefore, formulating with cheaper and more practical protein sources should be relatively straightforward.

Conclusions and Recommendations

Amino acid research on YTK is virtually non-existent. A pragmatic approach quantifying the absolute requirement and understanding of interactions of related amino acids is required to ensure that diets are formulated to meet the EAA requirement for YTK. Further, requirements should be assessed within the context of environment and ontogeny. This is particularly important as we move towards fishmeal replacement diets for YTK. This study identified that the minimum requirement for histidine for YTK is < 7.45 g kg^{-1} diet. Although an absolute histidine requirement was not quantified, current industry feeds available for YTK should easily meet this specification. The results of Experiment 1 were inconclusive and raise questions over the physiological capacity of juvenile YTK to utilise high levels of crystalline amino acids, this is an area that requires further investigation. YTK are very efficient at utilising non fishmeal proteins and this is an area that requires further research. Zero or very low fishmeal diets for YTK are quite plausible, however longer term studies are required to demonstrate their efficacy.

Findings

- Dietary histidine requirement of juevenile YTK is < 7.45 g kg^{-1} diet. As indicated above, this value is based on the formulated value for the basal diet used in Experiment 2.
- It is possible that diets containing high levels of crystalline amino acids may not be effectively utilised by YTK. This response needs to be examined if further amino acid requirement studies are contemplated with YTK.
- YTK perform well over the short term on fishmeal free diets containing elevated dietary levels of brewer's yeast (20%) and krill meal (20%).
- It is unlikely that current commercial diets for juvenile YTK cultured under normal conditions are deficient in histidine if they contain > 7.45 g histidine kg^{-1} diet.
- There is great potential for zero or low fishmeal diets for YTK provided:
    a) diets are nutritionally balanced and;
    b) diets maintain a high degree of palatability.
- Research on the use of crystalline amino acids in diets for YTK is required.
- Further essential amino acid requirement studies are required to ensure diets are formulated to appropriate specifications for YTK.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, New South Wales Department of Primary Industries (NSW DPI) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds. The authors thank Dr Basseer Codabaccus, Ian Russell and Justin Tierney for their technical assistance. Dr Michael Salini (Ridley) supplied several of the raw ingredients.

References

Abidi, S.F., Khan, M.A., 2004. Dietary histidine requirement of fingerling Indian major carp, *Labeo rohita* (Hamilton). Israeli Journal of Aquaculture-Bamidgeh 56, 200-208.

ACEC, 2015. A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research, 4th Edition, NSW DPI Animal Care and Ethics Committee, pp. 56.

Ahmed, I., 2013. Dietary amino acid l-histidine requirement of fingerling Indian catfish, *Heteropneustes fossilis* (Bloch), estimated by growth and whole body protein and fat composition. J. Appl. Ichthyol. 29, 602-609.

Ahmed, I., Khan, M.A., 2005. Dietary histidine requirement of fingerling Indian major carp, *Cirrhinus mrigala* (Hamilton). Aquac. Nutr. 11, 359-366.

Akiyama, T., Arai, S., Murai, T., Nose, T., 1985. Threonine, histidine and lysine requirements of chum salmon fry. Bulletin of the Japanese Society of Scientific Fisheries 51, 635-639.

Alam, Teshima, Ishikawa, Koshio, Yaniharto, 2001. Methionine requirement of juvenile Japanese flounder *Paralichthys olivaceus* estimated by the oxidation of radioactive methionine. Aquac. Nutr. 7, 201-209.

Bender, D.A., 2012. Amino acid metabolism. Third edition. John Wiley & Sons. Pp. 478.

Booth, M.A., Allan, G.L., Pirozzi, I., 2010. Estimation of digestible protein and energy requirements of yellowtail kingfish *Seriola lalandi* using a factorial approach. Aquaculture 307, 247-259.

Breck, O., Bjerkås, E., Campbell, P., Rhodes, J., Sanderson, J., Waagbø, R., 2005. Histidine nutrition and genotype affect cataract development in Atlantic salmon, *Salmo salar* L. J. Fish Dis. 28, 357-371.

Castillo, S., Halligan, S., Gatlin, D.M., 2015. Growth responses of juvenile red drum *Sciaenops ocellatus* to dietary phenylalanine and tyrosine can be used to calculate the total aromatic amino acid requirement. The Journal of Nutrition 145, 2341-2346.

Espe, M., Lied, E., 1994. Do atlantic salmon (*Salmo salar*) utilize mixtures of free amino-acids to the same extent as intact protein-sources for muscle protein-synthesis. Comparative Biochemistry and Physiology a-Physiology 107, 249-254.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. The Journal of Biological Chemistry 226, 497-509.

Fountoulakis, M., Lahm, H.W., 1998. Hydrolysis and amino acid composition analysis of proteins. J. Chromatogr. A. 826, 109-134.

Gao, Y.J., Liu, Y.J., Chen, X.Q., Yang, H.J., Tian, L.X., 2016. Total aromatic amino acid requirement of juvenile grass carp (*Ctenopharyngodon idella*). Aquac. Nutr. 22, 865-872.

Han, Y., Koshio, S., Ishikawa, M., Yokoyama, S., 2013. Interactive effects of dietary arginine and histidine on the performances of Japanese flounder *Paralichthys olivaceus* juveniles. Aquaculture 414-415, 173-182.

Kim, K.-I., Kayes, T.B., Amundson, C.H., 1992. Requirements for lysine and arginine by rainbow trout (*Oncorhynchus mykiss*). Aquaculture 106, 333-344.

Klein, R.G., Halver, J.E., 1970. Nutrition of salmonoid fishes: Arginine and histidine requirements of Chinook and coho salmon. J. Nutr. 100, 1105-1109.

Li, Y., Cheng, Z.Y., Mai, K.S., Ai, Q.H., Shi, J.H., 2014. Dietary histidine requirement for juvenile large yellow croaker, *Pseudosciaena crocea* R. Israeli Journal of Aquaculture-Bamidgeh 66, 1-7.

Liao, S.-M., Du, Q.-S., Meng, J.-Z., Pang, Z.-W., Huang, R.-B., 2013. The multiple roles of histidine in protein interactions. Chemistry Central Journal 7, 44.

Marcouli, P.A., Alexis, M.N., Andriopoulou, A., Iliopoulou-Georgudaki, J., 2006. Dietary lysine requirement of juvenile gilthead seabream *Sparus aurata* L. Aquacult. Nutr. 12, 25-33.

Masumoto, T., 2002. Yellowtail, *Seriola quinqueradiata*. In: Webster, C.D. and Lim, C.E. (Eds). Nutrient requirements and feeding of finfish for aquaculture. Eds,. CABI Publishing, Wallingford, Oxon, New York, USA. pp. 131-146.

Moon, H.Y., Gatlin, D.M., 1991. Total sulfur amino acid requirement of juvenile red drum, *Sciaenops ocellatus*. Aquaculture 95, 97-106.

Nose, T., 1979. Summary report on the requirements of essential amino acids for carp. in: Halver, J.E.T., K. (Ed.), Fish Nutrition and Fishfeed Technology. Heenemann GmbH, Berlin, Germany, pp. 145-156

Nunes, A.J.P., Sa, M.V.C., Browdy, C.L., Vazquez-Anon, M., 2014. Practical supplementation of shrimp and fish feeds with crystalline amino acids. Aquaculture 431, 20-27.

Ogata, H., 2002. Muscle buffering capacity of yellowtail fed diets supplemented with crystalline histidine. J. Fish Biol. 61, 1504-1512.

Olson, J.S., Mathews, A.J., Rohlfs, R.J., Springer, B.A., Egeberg, K.D., Sligar, S.G., Tame, J., Renaud, J.-P., Nagai, K., 1988. The role of the distal histidine in myoglobin and haemoglobin. Nature 336, 265-266.

Peachey, B.L., Scott, E.M., Gatlin III, D.M., 2018. Dietary histidine requirement and physiological effects of dietary histidine deficiency in juvenile red drum *Sciaenop ocellatus*. Aquaculture 483, 244-251.

Peres, H., Oliva-Teles, A., 2005. The effect of dietary protein replacement by crystalline amino acid on growth and nitrogen utilization of turbot *Scophthalmus maximus* juveniles. Aquaculture 250, 755-764.

Pirozzi, I., Gamble, S., Siqila, A.M., Booth, M., 2018. The utilisation of soy protein in aquafeeds by gold-spot grouper *Epinephelus coioides*. Oral presentation. 18th International Symposium on Fish Nutrition and Feeding (ISFNF). Las Palmas de Gran Canaria - Canary Island, Spain. June 3rd-7th, 2018.

Rodehutscord, M., Becker, A., Pack, M., Pfeffer, E., 1997. Response of rainbow trout (*Oncorhynchus mykiss*) to supplements of individual essential amino acids in a semipurified diet, including an estimate of the maintenance requirement for essential amino acids. The Journal of Nutrition 127, 1166-1175.

Rodehutscord, M., Mandel, S., Pack, M., Jacobs, S., Pfeffer, E., 1995. Free amino acids can replace protein-bound amino acids in test diets for studies in rainbow trout (*Oncorhynchus mykiss*). J. Nutr. 125, 956-963.

Santiago, C.B., Lovell, R.T., 1988. Amino acid requirements for growth of Nile tilapia. The Journal of Nutrition 118, 1540-1546.

Simmons, L., Moccia, R.D., Bureau, D.P., Sivak, J.G., Herbert, K., 1999. Dietary methionine requirement of juvenile Arctic charr *Salvelinus alpinus* (L.). Aquac. Nutr. 5, 93-100.

Waagbo, R., Trosse, C., Koppe, W., Fontanillas, R., Breck, O., 2010. Dietary histidine supplementation prevents cataract development in adult Atlantic salmon, *Salmo salar* L., in seawater. Br. J. Nutr. 104, 1460-1470.

Watanabe, T., 2009. Requirements of nutrients in fish. Protein. In: Watanabe, T. (Ed.), Nutrition and feeding in fish and crustaceans, pp. 61-106.

Wilson, R.P., Poe, W.E., Robinson, E.H., 1980. Leucine, isoleucine, valine and histidine requirements of fingerling Channel catfish. J. Nutr. 110, 627-633.

Zehra, S., Khan, M.A., 2014. Dietary phenylalanine requirement and tyrosine replacement value for phenylalanine for fingerling *Catla catla* (Hamilton). Aquaculture 433, 256-265.

Table 3.1.5.2.1. Literature values for the histidine requirement of selected juvenile fish species.

Common name	**Species name**	**Histidine requirement** (% diet)	**Histidine requirement** (% crude protein)	**Dietary crude protein of test diet (%)**	**Reference**
Channel catfish	*Ictalurus punctatus*	0.4	1.5	24	Wilson et al. (1980)
Chinook salmon	*Oncorhynchus tshawytscha*	0.7	1.8	40	Klein and Halver (1970)
Chum salmon	*Oncorhynchus keta*	0.7	1.6	40	Akiyama et al. (1985)
Coho salmon	*Oncorhynchus kisutch*	0.7	1.8	40	Klein and Halver (1970)
Common carp	*Cyprinus carpio*	0.8	2.1	48	Nose (1979)
Indian catfish	*Heteropneustes fossilis*	0.54	1.35	40	Ahmed (2013)
Indian major carp	*Cirrhinus mrigala*	0.9	2.1	40	Ahmed and Khan (2005)
Indian major carp	*Labeo rohita*	0.82-0.90	2.05-2.25	40	Abidi and Khan (2004)
Japanese flounder	*Paralichthys olivaceus*	1.56	3.6	43	Han et al. (2013)
Japanese yellowtail	*Seriola quinqueradiata*	0.65-0.85	1.49-1.95	43	Masumoto (2002)
Nile tilapia	*Oreochromis niloticus*	1	1.7	28	Santiago and Lovell (1988)
Rainbow trout	*Oncorhynchus mykiss*	0.55	1.1	34	Rodehutscord et al. (1997)
Red drum	*Sciaenops ocellatus*	0.59	1.6	37	Peachey et al. (2018)
Yellow croaker	*Larimichthys polyactis*	0.9	2	44	Li et al. (2014)
	Average±SD	**0.78±0.28**	**1.88±0.59**		

Table 3.1.5.2.2. Experiment 1 - formulation of semi-purified diets and proximate composition (dry matter basis).

Ingredient (%)	**Diet1**	**Diet2**	**Diet3**	**Diet4**	**Diet5**	**Diet 6**
Fishmeal	10.0	10.0	10.0	10.0	10.0	10.0
Dehulled lupins	6.00	6.00	6.00	6.00	6.00	6.00
Gelatin	5.00	5.00	5.00	5.00	5.00	5.00
Fish Oil	16.0	16.0	16.0	16.0	16.0	16.0
Dextrin	15.0	15.0	15.0	15.0	15.0	15.0
Amino acid mix*	27.5	27.5	27.5	27.5	27.5	27.5
Choline chloride (70%)	0.40	0.40	0.40	0.40	0.40	0.40
Diatomaceous earth	13.2	13.2	13.2	13.2	13.2	13.2
Glutamic acid	2.00	2.00	2.00	2.00	2.00	2.00
NaH_2PO_4	0.50	0.50	0.50	0.50	0.50	0.50
Rovimix Stay-C (35)	0.30	0.30	0.30	0.30	0.30	0.30
Taurine	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin mineral premix	1.00	1.00	1.00	1.00	1.00	1.00
Y_2O_3	0.10	0.10	0.10	0.10	0.10	0.10
Glycine	**2.00**	**1.75**	**1.50**	**1.25**	**1.00**	**0.00**
Histidine	**0.00**	**0.25**	**0.50**	**0.75**	**1.00**	**2.00**
Measured proximate composition						
Dry Matter (%)	88.36	88.75	88.40	88.35	88.36	88.95
Ash (%)	17.75	17.80	17.84	17.77	17.91	17.69
Protein (%)	47.81	47.71	43.30	45.70	44.28	47.28
Lipid (%)	14.06	14.48	14.36	14.85	14.31	14.87
Energy (MJ kg^{-1})	21.38	21.43	21.33	21.53	21.57	21.28

*Contained the following crystalline amino acids (%); Arg 13.7, Iso 9.9, Leu 16.6, Lys 18.8, Met 8.1, Phe 10.0, Thr 10.0, Val 10.9, Try 2.1

Table 3.1.5.2.3. Control diet (Diet 7) formulation and proximate composition (dry matter basis).

Ingredient	**(%)**
Fishmeal	40.0
Poultry meal	7.0
Meat meal	5.0
Blood meal	7.0
Lupins	10
Wheat flour	15.0
Fish oil	6.0
Poultry oil	6.0
Vitamin mineral premix	0.5
Rovimix Stay-C (35)	0.05
Taurine	1.0
NaH_2PO_4	0.5
Choline chloride (70%)	0.3
Diatomaceous earth	1.65
Measured proximate composition	
Dry matter (%)	12.69
Ash (%)	12.69
Protein (%)	51.18
Lipid (%)	16.77
Energy (MJ kg^{-1})	22.61

Table 3.1.5.2.4. Proximate and amino acid composition of protein sources used in Experiment 1 and Experiment 2 (dry matter basis).

Parameter	**Blood Meal**	**Brewer's Yeast**	**Dehulled Lupins**	**Feather Meal**	**Fishmeal**	**Gelatin**	**Krill Meal**	**Meat Meal**	**Poultry Meal**	**Sodium Caseinate**
Dry matter (%)	91.98	97.03	93.20	92.68	92.12	89.40	93.42	94.10	92.92	96.70
Ash (%)	1.76	5.57	4.50	3.24	18.15	1.14	12.33	35.78	13.70	3.75
Energy (MJ kg^{-1})	24.30	19.92	21.50	23.90	19.87	21.10	23.60	16.10	22.10	23.90
Protein (%)	97.77	47.90	45.00	90.74	71.98	98.14	61.55	50.19	67.94	92.81
Fat (%)	0.47	2.80	9.30	7.08	8.97	0.72	20.21	12.10	12.43	0.37
Amino acids (%)										
Alanine	7.09	2.76	1.30	3.97	3.94	8.22	3.18	3.36	4.04	2.65
Arginine	4.02	2.42	4.46	5.96	3.91	7.66	3.65	3.30	4.58	3.54
Aspartic acid	9.06	4.68	4.28	5.77	3.94	5.25	6.38	3.17	5.13	5.97
Cysteine	0.00	0.53	0.62	4.28	0.58	0.15	0.51	0.00	0.11	0.45
Glutamic acid	8.07	7.54	8.48	9.32	8.17	10.11	7.96	5.42	8.29	19.43
Glycine	3.86	2.06	1.66	6.36	4.20	21.77	2.78	6.74	5.87	1.69
Histidine	5.62	0.97	1.00	0.65	1.78	0.67	1.15	0.76	1.46	2.69
Isoleucine	0.81	1.99	1.82	3.86	2.87	1.46	2.88	1.22	2.67	4.86
Leucine	11.59	3.13	2.97	6.80	4.91	2.78	4.67	2.49	4.84	8.53
Lysine	8.71	3.46	1.91	1.99	4.99	3.43	4.16	2.34	3.86	7.21
Methionine	1.49	0.74	0.19	0.59	1.95	0.81	1.79	0.65	1.21	2.68
Phenylalanine	6.72	2.03	1.56	4.07	2.68	1.84	2.98	1.41	2.71	4.70
Proline	3.59	2.19	1.59	11.42	2.72	12.77	3.23	3.96	4.35	9.94
Serine	5.11	2.35	2.11	9.20	2.58	3.10	2.71	1.67	3.46	5.13
Taurine	0.00	0.00	0.00	0.00	0.58	0.00	0.51	0.10	0.29	0.00
Threonine	4.89	2.29	1.50	3.83	2.80	1.69	2.86	1.36	2.69	3.87
Tyrosine	2.90	1.60	1.32	2.45	1.95	0.51	2.69	0.87	1.88	4.92
Valine	8.72	2.34	1.77	5.34	3.30	2.33	2.94	1.89	3.72	6.53

Table 3.1.5.2.5. Measured amino acid content (dry matter basis) of diets used in Experiment 1. Two commercial diets and fishmeal also included for comparison of amino acid profiles. Diets 1-6 were subsequently fortified with tryptophan to approximately 0.5% and rerun. Diets 1-6 = Semi-purified diets; Diet 7 = Control diet; Comm. A and B = Commercial YTK diets, and fishmeal also included for comparison

	Amino Acid (%)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Comm. A	Comm. B	Fish meal
Essential Amino Acids	Arginine	4.26	4.38	4.35	4.39	4.36	4.32	2.89	2.88	2.82	3.91
	Histidine	0.27	0.52	0.7	0.97	1.17	2.07	1.43	1.5	1.37	1.78
	Isoleucine	2.9	2.95	2.9	2.88	2.9	2.94	1.74	2.06	2.00	2.87
	Leucine	4.73	4.72	4.7	4.6	4.56	4.78	3.8	3.6	3.44	4.91
	Lysine	4.32	4.43	4.48	4.51	4.55	4.41	3.29	3.36	3.18	4.99
	Methionine	2.11	2.2	2.08	2.05	1.98	2.14	1.03	1.08	1.07	1.95
	Phenylalanine	2.91	2.93	2.93	2.88	2.89	2.92	2.12	2.05	1.97	2.68
	Threonine	2.86	2.82	2.87	2.81	2.84	2.87	1.97	1.94	1.88	2.8
	Tryptophan	0.12	0.12	0.12	0.12	0.12	0.14	0.49	0.48	0.46	0.73
	Valine	3.4	3.42	3.36	3.27	3.29	3.4	2.51	2.38	2.26	3.3
	Taurine	0.97	0.91	0.87	0.83	0.83	0.86	1.25	0.71	0.83	0.58
Non-essential Amino Acids	Alanine	0.95	0.94	0.93	0.98	0.95	0.95	2.78	2.61	2.49	3.94
	Aspartic acid	1.08	1.11	1.13	1.15	1.15	1.12	4.00	4.23	3.95	5.68
	Cysteine	0.09	0.11	0.12	0.11	0.09	0.09	0.55	0.55	0.48	0.58
	Glutamic acid	3.58	3.67	3.67	3.72	3.68	3.63	6.24	6.67	6.45	8.17
	Glycine	3.56	3.24	2.98	2.89	2.57	1.67	3.1	2.72	2.62	4.2
	Hydroxyproline	0.65	0.61	0.59	0.67	0.62	0.62	0.59	0.32	0.31	0.61
	Proline	1.05	1.01	1	1.08	1.03	1.02	2.32	2.13	2.06	2.72
	Serine	0.54	0.54	0.56	0.57	0.57	0.55	2.07	2.01	1.92	2.58
	Tyrosine	0.28	0.31	0.32	0.32	0.32	0.31	1.29	1.27	1.22	1.95
	Met+Cys	2.2	2.31	2.2	2.16	2.07	2.23	1.58	1.63	1.55	2.53
	Phe+Tyr	3.19	3.24	3.25	3.2	3.21	3.23	3.41	3.32	3.19	4.63
	∑AA	40.63	40.94	40.66	40.8	40.47	40.81	45.46	44.55	42.78	60.93

Table 3.1.5.2.6. Formulation of practical diets used in Experiment 2 - Intact protein sources (dry matter basis) and measured proximate analyses.

Ingredients (%)	**P.Diet1**	**P.Diet2**	**P.Diet3**	**P.Diet4**	**P.Diet5**
Brewer's yeast	20	20	20	20	20
Krill meal	20	20	20	20	20
Feather meal	15.5	15.5	15.5	15.5	15.5
Lupins	6.53	6.53	6.53	6.53	6.53
Gelatin	9	9	9	9	9
Sodium caseinate	5.66	5.66	5.66	5.66	5.66
Fish Oil	12	12	12	12	12
Maize starch	5	5	5	5	5
Lysine	0.57	0.57	0.57	0.57	0.57
Methionine	0.97	0.97	0.97	0.97	0.97
Choline chloride (70%)	0.4	0.4	0.4	0.4	0.4
NaH_2PO_4	0.75	0.75	0.75	0.75	0.75
Rovomix Stay-C (35)	0.3	0.3	0.3	0.3	0.3
Taurine	0.72	0.72	0.72	0.72	0.72
Vitamin mineral premix	0.5	0.5	0.5	0.5	0.5
Y_2O_3	0.1	0.1	0.1	0.1	0.1
Glutamic acid	**2.0**	**1.5**	**1.0**	**0.5**	**0.0**
Histidine	**0.0**	**0.5**	**1.0**	**1.5**	**2.0**
Measured proximate composition					
Ash (%)	5.01	4.99	5.15	4.99	5.03
Protein (%)	58.26	57.58	57.43	57.88	58.61
Lipid (%)	18.83	18.49	24.86	18.93	19.80
Energy (MJ kg^{-1})	24.39	24.72	23.86	23.98	24.15

Table 3.1.5.2.7. Measured amino acid content (% dry matter basis) of diets used in Experiment 2.

	Amino Acid (%)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Essential Amino Acids	Arginine	3.22	3.25	2.81	3.63	3.37
	Histidine	0.75	1.16	1.60	1.95	2.42
	Isoleucine	2.25	2.26	2.18	2.24	2.30
	Leucine	3.75	3.76	3.59	3.81	3.63
	Lysine	2.82	3.02	2.72	3.33	3.38
	Methionine	1.74	1.68	1.63	1.69	1.69
	Phenylalanine	2.25	2.23	2.18	2.29	2.21
	Threonine	2.03	2.07	2.00	2.12	2.05
	Valine	2.71	2.70	2.62	2.75	2.68
	Taurine	0.74	0.75	0.73	0.72	0.74
Non-essential Amino Acids	Alanine	3.11	3.02	2.95	3.18	3.02
	Aspartic acid	4.56	4.60	4.46	4.64	4.50
	Cysteine	1.13	1.15	1.09	1.07	1.03
	Glutamic acid	9.58	9.30	8.75	8.49	7.69
	Glycine	4.77	4.55	4.65	4.90	4.51
	Proline	4.19	4.06	4.04	4.54	4.22
	Serine	3.45	3.58	3.19	3.47	3.31
	Tyrosine	1.68	1.72	1.72	1.69	1.68
	Met+Cys	2.87	2.83	2.72	2.76	2.72
	Phe+Tyr	3.93	3.95	3.90	3.98	3.89
	∑AA	54.71	54.86	52.91	56.51	54.43

Table 3.1.5.2.8. Water quality (mean ± SD).

Experiment	RAS	Temperature (°C)	DO (mg L^{-1})	pH	Salinity (‰)	NH_3
1a	1	17.1 ± 1.8	8.1 ± 0.4	7.6 ± 0.3	35.4 ± 1.1	1.2 ± 0.6
1a	2	22.7 ± 1.3	7.6 ± 0.5	7.6 ± 0.3	35.4 ± 0.8	1.2 ± 0.6
1b	1	22.2 ± 0.4	7.2 ± 0.5	7.5 ± 0.1	31.6 ± 0.5	0.5 ± 0.4
2	1	19.9 ± 0.4	13.4 ± 3.0	7.6 ± 0.2	32.7 ± 0.4	0.5 ± 0.2

Table 3.1.5.2.9. Summary data of histidine feed Experiment 1 after 6 weeks. Diets 1-6 semi-purified test diets. Diet 7 is the control diet. No Significant differences ($P > 0.05$) were found among Diets 1-6.

Parameter	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**	**Diet 7**	**Pooled SEM**
17 °C								
Initial weight	120.7	121.3	119.8	125.4	121.0	115.1	171.5	0.5
Final weight	184.4	189.6	185.1	177.7	186.8	181.7	279.9	4.7
Survival (%)	86.7	96.7	100.0	100.0	100.0	100.0	80.0	2.4
Weight gain (%)	60.1	63.0	58.6	53.9	61.0	56.7	140.0	3.8
SGR (% d^{-1})	1.15	1.19	1.12	1.05	1.16	1.09	2.13	0.06
FCR	2.27	2.15	2.62	3.16	2.29	2.66	1.26	0.23
22 °C								
Initial weight	115.9	115.9	116.0	115.7	116.1	116.3	116.0	0.4
Final weight	202.8	206.0	210.8	199.5	216.3	217.4	446.8	9.1
Survival (%)	76.7	73.3	73.3	83.3	70.0	86.7	66.7	5.5
Weight Gain (%)	75.1	77.8	81.8	72.5	86.3	87.0	285.2	8.0
SGR (% d^{-1})	1.32	1.37	1.42	1.29	1.47	1.49	3.21	0.09
FCR	3.04	2.56	2.65	3.09	2.48	2.56	1.47	0.25

Table 3.1.5.2.10. Summary data of histidine feed Experiment 1 with tryptophan supplemented diets; terminated after 3 weeks. Diets 1-6 fortified with tryptophan. Diets 7 and 8 are equivalent in composition to Diets 4 and 6, however, without additional tryptophan supplementation.

Parameter	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**	**Diet 7**	**Diet 8**	**Pooled SEM**
Initial weight	187.1	190.7	182.7	184.1	184.6	187.0	186.5	186.4	2.4
Final weight	212.2	219.9	212.1	213.4	210.0	212.4	221.3	215.2	3.6
Survival (%)	95.2	95.2	100.0	100.0	95.2	95.2	100.0	100.0	2.7
Weight gain (%)	13.4	15.4	16.1	15.9	13.7	13.5	18.7	15.6	1.8
SGR (% d^{-1})	0.60	0.68	0.71	0.70	0.61	0.60	0.82	0.69	0.08
FCR	4.96	4.75	3.76	4.05	4.88	5.60	3.71	4.34	0.66

Table 3.1.5.2.11. Diet proximate and amino acid composition (dry matter basis) and corresponding apparent digestibility coefficients (ADC). ADC data shown as mean ± SD; *n* = 2. Diet values vary slightly from those presented in other tables as these were analysed at a different laboratory and/or at a different time.

Parameter	Diet 3 composition	Apparent digestibility (ADC)
Proximates		
Dry matter	92.17	0.29 ± 0.02
Protein	41.09	0.79 ± 0.02
Fat	16.95	0.78 ± 0.11
Ash	18.30	-0.77 ± 0.25
OM	81.70	0.52 ± 0.03
NFE	23.66	-0.15 ± 0.15
Energy (MJ kg^{-1})	20.67	0.68 ± 0.02
Yttrium (mg kg^{-1})	855.80	
Amino Acids(mg kg^{-1})		
Alanine	38.90	0.93 ± 0.02
Arginine	47.94	0.93 ± 0.03
Aspartic acid	14.32	0.54 ± 0.11
Cystine	1.05	-0.09 ± 0.25
Glutamic acid	44.29	0.82 ± 0.05
Glycine	29.70	0.84 ± 0.05
Histidine	6.42	0.85 ± 0.04
Isoleucine	14.80	0.85 ± 0.04
Leucine	52.73	0.93 ± 0.02
Lysine	45.19	0.91 ± 0.03
Methionine	19.79	0.86 ± 0.03
Phenylalanine	32.43	0.93 ± 0.02
Proline	11.68	0.72 ± 0.09
Serine	7.98	0.64 ± 0.08
Threonine	31.42	0.91 ± 0.02
Tyrosine	4.79	0.58 ± 0.12
Valine	7.20	0.67 ± 0.09
Taurine	26.23	0.80 ± 0.02
∑AAs	410.64	0.93 ± 0.02

Table 3.1.5.2.12. Experiment 2 – Intact proteins sources; performance indices of YTK fed one of five diets supplemented with 0.0 to 2.0% histidine. Different superscript letters within columns indicate significant difference among treatments ($P < 0.05$). Histidine intake calculated based on expected histidine content of diets.

Diet	Initial weight (g)	Initial length (mm)	Initial K	Final weight (g)	Final length (mm)	Final K	Growth (g fish^{-1} day^{-1})	SGR (%)	Feed intake (g fish^{-1} day^{-1})	FCR	Histidine intake (g fish^{-1} day^{-1})
P.Diet1	80.1	184.2	1.28	325.5	285.2	1.40	5.01	2.86	5.09	1.02	0.046^{a}
P.Diet2	79.9	183.0	1.31	320.0	282.8	1.40	4.90	2.82	5.13	1.05	0.072^{b}
P.Diet3	79.9	182.5	1.32	328.4	284.5	1.41	5.07	2.88	5.12	1.01	0.097^{c}
P.Diet4	80.4	184.0	1.29	350.1	294.6	1.36	5.50	3.00	5.68	1.03	0.136^{d}
P.Diet5	80.6	184.6	1.28	332.6	283.2	1.54	5.14	2.89	5.12	1.00	0.148^{d}
Pooled SEM	0.42	0.75	0.01	12.40	4.15	0.04	0.25	0.08	0.17	0.02	0.003
F-ratio	*0.41*	*1.00*	*0.90*	*0.62*	*0.95*	*1.07*	*0.61*	*0.60*	*1.62*	0.60	*171.01*
P-value	*0.80*	*0.45*	*0.50*	*0.66*	*0.48*	*0.42*	*0.67*	*0.67*	*0.24*	0.67	*0.00*

3.1.5.3. Manuscript - Sulphur amino acid requirements of juvenile Yellowtail Kingfish (Seriola lalandi).

Caroline Candebat[b], Mark Booth[a], Basseer Codabaccus[b,c] and Igor Pirozzi[a,b]

[a]New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Locked Bag 1, Nelson Bay, NSW 2315

[b]Centre for Sustainable Tropical Fisheries and Aquaculture and College of Science and Engineering, James Cook University, 1 James Cook Dr, Townsville City, QLD 4811

[c]Institute for Marine and Antarctic Studies, University of Tasmania. Private Bag 49, Hobart, TAS, 7001, Australia

This manuscript may be referenced as: Candebat, C., Booth, M., Codabaccus, B. and Pirozzi, I. (2019). 3.1.5.3. Manuscript - Sulphur amino acid requirements of juvenile Yellowtail Kingfish (*Seriola lalandi*). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.209-233.

Abstract

Dietary requirements of Yellowtail Kingfish (*Seriola* lalandi; YTK) for the sulphur amino acids methionine and cysteine are currently unknown and information concerning taurine is limited. Two feeding experiments were undertaken with juvenile YTK at an average water temperature of 22 °C to quantify 1) the taurine requirement and potential sparing effect of methionine (TauMet experiment) and; 2) the methionine requirement and potential sparing effect of cysteine (MetCys experiment). A factorial dose-response design was implemented in both experiments. TauMet experiment: a 7 taurine × 2 methionine level design in which dietary taurine content was increased from 1.6 to 20.4 g kg^{-1} and dietary methionine content averaged 10.9 g kg^{-1} and 17.2 g kg^{-1}, respectively. MetCys experiment: a 5 methionine × 2 cysteine level design in which dietary methionine content was increased from 7.9 to 25.2 g kg^{-1} and dietary cysteine content averaged 5.5 g kg^{-1} and 13.9 g kg^{-1}, respectively. All diets in the MetCyst experiment contained approximately 7 g taurine kg^{-1}. Results from the TauMet experiment demonstrated that the specific growth rate (SGR) and feed conversion ratio (FCR) of YTK was highly dependent on dietary taurine content when diets contained the lowest level of methionine (i.e. 10.9 g kg^{-1} diet). Methionine was able to spare dietary taurine when it was provided at 17.2 g kg^{-1} diet. Breakpoint analysis of the SGR response of YTK fed the low methionine series of diets indicated the digestible taurine requirement of YTK was 1.98 g kg BW^{-1} d^{-1} at an average digestible methionine intake of 3.4 g kg BW^{-1} d^{-1}. This equates to a dietary taurine requirement of 7.7 g kg^{-1} diet with a methionine content of 10.9 g kg^{-1} diet. Fish exposed to taurine and methionine deficiencies did not exhibit signs of green liver. Results from the MetCys experiment demonstrated that the SGR of YTK was highly dependent on dietary methionine content when diets contained the lowest level of dietary cysteine (i.e. 5.5 g cysteine kg^{-1} diet). Breakpoint analysis of the SGR response indicated the digestible methionine requirement of juvenile YTK was 6.7 g kg BW^{-1} d^{-1} at an average digestible cysteine intake of 1.7 g kg BW^{-1} d^{-1}. This equates to a dietary methionine content of 18.7 g kg^{-1} diet at an average dietary cysteine content of 5.6 g kg^{1} diet. Therefore, the dietary level of total sulphur amino acids (methionine+cysteine) is approximately 24.3 g kg^{-1} diet and the daily digestible intake of total sulphur amino acids is approximately 8.4 g kg BW^{-1} d^{-1}. Based on the SGR response of YTK cysteine can spare at least 48.2% of the dietary methionine requirement in YTK considering molecular weight and digestibility of methionine and cysteine. The decline of SGR in YTK when dietary methionine content exceeded 18.7 g kg^{-1} diet when cysteine was present at 5.5 g kg^{-1} diet indicated a maximum methionine threshold, possibly induced through the buildup of excess methionine derivates via transamination or transulfuration pathways. YTK exposed to sub-optimal methionine and cysteine exhibited bilateral opaque eyes which may be indicative of cataracts, but they showed no visual evidence of green liver.

The results suggest the dietary specification of methionine currently relied on in commercial aquafeeds for YTK may be inadequate. This study provides new data on the taurine, methionine and cysteine requirements of juvenile YTK and will facilitate the formulation of better diets for this species.

Introduction

Sulphur-containing amino acids (SAA), which include methionine, cysteine and taurine (a β-sulphonic amino acid) allow normal growth and functioning of fish (Halver, 2002). Unlike methionine and cysteine, taurine is not incorporated into proteins. SAA (excluding taurine) initiate protein biosynthesis and are vital as building blocks for all proteins. SAA are nonpolar and hydrophobic and typically located internally within proteins. They are also a primary source of methyl and sulphur groups (Bertolo and McBreairty, 2013). Products of SAA metabolism are vital for the digestion of lipids by conjugating with bile acid (Kim et al., 2015). Furthermore, SAA and products serve as antioxidants, preventing cell damage and are cell volume regulators, maintaining cells hydration (Yancey, 2005).

Methionine is an essential amino acid and cannot be synthesized *de novo* in sufficient quantities to meet requirements. Therefore, adequate amounts of dietary methionine must be provided in aquafeeds to meet the metabolic demand of the species. Cysteine is a conditionally essential amino acid, and is a derivate of methionine metabolism from which it receives its sulphur atom (Brosnan and Brosnan, 2006). In fish, cysteine has the potential to spare 33 to 60% of methionine requirement (Abidi and Khan, 2011; Harding et al., 1977; Moon and Gatlin, 1991).

Taurine is derived from the trans-adenylation, methylation, sulphuration and hydrolyzation pathway of methionine and cysteine providing the substrate for the taurine synthesis (Brosnan and Brosnan, 2006; National Research Council, 2011; Wu, 2009). Taurine has many functional roles in the physiology of animals enabling key metabolic pathways. It is an intracellular organic osmolyte, regulating cell volume (Wijayasinghe et al., 2017). Taurine conjugates with bile acids and forms bile salt, which is essential for lipid utilization and digestion (Bellentani et al., 1987). It is a substrate for the development, functionality and cell protection of the central nervous, retinal and muscular system (Wu and Prentice, 2010).

The relatedness, interactions and sparing effects of SAA imply that quantifying the requirement of a species for one essential SAA must be done within the context of the concentration of other SAA present in the diet. This is why methionine and cysteine requirement are often expressed in terms of total sulphur amino acid (TSAA) requirement (National Research Council, 2011). Neither methionine nor cysteine has been studied in YTK. Current recommended levels of dietary methionine for Australian YTK are based on the work by Ruchimat et al. (1997) for the closely related Japanese Yellowtail (*Seriola quinqueradiata*) at 11.1 g kg^{-1} diet; however, the degree of interaction of dietary SAA compounds is not known for either species. The dietary taurine requirement of California yellowtail (*S. lalandi*) was determined by Salze *et al.* (2017) using experiment diets with a methionine content of 11 g kg^{-1} diet. Taurine requirement was reported in that study at 2.6 to 10.2 g kg^{-1} diet. However, that study used high soy protein diets which are ingredients that are not used at high inclusions in the Australian YTK industry. In juvenile Japanese Yellowtail deficiencies in dietary taurine are associated with green liver syndrome, inferior growth performance (Takagi et al., 2010) and increased susceptibility to diseases (Li et al., 2007).

With a concerted effort by aquafeed manufacturers around the world to use low or zero fishmeal inclusions in feed formulations, the increasing utilization of plant proteins and rendered animal products will mean that the TSAA of diets may become limiting unless diets are formulated to deliver a balanced suite of essential nutrients. However, to achieve this goal a comprehensive understanding of the quantitative nutrient requirements of the animal is required. The objective of this study is to 1) determine the requirement for taurine and methionine in juvenile YTK; and 2) to better understand the interaction among the sulfur amino acids methionine, cysteine and taurine, also in juvenile YTK.

Methods

Two experiments were performed under the NSW DPI Fisheries Animal Care and Ethics (ACEC) Research Authority known as 'Aquaculture Nutrition ACEC 93/5–Port Stephens'. Care, husbandry and termination of fish were carried out according to methods outlined in 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2017).

Experiment design

TauMet experiment

A factorial dose-response approach was applied to quantify the dietary taurine requirement of juvenile YTK relative to dietary methionine content. The design used 7 incremental levels of taurine ranging from 1.6 to 20.4 g kg^{-1} crossed with two levels of dietary methionine (i.e. 10.9 or 17.2 g kg^{-1} diet) (Table 3.1.5.3.1 and Table 3.1.5.3.2). All diets were isonitrogenous (≈500 g crude protein kg^{-1} diet) and isoenergetic (≈22 MJ gross energy kg^{-1} diet) (Table 3.1.5.3.2), formulated to meet the protein and energy requirements of small YTK using practical ingredients (Booth et al., 2010). Diets were formulated using a blend of prime fishmeal and fisheries by-product meal in order to reduce the residual taurine content of the basal formula as low as possible while maintaining the palatability of the diets. Diets were supplemented with crystalline methionine and taurine to achieve dietary specifications. Other protein sources included blood meal, corn gluten meal, dehulled lupin meal, feather meal and poultry meal (Table 3.1.5.3.1).

Fourteen fish (53.3 ± 0.4 g $fish^{-1}$) were stocked into 200 L experiment tanks with water supplied via a recirculating aquaculture system (RAS). Triplicate groups of fish (n = 3) were randomly allocated to each experimental diet and hand fed to apparent satiation twice per day (AM:PM) during weekdays and once per day (AM) on weekends for 45 days. The trial was terminated at week seven of the planned eight weeks due to a pump failure causing a loss of several tanks of fish (29.4%). There were sufficient replicate tanks of fish to proceed with a minimum of n = 2 subsamples for carcass composition and digestibility analyses with exceptions as indicated in sections on Apparent digestibility and Compositional analysis below.

MetCys experiment

A factorial dose-response approach was applied to quantify the dietary methionine requirement of juvenile YTK relative to dietary cysteine content. The design used 5 incremental levels of methionine ranging from 7.9 to 25.2 kg^{-1} diet crossed with two levels of dietary cysteine (i.e. 5.5 or 13.9 g kg^{-1} diet). All diets were isonitrogenous (≈600 g crude protein kg^{-1}) and isoenergetic (≈ 22 MJ gross energy kg^{-1}) and prepared using practical raw ingredients (Table 3.1.5.3.3 and Table 3.1.5.3.4). The diet specifications were achieved using a low quantity of prime fishmeal and other protein sources including blood meal, dehulled lupin meal, feather meal, gelatin, soy protein concentrate (SPC) and sodium caseinate. Diets were also supplemented with crystalline methionine and cysteine in order to achieve the target specifications (Table 3.1.5.3.3).

Twelve fish (52.6 ± 4 g $fish^{-1}$) were stocked into 200 L experiment tanks with water supplied via a RAS. Triplicate groups of fish (n = 3) were randomly allocated to each experimental diet and hand fed to apparent satiation twice per day (AM:PM) during weekdays and once per day (AM) on weekends for 54 days.

In either study all uneaten pellets were collected, stored frozen and dried to accurately calculate dry-basis feed intake per tank. Faecal samples were collected from fish at the end of the experiments to allow the apparent digestibility of diets to be determined. Additionally, representative samples of whole fish (n = 5) were sampled at the beginning of each trial for compositional analysis. Final carcass samples, liver and viscera tissues and faeces were collected at the end of each experiment for chemical analysis

Fish handling and experiment system

All fish were progeny of wild-caught YTK broodstock held at the Port Stephens Fisheries Institute (PSFI), NSW, Australia. Prior to stocking, juvenile YTK were fed two to three times daily with 3-4 mm floating pellets (Ridley; crude protein 50%, crude fat 14%, and crude fibre 4%) and held at water temperatures between 15-19 °C.

Both requirement trials were done indoors under controlled conditions in a laboratory housing a recirculating aquaculture system (RAS). The RAS consisted of two × 1,300 L sumps, two large sand filters, two protein skimmers and two × 750 L bio-filters. Effluent water was continuously exchanged with filtered and disinfected estuarine water. YTK received a prophylactic hydrogen peroxide treatment (150 ppm for 30 min) against fluke at week three and week six (TauMet study only). No fluke was detected throughout the trials. The oxygen concentration of the RAS was be maintained above 6 mg oxygen L^{-1} (100-150% saturation) using industrial oxygen (BOC). In the TauMet experiment oxygen was injected directly into the main supply manifolds of the RAS and in the MetCys trail oxygen was diffused directly into the 200 L experiment tanks via a single submerged air-stone. The 200 L tanks were partially covered with plastic oyster mesh and black plastic to prevent fish from escaping and ensure minimal disturbance during the experiments. The laboratory photoperiod was controlled to provide 12 h of light and 12 h of darkness using dimmed, overhead LED lighting. The water quality of the RAS was monitored daily with an electronic water quality meter (Horiba and Hach).

Water quality in the TauMet experiment was; water temperature (23.3 ± 0.6 °C), salinity (33.3 ± 4.6‰), dissolved oxygen (7.0 ± 1.0 mg L^{-1}) and pH (8.3 ± 0.5), TAN (≤ 0.7 mg L^{-1}). Water quality in the MetCys experiment was; water temperature (21.2 ± 0.6 °C), salinity (32.9 ± 3.2‰), dissolved oxygen (12.1 ± 3.0 mg L^{-1}) and pH (7.4 ± 0.4), TAN (≤ 0.25 mg L^{-1}).

Diet manufacture

All diets were made at PSFI using laboratory scale equipment. Prior to pellet making all raw materials were finely ground in a high speed hammer mill (Raymond Laboratory Mill, Transfield Technologies, Rydalmere, NSW, Australia; 1.6 mm screen). Wheat flour was autoclaved for 2 min at 121 °C prior to inclusion in the dry mash. The raw materials and supplements were then dry mixed in a Hobart mixer (Hobart Mixer; Troy Pty Ltd, Ohio, USA) before the addition of oil and fresh water to form a moist dough. The dough was then screw pressed into 6 mm pellets (Dolly, La Monferrina, Castell'Alfero, Italy). The moist pellets were then dried at approximately 60 °C to a moisture content of < 10%.

Major response variables and calculations

The following performance variables were used to determine the taurine and methionine requirements of rapidly growing juvenile YTK. Variables are based on a comparative-slaughter assay approach.

- $\text{Initial weight of fish (g)} = \text{individual weight of fish at stocking}$
- $\text{Harvest weight of fish (g)} = \text{individual weight of fish at harvest}$
- $\text{Specific growth rate (SGR)}(\%\ d^{-1}) = \frac{[\text{Ln (final weight)} - \text{Ln (initial weight)}]}{\text{days}} \times 100$
- $\text{Condition factor K} = [\text{individual weight of fish(g)} - \text{fork length of fish (mm)}] \times 10^5$
- $\text{Food conversion ratio (FCR)} = \text{feed intake per tank (g)} - \text{wet weight gain per tank (g)}$
- $\text{HSI} = \left(\text{liver weight (g)} \div \text{whole weight of fish (g)}\right) \times 100$
- $\text{VSI} = \left(\text{viscera weight} \div \text{whole weight of fish (g)}\right) \times 100,$
  $; \text{where viscera includes liver} + \text{organs} + \text{intraperitoneal fat}$
- $\text{Intraperitoneal fat ratio (IPR)} = \left(\text{amount of visceral fat} \frac{\text{g}}{\text{whole}} \text{weight of fish (g)}\right) \times 100$

Cysteine sparing

The sparing of cysteine was defined as the quantity which can substitute for methionine. Theoretically, the difference between TSAA requirement and the minimum obligatory methionine (MOM) requirement

should equal the potential of cysteine to spare methionine requirement, considering the molecular weight difference of methionine and cysteine (i.e. 0.8 Cys = 1.0 Met) (Ball et al., 2006). Therefore, the cysteine sparing potential can be calculated as;

- $Cysteine\ sparing\ potential = TSAA\ - MOM$

Apparent digestibility

Following the conclusion of the feeding trials the apparent digestibility of the diets was determined using stripping techniques. Prior to stripping, fish were anaesthetised using 5-25 mg L^{-1} Aqui-S™. Faeces were collected from the posterior intestine by applying gentle abdominal pressure. Contamination with urine or mucous were minimized and samples were immediately stored at -20 °C. This procedure was repeated twice a week until approximately 3 g dry faecal matter was obtained. Faecal material from the TauMet experiment was collected from duplicate groups of three to four YTK. Only faeces from fish fed diet 1, 4, 7, 8, 11 and 14, were collected representative for fish fed low, medium or high relative levels of dietary taurine at standard or relatively high inclusion levels of methionine. Faecal material from the MetCys experiment was collected from triplicate groups of four to six YTK. Only faeces from fish fed diet 1, 3, 5, 6, 8 and 10 were collected representative for fish fed low, medium or high relative levels of dietary methionine at standard or relatively high inclusion levels of cysteine. Diet and faecal samples were analysed for dry matter, nitrogen, gross energy, fat, ash and amino acids. Aparent digestibility coefficients (ADCs) were calculated according to the equation described by Cho et al. (1982), with the exception that yttrium was used as the internal marker

$$ADC(\%) = 100 \times [1 - \left(\frac{F}{D} \times \frac{D_{marker}}{F_{marker}}\right)]$$

where; F= % nutrient in faeces; D= % nutrient in diet; D_{marker} = % marker in diet; F_{marker} = % marker in faeces.

Compositional analyses

Dry matter, protein, lipid, ash and gross energy of experimental diets, whole-body and faecal sample (including yttrium) analyses were conducted by CSIRO (Agriculture and Food, Carmody Road, St Lucia, QLD 4067, Australia) according to routine methods outlined in AOAC (2005). Crude protein content was determined by multiplying the nitrogen content of each sample by 6.25. Crude lipid was determined by extraction using chloroform:methanol (2:1) (Folch et al., 1953). Amino acid profile analysis was performed according to standard operating procedure SOP QAAA-001 of the Australian Proteome Analysis Facility (APAF; Macquarie University, Sydney Australia). Duplicate groups of whole carcass from each TauMet diet was collected, except for YTK fed diet 6 and 8 (n = 1). Triplicate groups of whole carcass from each MetCys diet was collected.

Data analyses

Raw data were processed via Microsoft Excel 2016 and further modelled via GraphPad Prism ver. 6 (La Jolla, CA, USA).

The dose-response experiments were designed to evaluate the performance of YTK fed diets below (deficiency); at (optimal); or above (excess), their methionine or taurine requirement. FCR and SGR were selected as the response variables in this report. Non-linear or multiphasic/linear regression analyses were fitted to the response data. Curves were visually assessed for the quality of their fit to the data. Statistical models were cross-validated for quality and fit via Akaike information criterion (AICc), absolute sum of squares, and R squared. The model with the strongest likelihood from the following was then selected as the most representative:

1. $y = A - b * s * \ln(1 + \exp\left(\frac{c-x}{s}\right))$ (Koops and Grossman, 1993)

2. $y = intercept + slope * X$

3. $y = a + bX + cX^2$

Statistical analyses

Raw data were processed via Microsoft Excel 2016 and further statistically analysed, using the R language and the R software environment for statistical computing (2.13.) with the packages multcompView, ggplot2, car, and PMCMR.

All response variables were subject to the Shapiro-Wilk normality test and Levene's test for homogeneity of variance. If assumptions were not met, data were log, sqrt, or inverse transformed and then again tested for normality and homogeneity. If assumptions of ANOVA were not met after transformation a Kruskal Wallis test was applied. Normal data were analysed using two-way ANOVA. Tukey HSD test was conducted on significant terms.

Results

TauMet experiment - growth and feeding performance

There were significant interactions ($P < 0.05$) between the level of dietary taurine and dietary methionine in most growth and feeding responses (Table 3.1.5.3.5).

Harvest body weight, weight gain, SGR and feed efficiency increased significantly ($P < 0.05$) with increasing taurine content when the methionine content of the diets was approximately 11.0 g kg^{-1} diet, with no significant difference in performance after the taurine content reached 4.8 – 8.5 g taurine kg^{-1} diet. SGR plateaued at 2.6-2.7% d^{-1} (Figure 3.1.5.3.1; Table 3.1.5.3.5).

No significant difference was observed among YTK fed increasing levels of taurine when methionine was supplied at approximately 17.2 g kg^{-1} diet. Furthermore, YTK fed the series of diets high in methionine recorded improved growth and feeding efficiency compared to YTK fed the series of diets with the low methionine content (i.e. 10.9 g kg^{-1} diet). The harvest body weight of YTK fed the highest level of methionine increased by 22% (207.8-228.8 g); weight gain increased by 32% (289.6%- 330.8%), SGR increased by 19% (2.8 % d^{-1}-3.1 % d^{-1}) and FCRs were generally better compared to YTK fed the low methionine diet (Figure 3.1.5.3.1; Table 3.1.5.3.5).

Despite the relatively poorer performance of YTK fed the taurine / methionine limited diets, there was no evidence of green liver syndrome in YTK from any of the dietary treatments.

TauMet experiment - morphometric indices

Morphometric indices from the TauMet trial are presented in Table 3.1.5.3.6. The effect of dietary taurine level on VSI and muscle ratio of YTK was dependent on the level of dietary methionine. The VSI ranged from 5.3 to 6.6%. The muscle ratio ranged from 32.9 to 37.5%. Condition factor *K* increased with increasing level of taurine in the high methionine series, with *K* values ranging from 1.3 to 1.5. The HSI in YTK was not influenced by taurine level or methionine level and ranged from only 0.8% to 0.9%. The intraperitoneal fat ratio was significantly influenced by the level of methionine, but not by the level of taurine and ranged from 0.3% to 0.6%.

TauMet experiment - whole carcass compositional analyses

Whole carcass compositional analyses are presented in Table 3.1.5.3.7. Protein and amino acid content remained reasonably consistent among YTK fed different dietary treatments. There were no significant interactions detected among treatments when considering each of the compositional parameters (Table 3.1.5.3.7).

TauMet experiment - regression analyses

Linear and non-linear (Koops and Grossman, 1993) regression models were applied to describe the relationships of SGR or FCR to incremental increases in taurine (content and intake) for each methionine series (Figure 3.1.5.3.1). Different models for each curve were applied to find the model with the best fit (Table 3.1.5.3.8). The applied models indicated that the most suitable taurine level for optimized growth and FCR in juvenile YTK was between 1.71 and 1.98g digestible taurine kgBW^{-1} d^{-1} when the average methionine content of the diet is 10.9 g kg^{-1} diet. Juvenile YTK were unresponsive to taurine intake in the diet series supplemented with 17.2 g methionine kg^{-1} diet, indicating that taurine intake was not influencing the growth and FCR of these fish.

MetCys experiment - growth and feeding performance

Fish growth and feeding responses from the MetCys study are presented in Table 3.1.5.3.9. There were significant interactions ($P < 0.05$) between the level of dietary methionine and the level of dietary cysteine with respect to growth and FCR of YTK. Harvest body weight, weight gain and SGR increased significantly ($P < 0.05$) with increasing methionine level within the low cysteine series. The SGR response peaked around 3.4 to 3.6% d^{-1}. Growth and feeding efficiency decreased significantly ($P < 0.05$) when dietary methionine level exceeded 18.01- 18.65 g kg^{-1} in the low methionine series (Figure 3.1.5.3.2). No significant differences were observed in feed intake among the dietary treatments. FCR ranged from 0.9-1.1 across all dietary treatments (Figure 3.1.5.3.2; Table 3.1.5.3.9).

Approximately 82.5% of YTK fed the diet providing the lowest content of methionine and cysteine, respectively (i.e MetCys Diet 1), had opaque eyes after eight weeks; both eyes were affected. No other YTK in this experiment exhibited this condition (Table 3.1.5.3.9).

MetCys experiment – morphometric indices

Results on morphometric indices from the MetCys trial indicate no significant interaction among any of the variables (Table 3.1.5.3.10). The condition factor *K* ranged from 0.90 to 2.64. The HSI ranged between 0.72% to 0.85% and the VSI ranged from 5.29% to 7.44%.

MetCys experiment – whole carcass compositional analyses

Whole carcass compositional analyses are presented in Table 3.1.5.3.11. Protein and amino acid content remained reasonably consistent among YTK fed different dietary treatments. There were no significant interactions ($P > 0.05$) detected among treatments when considering each compositional parameter (Table 3.1.5.3.11).

MetCys experiment – regression analyses

A second order polynomial regression was used to describe the relationship of SGR or FCR to incremental increases in methionine (content and digestible intake) for each cysteine series (Figure 3.1.5.3.2; Table 3.1.5.3.8). The applied models indicated that growth and feeding efficiency was optimized with a digestible methionine intake of 6.75 and 6.36 g kg BW^{-1} day^{-1} at an average digestible cysteine intake of 1.73 g kg BW^{-1} day^{-1}. This equates to a dietary methionine content of 18.65 and 18.01 g kg^{-1} diet at an average dietary cysteine level of 5.56 g kg^{-1} diet. Furthermore, a reduction in SGR and a worsening in FCR was observed in YTK fed diets where digestible methionine intake in fish exceeded these values indicating juvenile YTK may have an upper tolerable limit with respect to digestible intake of total sulphur amino acids.

YTK fed the high cysteine diet series recorded high variability in both SGR and FCR. No specific requirement for methionine could be established in this series of diets. The juvenile YTK fed the high cysteine series of diets generally performed poorly relative to YTK fed the low cysteine series of diets, however, this difference was not statistically significant (Figure 3.1.5.3.2; Table 3.1.5.3.9).

Discussion

TauMet experiment

This study has determined the taurine requirement of juvenile YTK and has revealed that dietary methionine can spare taurine when taurine content of the diet is relatively low. The results demonstrate that taurine and methionine have irreplaceable functions in the metabolism and health of YTK. Nevertheless, taurine can also be spared through the *de novo* synthesis of the precursor. There is no benefit of supplementing taurine into diets for YTK when methionine intake is adequate. However, if the residual dietary methionine content of a formulation approaches 10.9 g kg^{-1} diet, then taurine should be supplemented at a rate of ≥ 7.7 g taurine kg^{-1} diet.

The taurine requirement in juvenile Californian yellowtail (*S. lalandi*) was estimated by Salze *et al.* (2017) to be between 2.6 to 10.2 g kg^{-1} diet depending on the response variable or regression model they applied. The main protein sources used in their experimental diets were poultry meal and soy bean meal and, importantly, their diets contained 10.5 g methionine kg^{-1} and 6.3 g cysteine kg^{-1}, respectively. These results generally support the findings of the present study; taurine supplementation is required when dietary specification of methionine is approximately 10 g kg^{-1} in diets for YTK. Taurine is derived from the metabolic pathways of methionine and cysteine and this process is enzyme facilitated. If the respective organism has the enzyme suite available by which to synthesize taurine *de novo*, then dietary methionine can completely spare taurine as precursor (Li et al., 2009).

Sparing effects of sulphur amino acids on taurine requirements have also been demonstrated in other finfish species. Ferreira et al. (2015) demonstrated that supplementary taurine does not need to be supplied in diets for rock bream when TSAA levels are at 27 g kg^{-1} diet and they suggested that rock bream may have some capacity to biosynthesise taurine, but not at a rate that can meet requirements. It is not clear if YTK have a capacity for *de novo* synthesis of taurine. The rate of endogenously synthesized taurine in fish differs among species and depends on two controlling enzymes; cysteine dioxygenase (CDO) and cysteinesulfinate decarboxylase (CSD) (Yokoyama et al., 2001; Gaylord et al., 2007). Because of recent developments in the understanding of SAA metabolism, taurine's conventional definition as nonessential amino acid has been challenged and revaluated to be considered conditionally essential for many fish species (Takagi et al., 2008; Li et al., 2009; Salze et al., 2011).

In comparison to high quality fishmeal, taurine is low or absent in many alternative protein sources, especially in plant derived proteins sources such as soybean. Taurine is therefore routinely supplemented into aquafeeds. The current industry practice of supplying a minimum of about 11.0 g methionine kg^{-1} diet for Australian YTK is loosely based on the study of Ruchimat et al. (1997) who described the methionine requirement of Japanese Yellowtail to be 11.1 g methionine kg^{-1} diet which was quantified using break point analyses. Interestingly, if non-linear regression analyses (quadratic) is applied to the data of Ruchimat et al. (1997) a requirement value of 12.9 g kg^{-1} diet is obtained. Further, it is important to note that Ruchimat et al. (1997) qualifies this value in the presence of 3.1 g cysteine kg^{-1} diet i.e. a minimum TSAA specification of 14.2 g kg^{-1} diet. The TSAA contents of the high methionine series of diets in the TauMet study were on average 22.7 g kg^{-1} diet. It would therefore be pertinent to conduct follow up studies to quantify the cysteine requirement of YTK.

MetCys experiment

The MetCys experiment successfully determined the methionine requirement of juvenile YTK and revealed that dietary cysteine can spare a proportion of the TSAA requirement in YTK reared at or near their optimal water temperature. Additionally, the upper threshold for methionine was determined based on declines in SGR and FCR.

The methionine requirement of juvenile YTK is met when the digestible methionine intake reaches 6.36-6.75 g kg BW^{-1} d^{-1} at an average digestible cysteine intake of 1.73 g kg BW^{-1} d^{-1}. This equated to a relative methionine requirement of 18.01-18.65 g kg^{-1} diet at an average cysteine content of 5.56 g kg^{-1} diet; i.e. a TSAA (methionine+cysteine) content of 23.57-24.21g kg^{-1} diet. This is similar to that reported for Barramundi at 17.1-20.2 g kg^{-1} (Poppi et al., 2017). Other aquaculture finfish species appear to have a relatively lower TSAA requirement than juvenile YTK. The TSAA requirement (methionine+cysteine) in Red Drum (*Sciaenops ocellatus*) is 10.6 g kg^{-1} diet, which is similar to the TSAA requirement of 10

g kg^{-1} dry diet in juvenile Hybrid Striped Bass (*Morone chrysops* × *M. saxatilis*) (Moon and Gatlin, 1991; Keembiyehetty and Gatlin, 1993).

The methionine requirement was met at an SGR of about 3.4% d^{-1} and FCR of 0.9, followed by a deterioration in SGR and FCR when the digestible methionine intake or content exceeded the requirement. Interestingly, the level of cysteine and methionine in whole carcasses at higher SAA intake stayed the same, indicating that other substrates might be responsible for the compromised growth and feed efficiency. A declining growth rate at supra-optimal methionine level has been demonstrated in several vertebrates and is possibly induced through the build-up of S-adenosyl methionine (Regina et al., 1993; National Research Council, 2011). In Rohu (*Labeo rohita*), a methionine content above 12 g kg^{-1} diet caused significant growth depression (Abidi and Khan, 2011). Excess L-cysteine led to a mortality rate of 50% in chicks and severely depressed growth in rats and pigs (Dilger et al., 2007). The effect of excess cysteine has not been studied well in YTK and requires further investigations. Methionine and cysteine are both sulphur amino acids and interrelated, therefore, toxicity might be related to a substance both amino acids share.

The biosynthesis from methionine to cysteine is at a certain stage a "metabolic one-way street" in all animals. Consequently, only the methionine proportion designated for the *de novo* synthesis of cysteine can be spared by dietary cysteine intake. In juvenile Nile Tilapia, cysteine can spare 49% of the requirement for methionine (Nguyen and Davis, 2009). Similarly, cysteine can spare approximately 40% of dietary methionine in Red Drum (Moon and Gatlin, 1991), 51% of the methionine requirement in juvenile Yellow Perch (*Perca flavescens*) (Twibell et al., 2000) and 33-39% of the methionine requirement in fingerling Rohu (Abidi and Khan, 2011). In Channel Catfish (*Ictalurus punctatus*) cysteine can spare up to 60% of the methionine requirement (Harding et al., 1977). The present study has found that cysteine can spare at least 48.2- 49.6% of the methionine requirement in juvenile YTK using SGR and FCR as response variables.

A great proportion of juvenile YTK fed Diet 1 in the MetCys experiment were afflicted with opaque or cloudy eye syndrome, visible signs that might be indicative of bilateral cataracts. Similar symptoms have been recorded in Rainbow Trout, in which 95% of fish developed bilateral cataract when fed methionine deficient diets (Cowey et al., 1992). However, fish from this study fed diets deficient in methionine did not show any cataract if diets contained high amounts of cysteine. This suggests that the methionine proportion synthesized to cysteine is responsible preventing cataract and maintaining eye health. Poston et al. (1978) states that the sulfhydryl group of methionine, rather than the methyl group of methionine, is most important in preventing cataracts. Our results generally support this concept, that the sulphur atom of methionine is passed on to cysteine, while the methyl group of methionine is transferred to S-adenosyl methionine several steps before the synthesis of cysteine.

Conclusions and Recommendations

Results from the TauMet and MetCys studies indicate that the recommended levels of taurine, cysteine and methionine in aquafeeds for YTK need to be reassessed. Based on the combined results of each study we recommend an optimal level of 18.65 g methionine kg^{-1} diet when the cysteine content of diets is approximately 5.6 g kg^{-1} diet, crude basis. This value exceeds that of current industry practice at ≈ 11 g methionine kg^{-1} diet for YTK. If diets contain approximately 11.0g methionine kg^{-1} diet we recommend a minimum of 7.7 g taurine kg^{-1} diet, crude basis, in order to optimize growth. The choice of mathematical model applied to dose response data can significantly influence requirement values. Cysteine can spare methionine up to at least 48.2%; however, high levels of cysteine may depress growth. Further investigation into the interactive relationships among the sulphur containing amino acids and their impact on overall requirements is therefore required. These recommendations are relevant for the size and culture conditions undertaken in this study. We further recommend investigation of the impacts of ontogenetic and abiotic factors on TSAA requirements in YTK.

Findings

- Methionine can spare taurine in diets for YTK.

- Cysteine can spare a proportion of the total sulfur amino acid requirement.
- No dietary taurine supplementation is required if enough dietary methionine is provided.
- Juvenile YTK require a digestible taurine intake of 1.71 g kg BW^{-1} d^{-1} at an average methionine intake of 3.43 g kgBW^{-1} d^{-1} to optimise growth.
- YTK require a digestible methionine intake of 6.4-6.8 g kg BW^{-1} d^{-1} at an average digestible cysteine intake of 1.7 g kg BW^{-1} d^{-1} (alternatively; 13.9 g methionine kg^{-1} diet at an average cysteine content of 5.6 g kg^{-1} diet).
- Exceeding a digestible methionine intake of 6.8 g kg BW^{-1} d^{-1} at a digestible cysteine intake of 1.2-2.0 g cysteine kg^{-1} (alternatively; 18.65 g methionine kg^{-1} diet at a cysteine content of 5.0-6.8 g kg^{-1} diet) may depress growth rate in YTK.
- No green liver symptoms were observed in YTK exposed to low dietary contents of taurine and methionine. YTK exposed to severe dietary methionine deficiencies showed signs of cataract, but not green liver.
- The current industry dietary methionine specification for YTK (≈ 11 g kg^{-1}) may not be sufficient to meet the TSAA requirement in juvenile YTK.
- Methionine can spare the need for supplementary taurine.
- YTK have an upper methionine threshold which, if exceeded, may lead to growth depression.
- Cysteine can spare methionine requirement to approximately 50.7%.

Publications

Candebat, C., Booth, M., Codabaccus, B.M., Pirozzi, I., n.d. Methionine requirement and the sparing effect of cysteine in juvenile Yellowtail Kingfish (*Seriola lalandi*). Aquaculture (in preparation).

Acknowledgements

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, New South Wales Department of Primary Industries (NSW DPI) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

This research was also supported by the James Cook University Postgraduate Research Scholarship. Some of the raw materials used in this experiment were kindly provided by Ridley Aquafeed. The authors would like to thank Luke Vandenberg, Brendan Findlay, Ian Russel, Justin Tierney, Dylan Nash, Markus Armstrong, Angela Liu and Steve Gamble for their technical assistance.

References

Abidi, S.F., Khan, M.A., 2011. Total sulphur amino acid requirement and cystine replacement value for fingerling rohu, *Labeo rohita*: Effects on growth, nutrient retention and body composition. Aquac. Nutr. 17, 583–594.

ACEC, 2017. A guide to acceptable procedures and practices for aquaculture and fisheries research, 4th Edition, NSW DPI Animal Care and Ethics Committee.

AOAC, 2005. Official Methods of Ananlysis. AOAC International.

Ball, R., Courtney-Martin, G., Pencharz, P., 2006. The *in vivo* sparing of methionine by cysteine in sulfur amino acid requirements in animal models and adult humans. J. Nutr. 136, 1682S–1693S.

Bellentani, S., Pecorari, M., Cordoma, P., Marchegiano, P., Manenti, F., Bosisio, E., De Fabiani, E., Galli, G., 1987. Taurine increases bile acid pool size and reduces bile saturation index in the hamster. J. Lipid Res. 28, 1021–1027.

Bertolo, R.F., McBreairty, L.E., 2013. The nutritional burden of methylation reactions. Curr. Opin. Clin. Nutr. Metab. Care 16, 102–108.

Booth, M.A., Allan, G.L., Pirozzi, I., 2010. Estimation of digestible protein and energy requirements of yellowtail king fi sh Seriola lalandi using a factorial approach. Aquaculture 307, 247–259.

Brosnan, J.T., Brosnan, M.E., 2006. The sulfur-containing amino acids: An overview. Am. Soc. Nutr. J. 136, 1–11.

Cho, C.Y., Slinger, S.., Bayley, H.S., 1982. Bionenergetics of salmonid fishes : Energy intake, expenditure and productivity, comp.Biochem.Physiol.,. Comp. Biochem. Physiol. 73, 25–41. https://doi.org/10.1146/annurev.genom.5.061903.180008

Cowey, C.B., Cho, C.Y., Sivak, J.G., Weerheim, J.A., Stuart, D.D., 1992. Methionine intake in rainbow trout (*Oncorhynchus mykiss*), relationship to cataract formation and the metabolism of methionine. J. Nutr. 122, 1154–1163.

Dilger, R.N., Toue, S., Kimura, T., Sakai, R., Baker, D.H., 2007. Excess dietary L-cysteine, but not L-cystine, is lethal for chicks but not for rats or pigs. J. Nutr. 137, 331–8. https://doi.org/137/2/331 [pii]

Ferreira, F.M., Yun, H., Park, Y., Lee, S., Park, G., Bai, S.C., 2015. Dietary sulfur amino acids can spare taurine in rock bream Oplegnathus fasciatu. Fish. Aquat. Sci. 18, 249–255.

Folch, J., Lees, M., Sloane, G.H., 1953. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. https://doi.org/10.1016/j.ultrasmedbio.2011.03.005

Gaylord, T.G., Barrows, F.T., Teague, A.M., Johansen, K.A., Overturf, K.E., Shepherd, B., 2007. Supplementation of taurine and methionine to all-plant protein diets for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 269, 514–524.

Halver, J.E., 2002. Amino acids and proteins, in: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition. Academic Press-An Elsevier Science Imprint, p. 163.

Harding, D.E., Allen, O.W., Wilson, R.P., 1977. Sulfur amino acid requirement of channel catfish: L-methionine and L-cystine. J. Nutr. 107, 2031–2035.

Keembiyehetty, C.N., Gatlin, D.M., 1993. Total sulfur amino acid requirement of juvenile hybrid striped bass (*Morone chrysops*×*M. saxatilis*). Aquaculture 110, 331–339. https://doi.org/10.1016/0044-8486(93)90380-H

Kim, S.K., Kim, K.G., Kim, K.D., Kim, K.W., Son, M.H., Rust, M., Johnson, R., 2015. Effect of dietary taurine levels on the conjugated bile acid composition and growth of juvenile Korean rockfish *Sebastes schlegeli*. Aquac. Res. 46, 2768–2775.

Koops, W.J., Grossman, M., 1993. Multiphasic allometry. Growth. Dev. Aging 57, 183–92.

Li, P., Mai, K., Trushenski, J., Wu, G., 2009. New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. Amino Acids 37, 43–53.

Li, P., Yin, Y.-L., Li, D., Woo Kim, S., Wu, G., 2007. Amino acids and immune function. Br. J. Nutr. 98, 237.

Moon, H.Y., Gatlin, D.M., 1991. Total sulfur amino acid requirement of juvenile red drum, *Sciaenops ocellatus*. Aquaculture 95, 97–106.

National Research Council, 2011. Proteins and amino acids, in: Nutrient Requirements of Fish and Shrimp. National Academies Press, Washington, DC, p. 392. https://doi.org/https://doi.org/10.17226/13039

Nguyen, T.N., Davis, D.A., 2009. Re-evaluation of total sulphur amino acid requirement and determination of replacement value of cystine for methionine in semi-purified diets of juvenile Nile tilapia, *Oreochromis niloticus*. Aquac. Nutr. 15, 247–253.

Poppi, D.A., Moore, S.S., Glencross, B.D., 2017. Redefining the requirement for total sulfur amino acids

in the diet of barramundi (*Lates calcarifer*) including assessment of the cystine replacement value. Aquaculture 471, 213–222.

Poston, H.A., Rumsey, G.L., Ronald, N.Y., Riis, C., Poston, H.A., Riis, R.C., Rumsey, G.L., Ketola, H.G., 1978. Nutritionally Induced Cataracts in Salmonids Fed Purified and Practical Diets Environmental Parameters Affecting Fish Physiology in Water Reuse Systems. Mar. Fish. Rev. 40, 45–46.

Regina, M., Korhonen, V.., Smith, T.., Alakuijala, L., Eloranta, T.., 1993. Methionine toxicity in the rat in relation to hepatic accumulation of S-adenosylmethionine: Prevention by dietary stimulation of the hepatic transsulfuration pathway. Arch. Biochem. Biophys. 300, 598–607.

Ruchimat, T., Masumoto, T., Hosokawa, H., Shimeno, S., 1997. Quantitative methionine requirement of yellowtail (*Seriola quinqueradiata*). Aquaculture 150, 113–122.

Salze, G., Craig, S.R., Smith, B.H., Smith, E.P., McLean, E., 2011. Morphological development of larval cobia *Rachycentron canadum* and the influence of dietary taurine supplementation. J. Fish Biol. 78, 1470–1491.

Salze, G.P., Stuart, K.R., Jirsa, D.O., Davis, D.A., Drawbridge, M.A., 2017. Quantitative dietary taurine requirement for California yellowtail, *Seriola lalandi*. J. World Aquac. Soc. 49, 113–126.

Takagi, S., Murata, H., Goto, T., Endo, M., Yamashita, H., Ukawa, M., 2008. Taurine is an essential nutrient for yellowtail *Seriola quinqueradiata* fed non-fish meal diets based on soy protein concentrate. Aquaculture 280, 198–205.

Takagi, S., Murata, H., Goto, T., Hatate, H., Endo, M., Yamashita, H., Miyatake, H., Ukawa, M., 2010. Necessity of dietary taurine supplementation for preventing green liver symptom and improving growth performance in yearling red sea bream *Pagrus major* fed nonfishmeal diets based on soy protein concentrate. Fish. Sci. 76, 119–130.

Twibell, R.G., Wilson, K.A., Brown, P.B., 2000. Dietary sulfur amino acid requirement of juvenile yellow perch fed the maximum cystine replacement value for methionine. J. Nutr. 130, 612–616.

Wijayasinghe, Y.S., Tyagi, A., Poddar, N.K., 2017. Regulation of cell volume by osmolytes, in: Rajendrakumar Singh, L., Dar, T.A. (Eds.), Cellular Osmolytes: From Chaperoning Protein Folding to Clinical Perspectives. Springer Singapore, Singapore, pp. 195–228.

Wu, G., 2009. Amino acids: metabolism, functions, and nutrition. Amino Acids 37, 1–17.

Wu, J.-Y., Prentice, H., 2010. Role of taurine in the central nervous system. J. Biomed. Sci. 17, 1–6.

Yancey, P.H., 2005. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J. Exp. Biol. 208, 2819–2830.

Yokoyama, M., Takeuchi, T., Park, G.S., Nakazoe, J., 2001. Hepatic cysteinesulphinate decarboxylase activity in fish. Aquac. Res. 32, 216–220.

Table 3.1.5.3.1. TauMet experiment. Diet formulation for the taurine requirement experiment.

	Low methionine series							**High methionine series**						
Raw material (% DM)	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**	**Diet 7**	**Diet 8**	**Diet 9**	**Diet 10**	**Diet 11**	**Diet 12**	**Diet 13**	**Diet 14**
Blood meal	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Choline chloride (70%)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Corn gluten	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Dehulled lupin	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Diatomaceous earth	1.7	3.1	3.56	3.23	2.91	2.59	2.27	3.29	2.97	2.65	2.32	2	1.68	1.36
Fish oil	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0
Fishmeal Prime	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Fishmeal Low Tau	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Meat meal	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Methionine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	1.11	1.11	1.11	1.11	1.11	1.11	1.11
Poultry meal	12	12	12	12	12	12	12	12	12	12	12	12	12	12
Rovimix Stay-C (35)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Taurine	0.0	0.3	0.64	0.97	1.29	1.61	1.93	0	0.32	0.64	0.97	1.29	1.61	1.93
Vit-min premix	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Wheat flour	17.9	16	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4
NaH_2PO_4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Y_2O_3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Table 3.1.5.3.2. TauMet experiment. Measured proximate, amino acid compositions and apparent digestibility (Mean ± SE; *n* = 2) of experimental diets used in the taurine requirement experiment.

	Low methionine series							High methionine series						
Parameters (dry basis)	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**	**Diet 7**	**Diet 8**	**Diet 9**	**Diet 10**	**Diet 11**	**Diet 12**	**Diet 13**	**Diet 14**
Proximate values (g kg^{-1})														
Ash	117.0	115.8	119.3	118.6	117.4	116.2	115.4	116.1	116.6	117.2	114.9	113.5	111.0	110.0
Total lipid	144.5	149.0	158.4	154.1	152.8	151.5	157.7	178.3	161.3	155.8	160.6	139.9	144.6	154.3
Total nitrogen	77.7	79.1	79.7	77.7	80.1	78.2	79.2	77.2	79.1	78.0	78.9	80.5	78.1	77.5
Total protein	485.9	494.3	498.3	485.4	500.4	488.7	495.1	482.7	494.5	487.5	493.1	503.1	488.4	484.4
Gross energy (MJ kg^{-1})	21.9	22.0	22.2	22.2	22.1	21.9	22.9	21.8	22.0	22.1	22.3	22.2	22.2	22.7
NFE by diff.	252.5	240.9	223.9	241.9	229.5	243.5	231.8	222.9	227.6	239.5	231.4	243.5	256.0	251.4
Amino acid content (g kg^{-1})														
Alanine	30.0	29.3	29.3	28.0	28.3	28.9	28.0	29.4	28.3	29.0	27.6	27.9	28.6	30.2
Arginine	56.5	54.0	56.4	52.2	51.9	52.2	51.3	53.0	52.1	52.7	51.8	50.6	51.7	53.9
Aspartic acid +Asparagine	43.4	42.1	43.2	40.6	40.2	40.8	40.1	41.6	40.6	40.6	39.0	38.8	40.1	40.3
Cystine	6.8	6.1	5.6	5.4	5.6	5.8	5.7	5.9	5.4	5.5	5.2	5.6	5.6	5.8
Glutamic acid + glutamine	70.5	67.1	68.0	64.5	62.6	64.0	62.4	64.2	62.4	61.5	60.0	59.7	61.4	61.9
Glycine	27.6	27.2	27.1	25.7	26.4	26.2	26.4	30.1	26.1	27.8	26.0	25.6	26.7	28.4
Histidine	14.6	18.2	15.6	14.3	16.1	13.8	15.0	14.1	13.6	13.2	14.0	12.5	13.9	12.2
Isoleucine	17.0	16.1	16.5	15.9	16.2	16.5	16.4	16.4	16.2	16.3	15.8	15.6	16.0	16.5
Leucine	42.9	41.4	42.2	40.9	41.4	42.7	42.1	42.3	41.0	41.8	40.3	40.0	41.1	42.1
Lysine	29.2	26.6	30.2	27.0	26.6	26.0	27.6	26.9	27.0	24.2	25.4	23.3	23.6	23.4
Methionine	12.0	10.5	11.1	10.8	10.9	10.1	11.1	16.7	19.3	16.4	18.8	16.0	17.9	15.0
Phenylalanine	23.8	23.4	23.3	23.2	23.5	24.2	24.0	24.6	23.6	24.7	23.0	23.0	23.8	24.2
Proline	25.9	25.2	24.9	23.9	24.0	24.5	24.4	25.4	24.1	24.0	23.5	23.2	24.1	24.3
Serine	25.0	25.2	25.1	22.9	23.9	22.8	23.1	23.4	22.6	25.5	23.2	22.5	23.5	28.4
Taurine	1.6	4.8	8.5	11.9	15.0	17.3	20.4	1.6	5.1	8.1	11.7	13.9	18.3	20.0
Threonine	20.7	20.0	20.6	19.4	19.4	19.5	19.0	19.9	19.3	19.9	18.7	18.7	19.2	20.4
Tyrosine	14.5	15.1	16.1	14.4	15.8	15.0	14.5	14.3	14.9	15.2	14.8	14.5	15.0	15.2
Valine	26.6	25.8	26.3	25.5	26.0	26.8	26.3	26.8	25.9	26.8	26.1	25.9	26.6	27.3
SUM AA	488.6	477.9	490.0	466.8	473.9	477.1	477.7	476.6	467.4	473.2	464.8	457.3	477.1	489.6
Apparent digestibility (%)														
Crude protein	82.3±0.4	NA	NA	80.0±5.3	NA	NA	80.0±1.5	82.4±0.2	NA	NA	77.7±0.3	NA	NA	80.6±4.5
Crude lipid	92.0±1.0	NA	NA	92.2±1.9	NA	NA	90.5±1.3	93.0±0.3	NA	NA	90.2±0.8	NA	NA	92.2±2.1
Gross energy	70.3±4.1	NA	NA	67.6±8.3	NA	NA	65.4±2.6	71.9±0.0	NA	NA	67.1±0.3	NA	NA	66.9±3.2
Taurine	51.9±3.9	NA	NA	84.0±5.2	NA	NA	90.2±1.4	40.8±6.1	NA	NA	84.3±0.3	NA	NA	89.9±2.5
Methionine	77.8±1.8	NA	NA	76.0±11.1	NA	NA	81.5±1.2	91.0±1.9	NA	NA	90.3±0.2	NA	NA	90.9±1.4
Cysteine	59.9±1.4	NA	NA	47.8±17.9	NA	NA	41.1±1.5	57.4±0.4	NA	NA	40.2±1.9	NA	NA	47.5±13.9

NA; Not Assessed.

Table 3.1.5.3.3. MetCys Experiment. Diet formulation for the methionine requirement experiment.

Raw Materials	Low cysteine series					High cysteine series				
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**	**Diet 7**	**Diet 8**	**Diet 9**	**Diet 10**
Blood Meal	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Choline chloride (70%)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Dehulled lupins	19.9	19.9	19.9	19.9	19.9	19.9	19.9	19.9	19.9	19.9
Diatomaceous earth	5.2	5.1	5.1	5.1	5.1	4.4	4.5	4.7	4.9	4.5
Feather meal	2.9	2.9	2.9	2.9	2.9	3	3	3	3	3.2
Fish oil	10	10	10	10	10	10	10	10	10	10
Fish meal	12.6	13	13	13	13	13	13	13	13	13
Gelatin	14.3	13.5	12.7	12.7	12.7	13.5	12.4	12.4	12.4	11.9
Lysine	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Maize starch	2.5	2.6	2.4	1.9	1.3	2.6	2.7	2	1.2	1.3
NaH_2PO_4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Rovimix Stay-C	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Sodium caseinate	9.8	10.10	10.10	10.10	10.1	9.8	10.1	10.1	10.1	10.1
Soy Protein Concentrate	15.2	14.7	15.2	15.2	15.2	15.1	15.2	15.2	15.2	15.2
Vit-min premix	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Taurine	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Cysteine	0	0	0	0	0	1.11	1.11	1.11	1.11	1.11
Methionine	0	0.53	1.06	1.59	2.14	0	0.53	1.06	1.59	2.15
Y_2O_3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Table 3.1.5.3.4. MetCys experiment. Measured proximate, amino acid compositions and apparent digestibility (Mean ± SE; *n* = 3; dry matter basis) of experimental diets used in the methionine requirement experiment.

Parameter	Low cysteine series					High cysteine series				
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10
Proximate value (g kg^{-1})										
Ash	107.3	106.2	106.8	106.9	108.2	100.0	99.8	103.0	104.5	104.3
Total lipid	135.8	119.2	134.0	135.6	135.2	130.2	128.9	130.7	128.6	138.1
Total nitrogen	102.2	97.1	93.2	101.9	99.1	102.1	94.6	96.3	107.9	104.2
Total protein	638.8	607.2	582.6	636.6	619.5	637.9	591.5	601.7	674.4	651.1
Gross energy (MJ kg^{-1})	21.78	22.09	22.13	22.19	22.16	22.38	22.13	22.36	22.19	22.37
Amino acid (g kg^{-1})										
Alanine	30.6	29.7	29.2	29.7	28.7	29	30	29.3	29.6	28
Arginine	39	38.5	37.3	38.7	37.4	37.5	38.9	38.3	38.2	36.6
Aspartic acid	44.7	44	41.7	44	42.2	42.1	43.2	43.5	43.1	41.4
Cysteine	5.8	5.6	5	5.4	5.9	13.7	14.1	14.4	13.5	13.9
Glutamic acid	84.4	83.9	79.4	83.9	80.6	80.3	82.4	82.7	82.1	79
Glycine	53.1	50.2	51.5	50	48.2	50.2	52.2	48.9	51.1	47.2
Histidine	13.1	13.3	12.5	13.4	13.1	12.6	13.2	13.3	12.9	12.6
Hydroxyproline	18.5	16.9	18.3	16.8	16	17.3	18.1	16.4	17.8	15.7
Isoleucine	21.8	22.1	20.7	22.3	21.8	21	21.8	21.8	21.4	21.1
Leucine	39.8	40.4	37.8	40.5	39.5	38.4	39.8	40	39.2	38.3
Lysine	33.9	33.8	32.1	34	32.9	32.6	33.4	33.9	33.4	32.2
Methionine	8.8	12.7	16.5	22.4	24.7	7.9	12.7	17.6	21.2	25.2
Phenylalanine	23	23.2	21.8	23.4	22.8	22.2	23	23.1	22.6	22.1
Proline	45.8	44.7	44	44.6	43.1	43.8	45.4	43.8	44.6	42.1
Serine	26.9	27.1	25.5	27	26	26.2	26.8	26.9	26.6	25.6
Taurine	7	7	6.9	7.1	6.9	7	7.3	7.3	7.2	6.9
Threonine	20.2	20.4	19.2	20.5	19.8	19.6	20.2	20.3	19.9	19.3
Tryptophan	4.9	4.9	4.5	4.9	4.7	5.1	4.8	5.1	4.8	4.4
Tyrosine	15.2	15.3	14.3	15.5	15	14.5	15.2	15.5	15.2	14.7
Valine	26.4	26.8	25.1	26.9	26.3	25.4	26.5	26.4	25.9	25.5
SUM AA	562.9	560.5	543.3	571	555.6	546.4	569	568.5	570.3	551.8
Apparent digestibility (%)										
Crude Protein	74.83±1.84	NA	75.21±0.72	NA	76.17±1.75	74.47±0.52	NA	77.95±0.98	NA	75.13±3.93
Crude Lipid	77.70±1.41	NA	83.31±0.75	NA	81.5±2.12	80.2±0.88	NA	84.02±0.74	NA	80.79±1.62
Gross Energy	62.36±1.81	NA	68.56±0.23	NA	67.06±1.79	63.45±0.90	NA	70.18±1.58	NA	65.49±2.78
Taurine	50.88±4.07	NA	46.22±1.72	NA	37.22±0.44	51.57±4.70	NA	42.64±4.24	NA	49.59±5.56
Methionine	78.46±1.89	NA	84.60±1.12	NA	86.18±0.45	77.75±0.74	NA	87.69±0.86	NA	82.39±5.31
Cysteine	36.87±4.07	NA	45.98±1.27	NA	52.5±3.07	74.02±0.73	NA	77.49±0.26	NA	72.84±4.26

NA; Not Assessed.

Table 3.1.5.3.5. TauMet experiment. Growth and feed performance of juvenile Yellowtail Kingfish (Mean ± SE; *n* = 3) fed practical diets containing seven graded levels of taurine and two graded levels of methionine for 7 weeks. Different superscript letters indicate significant differences within row.

	Low Methionine Diets							**High Methionine Diets**							
Parameter*	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**	**Diet 7**	**Diet 8**	**Diet 9**	**Diet 10**	**Diet 11**	**Diet 12**	**Diet 13**	**Diet 14**	Tau × Met
IBW (g)	53.5±0.7	53.4±0.6	53.0±0.7	54.0±0.6	52.5±0.9	53.1±0.7	53.1±0.6	53.3±0.6	53.3±0.6	53.2±0.8	53.7±0.6	53.0±0.7	53.0±0.8	53.5±0.7	NS
HBW (g)	134.8±3.0^{a}	161.7±4.5^{b}	196.2±5.1^{cde}	187.9±4.5^{cd}	187.9±6.9^{cd}	191.1±.3^{cde}	179.7±6.2^{bc}	207.8±4.5^{def}	228.8±5.6^{f}	212.5±6.3^{def}	211.2±5.1^{def}	205.4±5.4^{def}	214.5±5.2^{ef}	230.3±5.9^{f}	<0.05
FI (g fish^{-1} day^{-1})	3.41±0.07^{a}	3.83±0.12^{ab}	4.56±0.16^{bcd}	4.45±0.19^{bcd}	4.54±0.10^{bcd}	4.44±0.04^{bcd}	4.20±0.28^{abc}	4.95±0.08^{cd}	5.00±0.10^{cd}	5.21±0.15^{d}	4.92±0.02^{cd}	5.07±0.09^{cd}	4.74±0.18^{cd}	5.12±0.13^{d}	<0.05
WG (%)	151.8±1.2^{a}	202.8±7.8^{ab}	269.9±3.8^{bcd}	247.8±11.6^{bcd}	260.7±8.6^{bcd}	259.8±8.4^{bcd}	237.5±31.9^{abc}	289.6±9.0^{cd}	329.3±19.0^{d}	299.8±17.1^{cd}	303.0±6.1^{cd}	292.5±22.0^{cd}	304.3±11.2^{cd}	330.8±9.3^{d}	<0.05
SGR (% d^{-1})	1.92±0.01^{a}	2.31±0.05^{ab}	2.72±0.02^{cde}	2.59±0.07^{bc}	2.62±0.09^{bcd}	2.67±0.05^{bcd}	2.60±0.13^{bc}	2.83±0.05^{cde}	3.03±0.08^{de}	3.09±0.08^{e}	2.85±0.05^{cde}	2.99±0.08^{e}	2.91±0.06^{cde}	3.04±0.05^{de}	<0.05
FCR	2.01±0.05^{a}	1.70±0.02^{b}	1.53±0.03^{bc}	1.60±0.02^{bc}	1.55±0.03^{bc}	1.55±0.10^{bc}	1.57±0.08^{bc}	1.54±0.03^{bc}	1.37±0.05^{c}	1.47±0.04^{bc}	1.42±0.03^{c}	1.44±0.03^{c}	1.41±0.02^{c}	1.39±0.03^{c}	<0.05
Survival (%)	100±0	100±0	100±0	100±0	97.6±2.4	100±0	100±0	100±0	100±0	100±0	97.6±2.4	97.6±2.4	100±0	100±0	NS

*IBW, Initial Body Weight; HBW, Harvest Body Weight; FI, Feed Intake; WG, Weight Gain; SGR, Specific Growth Rate; FCR, Feed Conversion Ratio.

Table 3.1.5.3.6. TauMet experiment. Morphometric indices of juvenile Yellowtail Kingfish (Mean ± SE; *n* = 3) fed practical diets containing respectively seven graded taurine level and two graded methionine level over 7 weeks. Different superscript letters indicate significant differences within row.

	Low Methionine Diets							**High Methionine Diets**							**Tau × Met**
Parameter*	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**	**Diet 7**	**Diet 8**	**Diet 9**	**Diet 10**	**Diet 11**	**Diet 12**	**Diet 13**	**Diet 14**	
HSI (%)	0.80±0.03	NA	0.86±0.02	0.84±0.01	NA	NA	0.80±0.02	0.83±0.02	NA	0.82±0.02	0.77±0.03	NA	NA	0.87±0.03	<0.05
VSI (%)	6.35±0.16^{ab}	NA	5.78±0.09^{bc}	6.08±0.08^{ab}	NA	NA	6.07±0.15^{ab}	6.64±0.11^{a}	NA	5.98±0.15^{abc}	5.33±0.23^{c}	NA	NA	5.80±0.12^{bc}	<0.05
K	1.28±0.02^{a}	NA	1.28±0.02^{a}	1.31±0.02^{a}	NA	NA	1.32±0.01^{a}	1.33±0.03^{a}	NA	1.38±0.01^{ab}	1.50±0.06^{b}	NA	NA	1.37±0.02^{a}	=0.05
MR (%)	32.88±1.09^{ab}	NA	36.49±0.87^{a}	36.35±1.18^{a}	NA	NA	37.49±0.42^{a}	35.35±1.46^{a}	NA	35.11±1.80^{a}	28.59±1.30^{b}	NA	NA	33.87±0.77^{ab}	<0.05
IFR (%)	0.43±0.04^{ab}	NA	0.33±0.04^{a}	0.42±0.05^{ab}	NA	NA	0.37±0.02^{a}	0.59±0.07^{ab}	NA	0.65±0.06^{b}	0.51±0.08^{ab}	NA	NA	0.57±0.04^{ab}	NS

*HSI, Hepatosomatic Index; VSI, Viserasomatic index; K, Condition factor; MR, Muscle ratio; IFR, Intraperitoneal Fat Ratio; NA, Not Assessed; Tau, taurine; Met, methionine.
NA; Not assessed.

Table 3.1.5.3.7. TauMet experiment. Whole carcass and amino acid composition of juvenile Yellowtail Kingfish. (Mean ± SE; *n* = 2 (Diet 6 and Diet 8; *n* = 1)). Different superscript letters indicate significant differences within row.

Parameter (DM basis)	Low Methionine Diets							High Methionine Diets							Tau × Met
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13	Diet 14	
Proximate values (g kg^{-1})															
Ash	152.5±4.3^{a}	139.9±5.0^{ab}	133.2±2.9^{ab}	145.2±4.3^{ab}	141.5±6.2^{ab}	127.97	142.9±1.0^{ab}	129.6	146.3±5.3^{ab}	130.1±2.4^{ab}	130.7±0.2^{ab}	130.1±2.8^{ab}	130.3±2.4^{ab}	118.9±4.4^{b}	NS
Total Lipid	131.5±2.5	144.4±15.0	137.8±4.7	128.9±9.6	127.6±14.1	130.3	123.0±1.4	171.8	137.2±12.7	137.0±5.5	148.6±0.003	151.1±2.5	138.9±4.3	179.7±6.2	NS
Total Nitrogen	117.3±0.3	117.9±1.3	117.9±0.001	120.0±1.8	118.1±1.2	123.6	120.1±0.4	112.9	115.6±3.0	120.1±0.7	116.8±1.4	117.5±1.2	118.7±0.7	114.7±0.4	NS
Total Protein	733.3±2.0	736.9±8.4	736.7±0.1	750.3±11.3	737.8±7.5	772.7	750.6±2.4	705.5	722.8±18.5	750.7±4.1	729.7±9.0	734.6±7.5	741.8±4.3	716.6±2.3	NS
Gross Energy (MJ kg^{-1})	21.8±0.2	22.4±0.4	22.2±0.2	22.1±0.2	22.3±0.7	22.0	21.9±0.1^{a}	23.6	22.5±0.2	22.2±0.1	22.8±0.2	22.7±0.2	22.4±0.02	23.7±0.2	NS
Amino acid (g kg^{-1})															
Alanine	42.5±0.3	42.9±1.9	42.5±1.0	42.2±0.2	43.8±1.0	43.7	41.8±0.1	41.1	40.9±1.1	42.5±0.3	42.3±0.0	41.3±0.6	41.5±0.3	39.5±0.1	NS
Arginine	69.4±1.1	76.1±0.4	73.2±1.5	78.9±1.3	73.7±0.3	74.7	72.3±2.5	69.7	70.5±2.2	77.2±1.0	77.4±0.6	72.9±2.1	75.1±0.04	70.4±1.5	NS
Aspartic acid	64.1±1.4	66.7±1.8	62.3±0.5	64.8±0.9	67.8±0.8	68.7	62.5±0.4	60.1	61.8±2.1	66.0±1.0	65.2±1.6	64.1±0.7	65.10±0.4	62.6±0.7	NS
Cysteine	7.90±0.35	7.55±0.25	7.15±0.11	7.57±0.19	7.55±0.04	8.4	6.90±0.00	6.10	6.50±0.28	7.20±0.28	7.50±0.07	6.80±0.07	7.10±0.435	7.05±0.18	NS
Glutamic acid	92.3±2.4	92.7±2.7	90.3±1.8	91.8±1.0	94.0±0.04	93.8	90.2±0.1	86.4	87.9±0.8	93.1±0.3	90.9±1.	89.1±1.6	93.7±0.7	87.6±0.7	NS
Glycine	47.8±1.4	46.6±2.7	48.1±1.5	47.2±1.3	49.4±2.7	50.5	49.4±0.4	46.9	48.4±1.1	48.7±1.2	45.8±2.4	47.1±0.8	48.6±0.6	43.3±0.8	NS
Histidine	36.8±0.2	42.7±0.1	43.1±1.1	39.7±0.6	38.8±1.6	43.0	39.3±0.9	37.2	40.2±2.2	38.3±1.7	39.7±0.9	39.2±2.2	41.3±0.4	36.0±0.00	NS
Isoleucine	29.6±0.8	29.9±1.2	29.3±0.4	30.8±1.3	31.5±0.7	30.7	28.2±0.3	28.1	27.7±0.6	29.7±1.2	29.5±1.5	29.9±0.6	29.6±0.9	28.7±0.2	NS
Leucine	47.5±1.4	49.5±1.6	47.9±0.1	51.1±2.3	50.1±1.0	47.8	46.1±0.2	44.5	43.6±1.0	47.9±1.5	47.7±1.8	48.6±0.9	46.9±1.0	48.2±0.4	NS
Lysine	50.9±0.4	54.1±0.9	54.9±1.5	56.9±2.3	56.7±2.3	53.7	52.2±0.7	51.2	48.7±1.9	52.4±0.5	57.4±1.6	53.3±0.3	57.5±0.6	49.5±2.0	NS
Methionine	19.8±0.04	20.5±0.1	19.5±0.3	19.7±0.5	20.6±0.2	22.0	19.8±0.5	19.6	19.1±0.2	17.9±0.1	20.1±0.6	19.8±0.4	20.6±0.5	18.4±0.6	NS
Phenylalanine	28.9±0.7	29.6±0.5	28.8±0.04	30.4±1.1	30.8±0.3	29.4	28.7±0.1	28.1	27.3±0.6	29.1±0.9	29.2±1.1	29.3±0.4	28.7±0.6	28.5±0.2	NS
Proline	32.3±0.6	32.8±1.7	33.0±1.0	33.0±0.3	34.6±1.6	34.3	33.6±0.4	32.4	32.7±0.7	33.3±0.1	32.0±0.4	32.8±0.6	33.5±0.5	30.3±0.3	NS
Serine	28.8±0.5	29.0±0.3	28.4±0.0	28.5±0.5	28.9±0.2	29.0	27.6±0.5	27.1	27.7±1.5	28.7±0.9	27.7±0.3	26.8±0.3	27.9±0.4	26.9±0.4	NS
Taurine	4.65±0.39^{a}	9.05±1.73^{ab}	10.60±0.21^{ab}	9.50±1.10^{ab}	11.70±1.56^{ab}	13.10	12.90±0.2^{b}	4.10	9.90±0.64^{ab}	11.05±0.67^{ab}	9.80±1.41^{ab}	11.90±0.42^{ab}	12.20±0.57^{b}	8.65±0.11^{ab}	NS
Threonine	29.9±0.6	31.1±0.1	30.1±0.6	30.9±0.4	31.5±0.6	32.4	30.5±0.4	28.9	29.0±0.9	30.9±0.2	30.6±0.5	29.9±0.5	30.8±0.5	29.2±0.5	NS
Tyrosine	24.0±1.2	24.8±0.3	22.8±0.3	25.0±0.8	24.8±0.5	26.0	23.3±0.4	22.9	22.4±0.3	24.1±0.5	24.5±0.3	23.5±0.5	24.6±0.2	24.0±0.1	NS
Valine	32.6±0.8	33.5±0.7	32.5±0.1	34.0±1.3	34.8±0.7	34.0	31.9±0.2	31.4	31.0±0.2	32.9±1.1	33.7±0.7	33.1±0.0	33.3±0.1	32.9±0.3	NS

Table 3.1.5.3.8. Measurements on the fit of selected models on SGR and FCR of Yellowtail Kingfish to determine dietary requirements of taurine and methionine.

Experiment	Response variable	Model	Predicted requirement	95% Confidence interval	Smoothness of curve	Goodness of fit	
			Digestible taurine intake (g kg BW^{-1} d^{-1})			Absolute Sum of Squares	R^2
TauMet	SGR	Segmental linear regression (second slope = 0)	1.98	1.62-2.62	0	0.31	0.82
		Koops and Grossman	1.60	0.44-NA	0.4	0.33	0.81
			1.98	1.60-NA	0.04	0.31	0.84
			1.98	1.61-2.64	0.004	0.31	0.82
	FCR	Segmental Linear Regression	1.71	1.45-2.35	0	0.14	0.79
		Koops and Grossman	1.71	1.51-1.61	0.05	0.14	0.79
	SGR	Linear Regression	-	-	0	-	0.05
	FCR	Linear Regression	-	-	0	-	0.10
			Digestible methionine intake (g kg BW^{-1} d^{-1})				
MetCys	SGR	Koops and Grossman	3.28	3.15-NA	0.52	0.34	0.85
		Segmental linear regression (second slope = -0.08)	5.34	NA-6.94	0	0.33	0.86
		Three segmental linear regression (second slope= 0)	5.21	3.03-5.21	0	0.32	0.86
		Second order polynomial	6.75	-	-	0.32	0.86
	FCR	Second order polynomial	6.36	-	-	0.03	0.68
		Segmental linear regression (second slope = 0.04)	5.92	3.25-7.93	0	0.03	0.68
		Three segmental linear regression (second slope =0)	5.72	2.96-NA	0	0.03	0.68
	SGR	Not applicable	-	-	-	-	-
	FCR	Not applicable	-	-	-	-	-

Table 3.1.5.3.9. MetCys experiment. Performance of juvenile Yellowtail Kingfish (Mean ± SE; *n* = 3) fed diets containing five graded levels of methionine and two graded levels of cysteine fed over 8 weeks. Different superscript letters indicate significant differences within row.

	Low Cysteine Diets					**High Cysteine Diets**					
Parameter	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**	**Diet 7**	**Diet 8**	**Diet 9**	**Diet 10**	**Met × Cys**
IBW (g)	52.9±0.5	52.9±0.1	52.2±.4	52.7±0.3	52.6±0.4	52.8±0.8	52.8±0.2	52.7 ±0.1	52.8±0.4	52.7±0.3	NS
HBW (g)	245.3±9.1^{a}	303.3±7.3^{bc}	342.4±9.4^{c}	315.7±2.8^{bc}	294.0±11.5^{abc}	275.9±7.3^{ab}	312.5±18.7^{bc}	312.1±13.4^{bc}	300.6±17.0^{bc}	290±21.5^{abc}	NS
FI (g fish^{-1} day^{-1})	3.72±0.11^{a}	4.59±0.14^{ab}	4.95±0.19^{b}	4.39±0.1^{ab}	4.64±0.12^{abc}	4.34±0.10^{ab}	4.50±0.10^{ab}	4.92±0.34^{b}	4.47±0.20^{ab}	4.50±0.34^{ab}	<0.05
WG (%)	299.0±41.7^{a}	457.0±7.8^{b}	518.4±7.4^{b}	465.0±23.7^{b}	444.0±31.4^{b}	422.5±11.3^{b}	460.5±57.5^{b}	442.9±35.9^{b}	455.3±43.0^{b}	388.88±37.9^{ab}	<0.05
SGR (% d^{-1})	2.53±0.26^{a}	3.18±0.03^{b}	3.43±0.08^{b}	3.32±0.03^{b}	3.13±0.11^{b}	3.06±0.04^{b}	3.17±0.19^{b}	3.12±0.12^{b}	3.16±0.15^{b}	2.93±0.14^{b}	<0.05
FCR	1.09±0.05^{a}	1.00±0.02^{ab}	0.93±0.03^{b}	0.90±0.05^{b}	1.05±0.03^{ab}	1.05±0.02^{ab}	0.96±0.05^{b}	1.04±0.06^{ab}	0.98±0.02^{b}	1.06±0.04^{ab}	<0.05
Survival (%)	85.4±5.2^{a}	97.2±2.8^{ab}	94.4±2.8^{ab}	94.4±5.6^{ab}	97.2±2.8^{ab}	100±0.0^{b}	94.4±5.6^{ab}	91.7 ±4.8^{ab}	97.2±2.8^{ab}	88.9±2.8^{ab}	<0.05
Opaque Eye (%)	82.5±6.9	0	0	0	0	0	0	0	0	0	NA

*IBW, Initial body weight; HBW, Harvest body weight; WG, Weight gain; SGR, Specific growth rate; FCR, Feed Conversion Ratio.

Table 3.1.5.3.10. MetCys experiment. Morphometric indices of juvenile Yellowtail Kingfish (Mean ± SE; *n* = 3) fed diets containing five graded levels of methionine and two graded levels of cysteine over 8 weeks. Different superscript letters indicate significant differences within row.

	Low Cysteine Diets					**High Cysteine Diets**					
Index*	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**	**Diet 7**	**Diet 8**	**Diet 9**	**Diet 10**	**Met × Cys**
K	2.01±0.37^{ab}	2.64±0.97^{a}	0.90±0.10^{b}	1.38±0.28^{ab}	1.82±0.6^{ab}	1.58±0.17^{ab}	1.11±0.25^{ab}	1.06±0.11^{ab}	1.40±0.48^{ab}	1.65±0.33^{ab}	NS
HIS (%)	0.81±0.02	0.74±0.24	0.72±0.02	0.77±0.03	0.79±0.02	0.79±0.04	0.78±0.02	0.77±0.02	0.81±0.03	0.85±0.05	NS
VSI (%)	5.50±0.24	5.29±0.73	6.48±0.28	5.70±0.46	6.08±0.41	5.56±0.23	6.38±0.40	7.44±1.19	6.35±0.51	6.22±0.38	NS

*K, Condition factor; HSI, Hepatosomatic index; VSI, Viscerosomatic index; NS; non-significant.

Table 3.1.5.3.11. MetCys experiment. Whole carcass and amino acid composition of juvenile Yellowtail Kingfish (Mean ± SE; *n* = 3).

Parameter (wet basis)	**Low Cysteine Diets**					**High Cysteine Diets**					**Met × Cys**
	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Diet 6**	**Diet 7**	**Diet 8**	**Diet 9**	**Diet 10**	
Proximate values (g kg^{-1})											
Ash	35.56±0.93	36.71±1.72	34.61±0.37	35.61±0.76	37.45±2.32	31.95±1.57	35.40±1.01	34.67±0.81	38.24±1.14	35.65±0.78	NS
Total lipid	62.08±1.85	63.61±5.16	67.49±4.97	66.40±4.51	57.75±2.56	71.47±1.34	67.38±2.70	65.96±2.70	64.96±4.07	65.38±6.05	NS
Total nitrogen	31.94±0.35	31.25±0.28	32.93±0.78	33.00±1.11	31.91±0.35	30.90±0.27	33.04±0.55	33.73±0.62	32.15±1.29	32.67±0.25	NS
Total protein	199.6±2.2	195.3±1.8	205.8±4.9	206.3±6.9	199.4±2.2	193.1±1.7	206.5±3.4	210.8±3.9	201.0±8.1	204.2±1.6	NS
Gross energy (MJ kg^{-1})	7.16±0.15	7.10±0.11	7.41±0.11	7.21±0.14	7.02±0.15	7.42±0.09	7.38±0.05	7.51±0.17	7.18±0.12	7.38±0.25	NS
Amino acid (g kg^{-1})											
Alanine	13.57±0.27	13.17±0.22	13.95±0.20	13.78±0.63	13.40±0.17	13.37±0.37	13.67±0.31	13.89±0.17	13.02±0.95	13.8±0.08	NS
Arginine	10.43±0.21	10.88±0.44	11.07±0.36	10.96±0.48	10.68±0.25	9.90±0.24	11.42±0.27	10.56±0.21	10.32±1.62	10.93±0.39	NS
Aspartic acid	18.08±0.30	18.32±0.20	18.96±0.35	18.6±0.40	18.47±0.35	17.73±0.47	19.07±0.08	18.85±0.18	18.18±1.24	18.56±0.06	NS
Cysteine	1.76±0.04	1.65±0.04	1.73±0.10	1.73±0.08	1.66±0.03	1.77±0.06	1.77±0.02	1.69±0.05	1.54±0.20	1.82±0.06	NS
Glutamic acid	27.22±0.49	27.69±0.25	28.66±0.44	28.27±0.81	27.93±0.49	26.41±0.70	28.23±0.33	28.98±0.39	27.53±1.77	28.54±0.15	NS
Glycine	16.46±0.22	16.37±0.14	16.41±0.28	16.12±0.59	15.90±0.12	15.96±0.42	16.35±0.33	16.17±0.25	15.49±1.40	15.84±0.26	NS
Histidine	5.48±0.15	5.93±0.18	5.87±0.06	5.69±0.04	5.48±0.20	5.21±0.10	5.76±0.06	6.00±0.11	5.62±0.44	5.40±0.14	NS
Isoleucine	7.49±0.08	7.85±0.06	8.29±0.16	8.01±0.04	7.95±0.22	7.41±0.22	8.12±0.03	8.06±0.16	7.94±0.44	8.09±0.09	NS
Leucine	12.61±0.18	12.94±0.08	13.40±0.32	13.26±0.19	13.16±0.36	12.55±0.32	13.43±0.04	13.28±0.17	12.80±0.85	13.25±0.09	NS
Lysine	15.19±0.75	15.83±0.22	16.75±0.16	16.29±0.15	15.53±0.69	15.11±0.56	16.08±0.26	16.01±0.40	16.36±0.93	15.76±0.43	NS
Methionine	5.58±0.07	5.51±0.12	5.86±0.07	5.81±0.23	5.62±0.13	5.54±0.13	5.81±0.10	5.85±0.25	5.33±0.64	5.93±0.08	NS
Phenylalanine	7.43±0.10	7.23±0.18	7.69±0.15	7.53±0.28	7.51±0.11	7.36±0.20	7.71±0.10	7.65±0.09	7.33±0.60	7.68±0.04	NS
Proline	9.83±0.12	9.78±0.08	9.82±0.21	9.84±0.26	9.66±0.17	9.39±0.20	9.93±0.12	9.77±0.19	9.22±0.92	9.59±0.04	NS
Serine	7.63±0.20	7.62±0.12	7.96±0.29	8.10±0.27	7.77±0.16	7.47±0.29	8.13±0.04	7.91±0.18	7.43±0.95	7.88±0.07	NS
Taurine	2.80±0.05	2.35±0.07	2.49±0.07	2.63±0.14	2.43±0.10	2.69±0.13	2.53±0.07	2.64±0.07	2.55±0.22	2.53±0.08	NS
Threonine	8.60±0.19	8.81±0.08	9.09±0.14	9.00±0.20	8.91±0.21	8.59±0.22	9.09±0.09	8.96±0.19	8.26±0.92	9.01±0.09	NS
Tyrosine	6.00±0.14	5.77±0.39	6.48±0.08	6.09±0.35	6.22±0.20	6.25±0.27	6.49±0.30	6.38±0.16	5.73±0.76	6.57±0.09	NS
Valine	8.51±0.07	9.03±0.03	9.29±0.19	9.08±0.07	8.88±0.22	8.42±0.22	9.22±0.04	9.28±0.20	8.90±0.43	9.03±0.09	NS

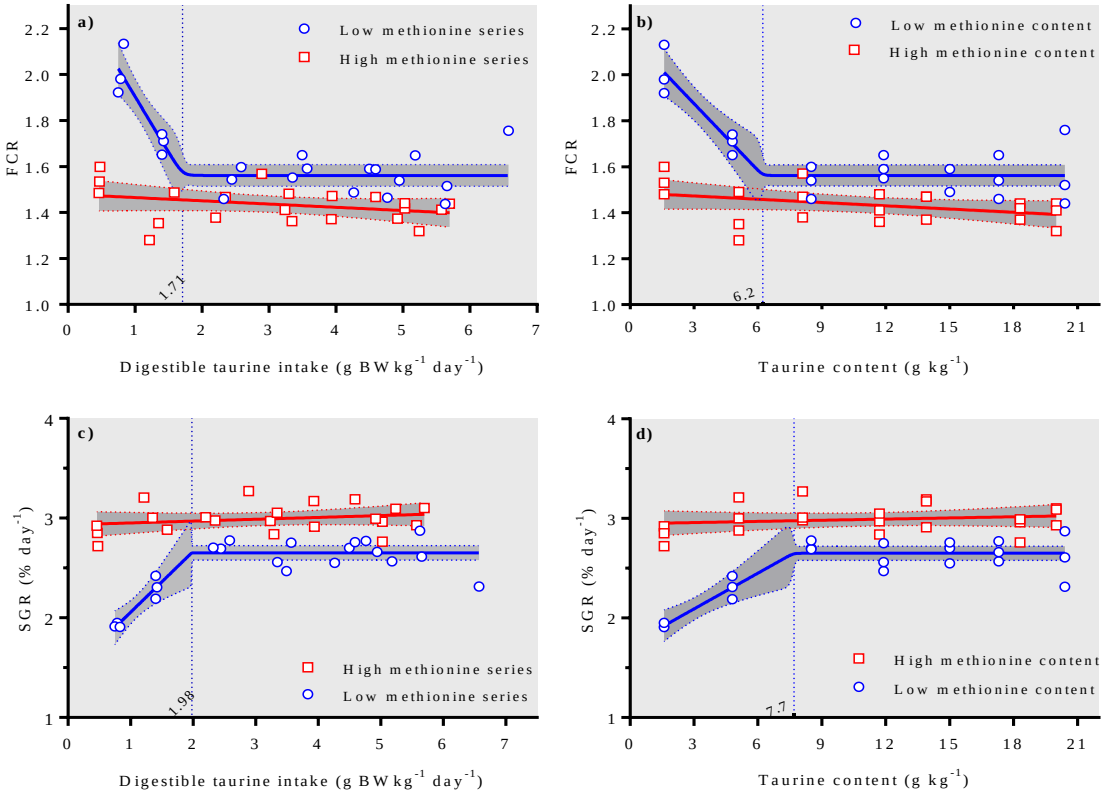


Figure 3.1.5.3.1. TauMet experiment: FCR response relative to digestible taurine intake (a) or dietary taurine content (b); SGR response relative to digestible taurine intake (c) or dietary taurine content (d). Regression model was selected according to the goodness of fit (Table 3.1.5.3.8). Dark grey areas indicate the 95% confidence interval. Dotted, vertical line indicates the minimum taurine requirement of Yellowtail Kingfish fed diets containing a low level of methionine.

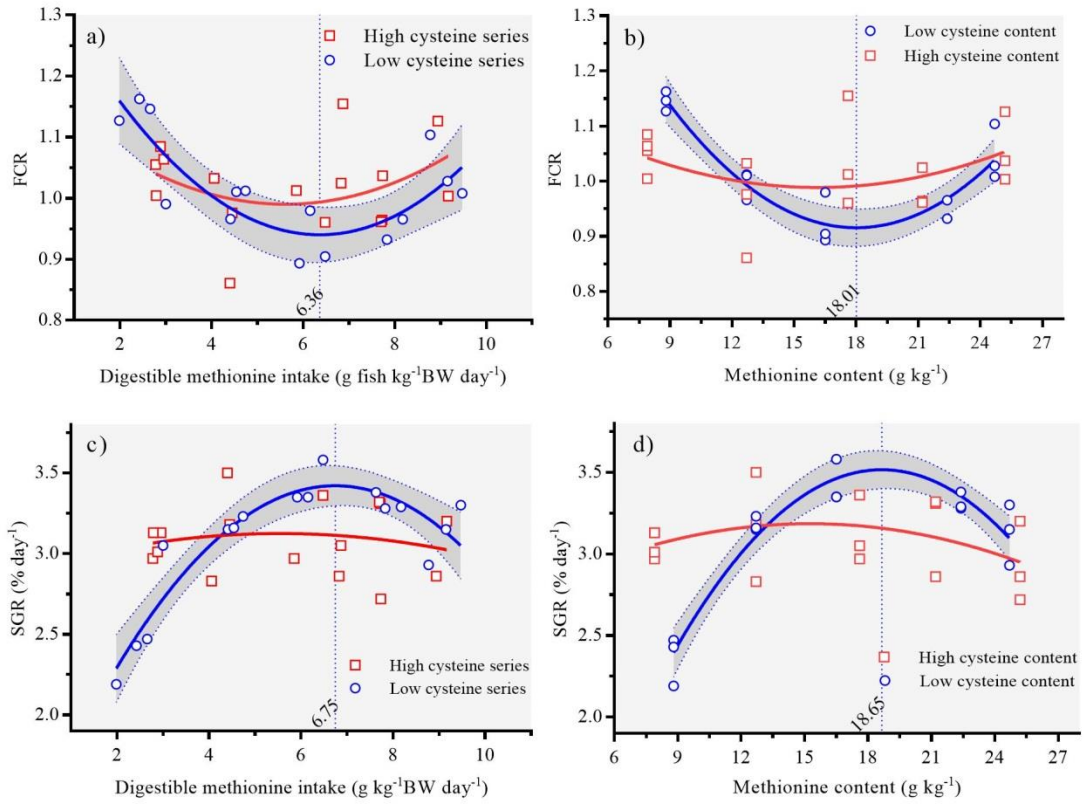


Figure 3.1.5.3.2. MetCys experiment: FCR response relative to the digestible methionine intake (a) and dietary methionine content (b); SGR response relative to the digestible methionine intake (c) and dietary methionine content (d). Regression model for fish fed diets low in cysteine (blue) were selected according to goodness of fit (Table 3.1.5.3.8). Dark grey areas indicate the 95% confidence interval for curves defining methionine requirement. Dotted, vertical lines indicate the lower and upper methionine requirement of Yellowtail Kingfish fed experimental diets containing low level of cysteine.

3.1.6. Chapter - Evaluation of bioactive ingredients/prebiotics that boost health of sub-adult Yellowtail Kingfish.

3.1.6.1. Manuscript - Use of prebiotic and probiotic supplements in diets for Yellowtail Kingfish Seriola lalandi; impacts on growth, digestibility, plasma biochemistry and hind-gut microbiome.

Mark Booth[a], Luke Vandenberg[a], Sarah Catalano[c], Andrew Oxley[c] and Igor Pirozzi[a,b]

[a] New South Wales Department of Primary Industries, Port Stephens Fisheries Research Institute, Taylors Beach 2316, NSW, Australia.

[b] College of Science and Engineering and Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville 4801, QLD, Australia.

[c] South Australian Research and Development Institute Aquatic Sciences Centre, West Beach, SA 5024, Australia

This manuscript may be referenced as: Booth, M., Vandenberg, L., Catalano, S., Oxley, A. and Pirozzi, I. (2019). 3.1.6.1. Manuscript - Use of prebiotic and probiotic supplements in diets for Yellowtail Kingfish *Seriola lalandi*; impacts on growth, digestibility, plasma biochemistry and hind-gut microbiome. In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.234-260.

Abstract

A 70 day growth experiment was done to evaluate the efficacy of four bioactive supplements in diets for juvenile (81.0 g) Yellowtail Kingfish (*Seriola lalandi*; YTK). Each bioactive was added to a negative control diet containing a high level of solvent extracted soybean meal (25.0%) and a moderate level of soy protein concentrate (5.0% diet). The negative control also contained a blend of fishmeal (25.0% of diet) as well as animal and vegetable proteins including blood meal, feather meal, poultry meal, corn gluten, maize starch and wheat flour. Fish oil (17.0% diet) was the major lipid source. The bioactive products evaluated were spent brewer's yeast (2.0% diet), inulin powder (1.0% diet), Protexin® powder (0.1% diet) and Pro(N8)ure®-IFS powder (0.1% diet). A positive control diet composed of prime fishmeal (55.0%) and fish oil (15.9% diet) was used for comparative purposes. In all, 6 diets were made. Multiple production responses were examined to evaluate the inclusion of the bioactives including specific growth rate (SGR), relative feed intake, food conversion ratio (FCR), condition factor, protein efficiency ratio (PER) and hepatosomatic index (HSI). In addition we examined the digestibility of diets, plasma biochemistry and impacts on the gut (rectal) microbiome. There were no significant differences among soy-based diets with respect to SGR, relative fed intake, FCR, condition factor, PER or HSI at the end of the study. A slight but statistically significant worsening in FCR and PER was recorded in YTK fed the fishmeal control diet compared to YTK fed the soy-based control diet however the biological driver for this is unclear. There were also no significant differences among diets with respect to levels of plasma cholesterol, triglycerides, total protein, glucose, lactate or aspartate aminotransferase (AST). The global level microbiome results also demonstrated no significant differences among diets with respect to species richness, evenness and diversity. However at the lower taxa level, varying abundances of certain operational taxonomic units (OTUs) may indicate health benefits to YTK with the addition of yeast or Pro(N8)ure® into a soybean meal diet although elucidation of key taxa, including definitive identification and pathogenicity tests, would be required to explore this notion further. Based on the results of this study there was no clear benefit of adding small amounts of spent brewer's yeast, inulin powder, Protexin® powder or Pro(N8)ure® powder to a soy-based control diet for YTK. The average SGR (2.28% d^{-1}), FCR (1.03:1.0) and PER (1.92) of YTK reared on the five soy-based diets

was extremely satisfactory considering the level of soybean meal and SPC used in these formulations. This indicates that higher levels of soybean meal or SPC may be appropriate in diets for juvenile YTK reared at water temperatures above 20 °C, especially when the diets are formulated to contain higher levels of methionine than previously thought necessary.

Introduction

The interest in the use of prebiotics and probiotics in aquaculture is increasing. The nomenclature surrounding these terms varies somewhat, but generally prebiotics are considered to be indigestible fibres (i.e. functional saccharides; e.g. inulin, oligofructose, xylooligosaccharide, fructooligosaccharide, mannanoligosaccharide, galactooligosaccharide, β-glucan etc.), which enhance beneficial gut bacteria resulting in improved health of the host. The benefits from use of prebiotics stem from the by-products derived or produced from the fermentation of the prebiotic substrate by intestinal bacteria. The fermentation process can also enhance colonies of commensal probiotic bacteria. Generally, probiotics are considered to be living microorganisms that can be ingested orally which lead to health benefits. Probiotics are live bacteria (e.g. *Lactobacillus*, *Bifidobacterium*) that exist in sufficient numbers, either due to ingestion or subsequent proliferation, which change the gut microflora of the host exerting beneficial health effects. The beneficial impacts on health manifest in a variety of ways including increases in growth, increased digestibility and upregulated immune response leading to increased resistance to stress and some diseases (Akhter et al., 2015; Dawood and Koshio, 2016).

Evaluating the influence of prebiotic and probiotic supplements is often done by measuring changes in basic biometric indices such as weight gain, feed conversion, digestibility and body composition. Changes in haematological and biochemical parameters are also commonly used to assess the effects of prebiotic and probiotic supplementation; measuring such parameters as haematocrit, triglyceride, cholesterol, total serum protein, albumin, globulin and glucose (Cerezuela et al., 2011). Upregulation of activity in amylases and proteases leading to improved digestion in fish has also been linked to the beneficial effects of prebiotics and probiotics. Importantly, prebiotics and probiotics are thought to positively modulate the immune system in fish, affecting lysozyme activity, alternative complement pathway, phagocytosis, respiratory burst activity, superoxide dismutase and mucus production (Cerezuela et al., 2011). As such, the upregulation of these immune parameters increases the health-status of the animal making it more robust to physical and biological stressors

From an aquaculture perspective the potential upregulation of the innate immune response in fish using prebiotics and probiotics is appealing because it offers alternative, non-invasive ways of controlling and protecting fish against various infectious agents (pathogens) that can cause wide spread disease and mortality. Gut health of YTK has been a particular issue in South Australia where an enteritis-like condition (a.k.a. winter syndrome, red intestine syndrome or subacute enteritis) has affected fish. It appears to be more prevalent in YTK fed aquafeeds containing plant proteins such as soybean meal and is exacerbated at water temperatures below < 18 °C (several examples cited in Stone et al., 2018). The enteritis is characterized by shortening of the mucosal folds, the reduction in absorptive capacity of enterocytes lining the epithelium and an enlarged lamina propria due to an infiltration of inflammatory cells, the presence of macrophages and eosinophilic granulocytes, and increased goblet cell proliferation (Stone et al., 2018). The problem appears multi-factorial in nature meaning that the root cause of the enteritis is often impossible to determine and even harder to prevent. Recently, researchers in South Australia have investigated the skin and gill microbiota of YTK in the hope that shifts in these communities may act as good indicators of changing gut health in farmed YTK (Legrand et al., 2018). These researchers also stressed the importance of finding other non-invasive ways to monitor the health of YTK as well as methods that allowed early detection and therapeutic intervention. Sadly, a diagnosis of enteritis in YTK is not often possible until it has reached a chronic state, after which therapeutic intervention is pointless.

A recent study on YTK explored the nutraceutical effect of grape seed extract for its potential to reduce the symptoms of sub-acute enteritis induced by feeding YTK on diets containing 30% soybean meal at low water temperature (Stone et al., 2018). The inclusion of grape seed extract at 20 mg kg^{-1} or 40 mg kg^{-1} of the diet did not reduce the symptoms of sub-acute enteritis or positively affect growth or FCR. Nonetheless, sub-acute enteritis or similar gut-related conditions in farmed YTK might be ameliorated by the use of other prebiotics or probiotics commonly used in other intensive animal production sectors.

For this reason the aim of the present study was to screen several commercially available prebiotic and probiotic sources for use in commercial feeds for YTK in the expectation they may 1) increase feed intake, growth rate, feed efficiency and digestibility of diets; 2) positively affect plasma biochemistry and 3) positively alter the composition of the hind-gut (rectal) microbiome.

Methods

This study was done with the approval of the NSW DPI Fisheries Animal Care and Ethics Committee (ACEC) under the research authority known as 'Aquaculture Nutrition ACEC 93/5–Port Stephens'. The care, husbandry and termination of fish was carried out according to methods outlined in 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2015).

Design of experiment and selected bio-actives

This experiment applies a similar approach to that of Stone et al. (2018) in that it uses a diet containing a high level of soybean meal (negative control) in order to place YTK under a perceived chronic nutritional stress. The soy-based control was formulated to include new research on the methionine and taurine requirements of juvenile YTK (see chapter 3.1.5). The negative control diet was supplemented with different, commercially available prebiotic and probiotic products to see if addition of these products affected feed intake, growth rate, food conversion ratio (FCR), digestibility, plasma biochemistry or gut health. The performance of YTK fed the negative control and the negative control augmented with prebiotic or probiotic products were compared to the performance of YTK fed a high fishmeal diet (positive control). All diets including the positive and negative control diets were formulated to be isoproteic, isolipidic and isoenergetic using the measured chemical composition of the constituent raw materials.

The four commercially available prebiotic and probiotic products evaluated in this experiment were:

- Spent brewer's yeast (Farmers Warehouse: http://www.farmerswarehouse.com.au/productDetail/ALL-ANIMAL-PRODUCTS/HORSES/HORSE-SUPPLEMENTS/Item/iO-Brewers-Yeast-4kg/20569),
- Inulin powder (pure chicory root inulin soluble fibre) (Bulk Powders Pty Ltd; www.bulkpowders.com.au/inulin-powder.html),
- Protexin® powder; a multi strain probiotic containing live bacteria; in feed formula containing 60×10^6 colony forming units; each gram contains; *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subspecies *bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*; *Bifidobacterium bifidum*; *Enterococcus faecium*; *Streptococcus salivarius* subspecies *thermophiles* (International Animal Health Products Pty Ltd; www.iahp.com.au/australia/protexin),
- Pro(N8)ure®-IFS powder; a heat stable prebiotic, dul strain probiotoc and enzmes; each kilogram contains 2.4×10^{11} colony forming units as *Bacillus subtilis* and *Bacillus licheniforms* (as PRO(N8)ure®-SL), 350 g Natuzyme (APVMA No. 50142), plus prebiotic (likely resistant starch) (International Animal Health Products Pty Ltd; www.iahp.com.au/australia/pron8ure).

Including the positive and negative control a total of 6 dietary treatments were evaluated in this study. The chemical composition of the major raw materials is presented in Table 3.1.6.1.1. The formulation and measured chemical composition of the diets is presented in Tables 3.1.6.1.2 and 3.1.6.1.3. Note that diets were supplemented with an inert marker (yttrium oxide) to allow the apparent digestibility of diets to be determined following the growth study.

All diets were made at PSFI using laboratory scale equipment. Prior to pellet making all raw materials were ground in a high speed turbine powder mill fitted with a 0.8 mm sieve (Model EFWB30B; Ernest Fleming Machinery and Equipment Pty Lt., Lane Cove West, 2066, NSW, Australia). Wheat flour was autoclaved for 2 min at 121 °C prior to inclusion in the dry mash. The raw materials and supplements were then dry mixed in a 120 L stainless steel vertical mixer (Flamingo 120 L; Ernest Fleming Machinery and Equipment Pty Lt., Lane Cove West, 2066, NSW, Australia), before the addition of a

oils and a suitable amount of fresh water. The damp mash was then pressed into 6 mm pellets using a stainless steel meat mincer (Dadaux TX-82; Barnco Sales, Ashfield 2131, NSW Australia). The moist pellets were gently dried for two days at approximately 50 °C in a specially constructed dehydrator until the dry matter content of pellets was ≥ 95%.

Fish handling and treatment

This experiment was done in a recirculating aquaculture system (RAS) fitted with 18 × 500 L cylindroconical polyethylene tanks (Polymaster FT500B; https://www.polymaster.com.au). The RAS was located in a greenhouse at PSFI and was maintained at a water temperature of approximately 20 °C using reverse-cycle refrigeration units. Each tank was fitted with an air stone diffuser (compressed air) and a mesh screen to prevent fish escaping. Each tank also contained an additional air stone diffuser which was connected to a high pressure oxygen reticulation system to ensure dissolved oxygen levels in all tanks remained > 6 mg L^{-1} at all times. The influent flow-rate to each tank was approximately 11 L min^{-1}. The RAS contained additional equipment to control water quality including dedicated bio-filters, particle filtration (Hydrotech drum filters) and foam fractionators (Aquasonic). Effluent water from the RAS was continuously removed and replaced with clean, pre-filtered and disinfected saltwater taken from the Tilligerry Estuary adjacent to PSFI. Photoperiod was allowed to follow the ambient autumn / winter cycle.

Water quality was monitored and recorded daily using electronic water quality meters and ammonia was measured regularly using off-the-shelf test kits. During the experiment the mean ± SD of pH, dissolved oxygen, water temperature and salinity were 7.5 ± 0.2 units, 8.5 ± 1.7, 19.8 ± 0.7 °C and 33.7±1.2 ppt. Ammonia ranged between 0.25 mg L^{-1} and 2.0 mg L^{-1}, but on most occasions was generally less than 0.5 mg L^{-1}.

Fish used in this experiment were progeny of wild caught broodstock held at the NSW DPI Port Stephens Fisheries Institute (PSFI). Fish were given a prophylactic bath in H_2O_2 to ensure the absence of skin and gill fluke before being transferred to the PSFI nutrition laboratories, but otherwise received no further treatment for fluke. Prior to the experiment all fish were reared on Huon Select 3 mm or 6 mm pellet (Skretting Australia). Fish were fasted for 24 h prior to entering the experiment.

Fifteen juvenile fish (81.0 ± 1.0 g; mean ± SD) were systematically stocked into each experiment tank following measurement of individual body weight and fork length (08/08/2018). A random sample of 5 initial fish were euthanised and frozen at this time for determination of chemical composition. Following the stocking procedure experiment tanks were randomly allocated to dietary treatments and fish commenced feeding on experimental diets the day after stocking. Fish were fed to apparent satiation once daily, seven days per week at approximately 11:00 h. The fish were on-grown without incident for 10 weeks (17/10/2018), after which they were reweighed and measured. At the same time 3 fish from each tank were randomly selected and killed (*ikijime*) in order to take a blood sample (i.e. plasma biochemistry), a swab from the anal vent (i.e. rectal microbiome sample), and to record the weight of the liver (i.e. hepatosomatic index). The 3 carcasses from each tank were then pooled and frozen. All remaining fish were returned to their respective tanks in preparation for collection of faecal material (i.e. digestibility of diets).

Digestibility of experimental diets

The digestibility of diets was determined by collecting faecal material from YTK using stripping techniques. The stripping technique was similar to that described by Booth and Pirozzi (2017). Briefly, sedated fish (Aqui-S®) were netted directly from their respective tank after which the ventral surface was wiped clean. A small amount of pressure was then applied to the abdomen using the thumb and forefinger to expel urinary products. The ventral area was cleaned again before firm abdominal pressure was applied to expel faecal material from the distal intestine. Faecal matter was expelled into a clean 70 mL container. Hands were rinsed clean between the handling of different fish and care was taken to ensure that the faecal samples were not contaminated by urine or mucous. Faecal samples were immediately stored in a freezer (-17 °C). Faecal samples were generally collected about 16 h after the

meal and fish were never stripped on consecutive days. Faecal samples from each tank were pooled and kept frozen at -17 °C until a sufficient amount of material was obtained for chemical analysis.

Apparent digestibility coefficients (ADCs) for dietary dry matter (DM), nutrients and energy were calculated according to the equation described by Cho et al., (1982), with the exception that yttrium was used as the inter marker;

- ADC of dry matter (%) = [1 - (concentration of Ytt in diet / concentration of Ytt in faeces)] × 100,
- ADC of nutrients or energy (%) = [1 - (concentration of Ytt in diet / concentration of Ytt in faeces × concentration of nutrient or energy in faeces / concentration of nutrient or energy in diet)] × 100.

Microbiome sampling

Gut (rectal swab) microbiome samples were collected at the end of the experiment to coincide with the weighing of fish. Rectal swabs were taken using sterile FLOQSwabs (Copan Flock Technologies) and immediately placed in a 15 mL falcon tube containing stabilising buffer (RNAlater™, Ambion), labelled and stored at 4 °C for 1-2 days and then for a month at -20 °C prior to RNA extraction. Samples were shipped to SARDI for preparation and extraction of the active bacterial community (RNA). The gut microbiota of YTK from the rectal swab sample was elucidated following similar procedures and sequencing methods outlined in Legrand et al., (2018).

RNA extraction of rectal microbiome samples

RNA was extracted on ice from stabilised samples according to the methods detailed in Szafranska et al. (2014). In brief, the tip was taken out of the stabilizing buffer and placed in a lysing matrix B tube (MP Biomedicals) containing 1 mL of cold (4 °C) RLT buffer + 1% β-mercaptoethanol. Samples were disrupted via bead-beating using the FastPrep-24™ 5G instrument (MP Biomedicals) at an intensity of 5.5 for 45 s, placed on ice for 3 min then disrupted a second time as described above prior to centrifugation at 14,000 × g for 10 min at 4 °C. The supernatant was transferred to 1.5 mL RNase-free Biopur centrifuge tubes (Eppendorf) and the RNeasy minikit was used to extract the RNA according to manufacturer's instructions. RNA was eluted in 30 µL of RNase free water, passed through the spin column twice to concentrate each sample and quantified using a NanoDrop 2000 spectrophotometer. To remove any source of potential contaminating gDNA, a routine DNase treatment was performed for all samples using the Turbo DNA-free™ kit (Life Technologies) following the manufacturer's instructions. All samples were precipitated with ethanol using standard procedures and RNA quantified using NanoDrop. Samples were stored at -80 °C prior to use in down-stream procedures.

cDNA synthesis, PCR amplification, AMPure bead-purification and Picogreen quantification

The RNA extracts were converted to cDNA to assess for the active (and likely resident) bacterial constituents using the Superscript™ III First Strand Synthesis System (Life Technologies) following the manufacturer's instructions and stored at -20 °C prior to PCR amplification. The V1-V2 hypervariable region of the 16S rRNA gene was amplified as described by Camarinha-Silva et al. (2014); though included a pre-enrichment of the V1-V2 target region by conducting a 20 cycle PCR reaction with primers 27F and 338R as described by Chaves-Moreno et al. (2015). Specifically, 2 µL of cDNA was used as template in the first round of PCR, with 1 µL aliquots from the first round of PCR used as template in a second 15 cycle PCR reaction to append the forward barcode and reverse adapter sequences complementary to the Illumina platform specific adaptors. One microlitre aliquots of the second PCR reaction were subsequently used as a template in a third 10 cycle PCR to append the Illumina multiplexing sequencing and index primers. PCR amplicons were visualised via agarose gel electrophoresis and products of the expected size (~438 bp) were purified using Agencourt AMPure XP beads (Beckman Coulter). Samples were quantified in duplicate using the Quant-iT™ Picogreen® dsDNA kit (Life Technologies) following the manufacturer's instructions. Approximately 100 samples

were pooled for each library in equimolar ratios and sequenced on the MiSeq platform (Illumina, San Diego, CA) using 250 nucleotide (nt) paired-end sequencing chemistry through the Australian Genome Research Facility (AGRF). As a sequencing control, amplicons generated from a single bacterial species (*Lactobacillus reuteri*) were included within each Illumina index within each of the libraries. The final list of samples that generated good-quality microbiomic libraries for this component of work are presented in Table 3.1.6.1.6.

Bioinformatics analysis

In total, 3,334,090 million sequence reads were derived from 52 samples (of the 54 that were collected). Two samples failed to amplify enough material to produce good-quality NGS libraries. This was accounted for in the experimental approach, by allowing for ample replication of fish. Sequence reads were paired using PEAR (version 0.9.5) (Zhang et al., 2014), where primers were identified and removed. Paired-end reads were quality filtered, with removal of low-quality reads, full-length duplicate sequences (after being counted) and singleton sequences using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010), USEARCH (version 8.0.1623) (Edgar, 2010; Edgar et al., 2011) and UPARSE software (Edgar, 2013). Reads were mapped to Operational Taxonomic Units (OTUs) using a minimum identity of 97%, and putative chimeras removed using the RDP-gold database as a reference (Cole et al., 2014). These OTUs were further filtered as conducted previously (Zhang et al., 2016) where only those that contributed to > 0.01% of the host-associated dataset (gut samples only) were used (see Table 3.1.6.1.2 for a summary of OTUs remaining post-filtering). Rarefaction curves were used to inspect (retrospectively) sampling depth for each gut sample (Appendix 3.1.6.1.1). Further interrogation of the resultant OTUs was conducted using the Seqmatch function of the RDP database (Wang et al., 2007) as well as SILVA (Quast et al., 2013), whereby lineages based on the SILVA taxonomy and best hits from RDP were assigned for each OTU alongside the corresponding RDP sequence similarity value (SeqMatch, S_ab score). The S_ab score represents the number of unique 7-base oligomers shared between an OTU and a known sequence contained in the RDP database divided by the lowest number of unique oligos in either of the two sequences. A S_ab score of 1.000 represents an identical match to the nearest database sequence, with values closer to 1.000 providing greater confidence in the identification OTU sequence.

Collection and analysis of plasma

Blood was collected from the caudal vein of each fish immediately after conducting the *ikijime* procedure. Approximately 2 mL of whole blood was drawn into a 5 mL syringe through a 19 × 1.5 inch gauge needle (Terumo Australia, Talavera Road, Macquarie Park 2113, NSW, Australia). Blood was immediately transferred into two 1 mL Greiner Bio-One™ MiniCollect™ tubes containing anti-clotting agent (#450537 lithium-heparin; http://www.interpath.com.au/). Blood samples were centrifuged for 10 min at 11,500 rpm after which plasma was withdrawn, transferred to a clean 0.5 mL Eppendorf tube and frozen (Lab-Co® High Speed Mini Centrifuge Cat# 400.003.050; Australian Scientific). Plasma samples were analysed for routine chemistry and cardiovascular risk markers by Pathology North Hunter Services, a NATA accredited Royal College of Pathologists Australia certified organisation (www.patholgynorth.com.au).

Chemical analysis of raw material, diet and faecal samples

Raw materials, diets and faecal samples were analysed for dry matter, crude protein, gross energy (bomb calorimetry), lipid and ash content, respectively. Diets and faecal material were also analysed for yttrium in order to determine apparent digestibility of the test diets. These analyses were done by CSIRO (Agriculture and Food, Carmody Road, St Lucia, QLD 4067, Australia). Crude protein was estimated by multiplying the nitrogen content of all samples by a generic factor of 6.25 (Rutherfurd, Moughan, 2018).

Biometric response variables

The following biometric performance variables were used to assess the effects of different feed treatments. The average value of all fish in each tank was used in calculations;

- Weight gain (g $fish^{-1}$) = final weight of fish (g) - initial weight of fish (g)
- Specific growth rate (% d^{-1}) = [Ln(final weight) – Ln(initial weight)] / 70 days × 100
- Condition factor K = [individual weight of fish (g) / fork length of fish $(mm)^3$] × 10^5
- Relative feed intake (g $kgBW^{-1}$ d^{-1}) = individual feed intake (g) / (GMBW/1000) / 70 days; where GMBW = geometric mean body weight of fish.
- Relative weight gain (g $kgBW^{-1}$ d^{-1}) = individual weight gain (g) / (GMBW/1000) / 70 days; where GMBW = geometric mean body weight of fish.
- Food conversion ratio (FCR) = dry basis feed intake per tank (g) / wet weight gain per tank (g)
- Hepatosomatic index (HSI %) = liver weight (g) / whole body weight (g)
- Protein efficiency ratio (%) = weight gain of fish (g) / dry basis protein intake of fish (g); note the sum of amino acids for each test diet was used to estimate biological protein content.

Statistical analyses

The effect of different diets on the performance of YTK dietary was examined using one-way ANOVA. ANOVA was done using NCSS 11 Version 11.0.13 (NCSS 11 Statistical Software (2016). NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss). ANOVA was considered significant when $P < 0.05$. The Tukey's multiple comparison procedure was used to discriminate between significantly different treatment means at the 95% confidence interval.

In order to explore for patterns across the global bacterial communities, a data matrix comprising the percent standardised abundances of OTUs was used to construct a sample-similarity matrix using the Bray-Curtis algorithm (Bray and Curtis, 1957), where samples were then ordinated using non-metric multidimensional scaling (nMDS) with 50 random restarts (Clarke et al., 2001). Significant differences between *a priori* pre-defined groups of samples were evaluated using permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations, allowing for type III (partial) sums of squares, fixed effects sum to zero for mixed terms, and exact p-values generated using unrestricted permutation of raw data (Anderson, 2001). Groups of samples were considered significantly different at $P < 0.05$. Pairwise tests in PERMANOVA were used to determine which *a priori* pre-defined categories (e.g. fishmeal control diet vs soybean meal control diet) were significantly different. The multivariate analyses, relative percent abundance of bacterial phyla and rarefaction curves were performed and calculated using PRIMER (v.7.0.11), PRIMER-E, Plymouth Marine Laboratory, UK (Clarke et al., 2001).

Conventional measures of species diversity, richness and evenness were calculated using algorithms for total OTUs (S), Pielou's evenness (J'), Shannon diversity (H') and Simpson (1-λ), while taxonomic diversity was calculated using algorithms for taxonomic distinctness: average taxonomic distinctness (avTD - delta+) and variation in taxonomic distinctness (varTD - lambda+) using PRIMER (v.7.0.11) (Clarke et al., 2001). These univariate indicators of diversity (S, J', H', 1-λ, avTD, varTD) were compared between *a priori* pre-defined groups of samples using one-way ANOVA and plotted in Prism v. 8.0.1 (Graphpad Software Inc.). Variables were considered to be significantly different at $P < 0.05$, for which a Tukey's post-hoc multiple comparisons test was then performed comparing the means of each group (Prism v. 8.0.1). For further presentation of the data, relative abundance plots of top contributing OTUs were constructed in Excel. This included plots of: i) the top 15 most abundant OTUs across the six diets; ii) the top 10 most abundant OTUs in the fishmeal positive control diet with corresponding abundance of these OTUs in the soybean meal negative control diet shown; and iii) the top 10 OTUs in the soybean meal negative control diet with the corresponding abundance of these OTUs in the four supplemented soybean meal bioactives diets shown. To obtain the identification of the closest cultured species for each of the most abundant OTUs, the corresponding sequence was blasted against the RDP isolate database only. A similarity (S_ab) score in parenthesis is presented for each OTU in these plots.

Results

Feed intake, growth and fish health were excellent during the experiment. However three underperforming fish died during the trial. Two fish died on the 13/09/2018; one from tank 8 (126.4 g; 233 mm FL; Pro(N8)ure®), and one from tank 9 (135.6g; 235mm FL; Protexin®). One fish died in tank 1 on the 29/9/2018 (183 g; 275 mm FL; brewer's yeast). All of the dead fish were extremely thin compared to the population of fish in the same tank, suggesting they were not consuming adequate feed.

The performance metrics of fish recorded at the end of the growth experiment are presented in Table 3.1.6.1.4. There were no significant differences among any of the dietary treatments with respect to harvest weight, harvest fork length, condition factor at harvest, SGR, relative feed intake, relative weight gain or HSI (all $P > 0.05$). There was a significant difference among the FCR of treatments, with the FCR of fish reared on the fishmeal control diet being significantly higher (worse) than fish reared on the soy-based control diet or the diet containing inulin ($F_{5,12} = 3.87$; $P = 0.025$). There was also a significant difference among the PER of different diets calculated using amino acid nitrogen, with the PER of fish reared on the fishmeal control diet being significantly lower than fish reared on the soy-based control diet or the diet containing inulin ($F_{5,12} = 4.02$; $P = 0.023$).

Digestibility of the diets was generally good (Table 3.1.6.1.2). There were no significant differences among the apparent digestibility of the diets for all proximate and energy values ($P > 0.05$; Table 3.1.6.1.2).

The plasma biochemistry of harvested fish is presented in Table 3.1.6.1.5. There were no significant differences among diets with respect to any of the listed parameters (all $P > 0.05$).

Sample information collected for microbiome analyses is presented in Table 3.1.6.1.6. A summary of sequenced sample parameters is presented in Table 3.1.6.1.7.

Global community structure

On a global community structure level, there was no significant difference between the positive control fish meal diet (FMC) samples compared to the negative control soybean meal diet (SBMC) samples (Figure 3.1.6.1.1, Table 3.1.6.1.8A). The addition of bioactives (e.g. yeast, inulin, Protexin® and Pro(N8)ure®) to the soybean meal diet did not significantly alter the global community structure compared to the negative control diet (Figure 3.1.6.1.1, Table 3.1.6.1.8B), and there was also no significant difference between the four bioactives trial diets (Figure 3.1.6.1.1, Table 3.1.6.1.8C).

Bacterial phyla

All six diets were primarily dominated by taxa from the bacterial phyla Proteobacteria, and to a lesser extent, Bacteroidetes (Figure 3.1.6.1.2). Phyla representation was similar between the two control diets (FMC vs SBMC), as well as between the five soybean diets including the negative control (SBMC) and four bioactives supplemented diets, although greater representation by Chloroflexi was evident in the Protexin® and Pro(N8)ure® diets compared to the soybean meal negative control diet (SBMC) (Figure 3.1.6.1.2).

Top OTUs

In general, a high level of species richness across all six diets was evident from the top 15 OTU plot, with additional taxa beyond the top 15 contributing to the relative abundance (Figure 3.1.6.1.3). Slight changes in taxa composition were observed between the two control diets, with greater contribution by OTU 2 (*Photobacterium damselae* subsp. *damselae*/*P. leiognathi*, S_ab score 1.000) and OTU 1 (*Vibrio* sp. V776/*Allivibrio finisterrensis*, S_ab score 1.000) in the soybean meal diet compared to fishmeal diet (Figures 3.1.6.1.3 and 3.1.6.1.4). OTU 2, with closest sequence similarity to *Photobacterium damselae* subsp. *damselae*/*P. leiognathi* (S_ab score 1.000) was also recorded in the four soybean meal bioactives diets (Figure 3.1.6.1.3), although it's relative abundance was lower in the yeast and Pro(N8)ure® supplemented soybean meal diets (Figure 3.1.6.1.5). An increase in abundance of two taxa, OTU 4

(*Thiothrix* sp., S_ab 0.785) and OTU 3 (*Jeongeupia* sp., S_ab 0.498) were also recorded in the yeast and Pro(N8)ure® supplemented soybean meal diets.

Therefore, characterisation of and determining the potential pathogenicity of OTU 2, OTU 3 and OTU 4 are warranted to assess if these taxon are beneficial organisms (e.g. OTU 3 and OTU 4 with increased abundance observed in the yeast and Pro(N8)ure® diets) or opportunistic pathogens (e.g. OTU 2 with decreased abundance observed in the yeast and Pro(N8)ure® supplemented diets), which may then provide support for the additional of yeast and/or Pro(N8)ure® into the diets of YTK to improve health outcomes.

Diversity indices

There was no significant difference in diversity indices between the six diets except for greater species diversity (delta+) recorded in the yeast and Protexin® supplemented soybean meal diets compared to the inulin supplemented soybean meal diet (Figure 3.1.6.1.6, Table 3.1.6.1.9).

Discussion

The selected bioactives (i.e. prebiotics and probiotics) examined in this study had little influence on harvest weight, condition factor, specific growth rate, relative feed intake and FCR of YTK when added to a soy-based control diet, at least at the inclusion levels tested here. The lack of effect on gross performance metrics is not uncommon in fish studies evaluating low dietary levels of prebiotics or probiotics (RingØ et al., 2010; Zhou et al., 2018), and if an effect occurs, it seems more likely to manifest in changes to the innate immune system brought about by shifts in the composition and functionality of the gut microbiome (Akhter et al., 2015; Dawood, Koshio, 2016; Zhou et al., 2018). The overall benefits of using prebiotics and probiotics in aquaculture diets or indeed aquaculture rearing systems is still poorly understood due to the varied responses recorded among different aquatic species to the same bioactive, the dietary supplementation level and or the duration of use (Grisdale-Helland et al., 2008).

The growth rate of fish in this study was reasonably uniform across all dietary treatments (i.e. SGR between 2.22-2.33% d^{-1}) and fish increased their body weight by an average of 393% in 70 days. The FCR and PER of the soy-based diets were also very consistent, ranging narrowly between 0.99-1.07:1.0 and 1.85-1.99, respectively. These results point to the nutritional adequacy of all diets in terms of supporting weight gain. However, the FCR and PER of fish fed the fishmeal control diet was significantly higher and lower, respectively, than fish fed the soy-based control diet. This result was contrary to what was expected insofar as most other studies have demonstrated that the growth and FCR of juvenile YTK is negatively affected when fed diets containing elevated levels of soybean meal or soy protein concentrate (Bowyer et al., 2013a; b; Takagi et al., 2013; Stone et al., 2018). This trend appears quite consistent across different studies and is exacerbated to some extent by low water temperature (Bowyer et al., 2014; Stone et al., 2018). The present study was run at a constant water temperature of 20 °C which is close to the preferred water temperature for this species (Pirozzi and Booth, 2009), therefore, it is unlikely water temperature had any bearing on the difference in the FCR and PER of the two control diets.

In line with our results other researchers have reported slightly better weight gains in juvenile Californian yellowtail (*S. lalandi*) fed diets containing high levels of non-GMO soybean meal (between 35-48%) compared to those fed a fishmeal control diet (45.0% fishmeal), albeit FCR of fish fed the diets containing non-GMO cultivars was marginally worse (Buentello et al., 2015). These authors concluded that the equivalent growth rates and FCR of fish fed the non-GMO cultivars was likely due to the lower anti-nutrient levels of the selected cultivars and the higher than usual level of taurine supplementation they used in their diets (1.5% was added to all diets). It should be noted that the test diets formulated in this present study incorporated new data on the methionine requirements of juvenile YTK reared under warm water conditions (see chapter 3.1.5). The elevated levels of methionine (≈ 2.0% diet) may have overcome previously unknown deficits associated with YTK research on the use of soybean meal or soy protein concentrate. The apparent digestibility coefficient values did not vary significantly among the diets including the control diets implying nutrients were similarly available to YTK. There was also no indication that the selected bioactive supplements directly influenced the

plasma biochemistry of YTK in this study. These data tend to support the similar growth demonstrated across all diet treatments.

Brewer's yeast was included at 2.0% of the diet in this study, a far higher inclusion than each of the other products. Brewer's yeast is a good source of nucleic acids and polysaccharides including ß-glucans. ß-glucans is reported to enhance the immune functions in many fish and is the factor within brewer's yeast thought responsible for enhancing immune function (Li and Gatlin, 2003). However, while eight weeks feeding of brewer's yeast (*Saccharomyces cerevisiae;* Brewtech®) at 1%, 2% and 4% of the diet enhanced weight gain or feed efficiency of juvenile Hybrid Striped Bass (*Morone chrysop* × *M. saxatilis*) and led to improved resistance to a *Streptococcus iniae* challenge, it did not improve these production responses significantly (Li and Gatlin, 2003). High dietary inclusion of brewer's yeast (between 11% and 54.8%) did not significantly improve growth rate or feed intake in Juvenile Sea Bass (*Dicentrarchus labrax*) compared to a fishmeal control diet, but it did improve feed conversion efficiency, PER and protein retention. Therefore, the lack of biometric responses to brewer's yeast and possibly the other bioactive products in this study could be due to an insufficient inclusion level.

Proprietary prebiotics such as GroBiotic®-A (partially autolysed brewer's yeast and other components including B-glucans) and other biactives such as galacto-oligosaccharide (GOS) and mannan-oligosacharide (MOS) have proved beneficial in enhancing the apparent protein, lipid, organic matter and energy digestibility of soybean based feeds for Red Drum (*Sciaenops ocellatus*) when included at 1% of the diet. However, addition of a similar level of inulin had little impact on protein digestibility and actually decreased lipid and energy digestibility in soybean based diets (Burr et al., 2008). The amount of brewer's yeast (2.0% diet) and inulin (1.0% diet) added to the soybean meal control diet in the present study was similar to the levels tested on Red Drum (Burr et al., 2008) and other species (RingØ et al., 2010)

This hypothesis may be more relevant in terms of the Protexin® and Pro(N8)ure® products which were added to the soy-based control diet at very low amounts compared to the yeast and inulin products. The added amounts of Protexin® and Pro(N8)ure® were based on the manufacturers recommendations, however these were not specific to fish, but rather made for animals such as cattle, horses, pigs and poultry. Further studies would need to be done to determine if higher amounts of the particular bioactives evaluated in this screening trial affected the production indices of YTK.

Fish fed the fishmeal control and soybean meal control diets had a similar microbiome global community structure and bacterial phyla profile. Diversity indices were also consistent between these two control diets, with high levels of species richness (total species), evenness (Pielou's, lambda+) and diversity (Shannon, Simpson and delta+). This suggests on a global level, that there is no major change in the microbiome composition of YTK fed either a diet containing fishmeal or soybean meal. Nonetheless, slight differences were observed at the lower taxa level, with an increase in abundance of OTU 1, with closest sequence similarity to *Vibrio* sp. V776/*Allivibrio finisterrensis* (S_ab score 1.000), and OTU 2, with closest sequence similarity to *Photobacterium damselae* subsp. *damselae*/*P. leiognathi* (S_ab score 1.000) in the soybean meal diet compared to fishmeal diet. Further investigation is warranted to determine the pathogenicity of these taxa (OTU 1 and OTU 2), as if both are found to be opportunistic pathogens (certain Vibrios and *Photobacterium* spp. are known to be opportunistic pathogens of fish), the result observed here may support the use of fishmeal over soybean meal diets in YTK production, as potentially detrimental taxa may be reduced while diversity and richness is still maintained on a fishmeal based diet.

On a global community structure level and in regards to the diversity indices that were evaluated (species richness, evenness and diversity), no significant differences were observed between the soybean meal negative control diet and four soybean meal treatment diets that were supplemented with bioactives, suggesting the additional of bioactives (either yeast, inulin, Protexin® or Pro(N8)ure®) into a soybean meal diet at the levels used in this study does not change the microbiome composition of YTK. Nonetheless, slight differences were observed at the lower taxa level, with the decrease of OTU 2 (*Photobacterium damselae* subsp. *damselae*/*P. leiognathi* - which may be an opportunistic pathogen) and increase in OTU 3 and OTU 4 (*Jeongeupia* sp. and *Thiothrix* sp. respectively - which may be beneficial taxa) in the yeast and Pro(N8)ure® supplemented soybean meal diets compared to the soybean meal control diet with no bioactives added. Further investigation on the additional of yeast and Pro(N8)ure® into the diets of YTK is, therefore, warranted/recommended following the characterisation of OTU 3 and OTU 4 (to determine their definitive identification as a weak sequence identity to

Jeongeupia sp. [S_ab 0.498] and *Thiothrix* sp [0.785] was recorded respectively, and to determine if they are beneficial taxa) and tests on the pathogenicity of OTU 2 (to determine if it is an opportunistic organism). As such, an increase in abundance of OTU 3 and OTU 4, along with a decrease in abundance of OTU 2, may indicate favourable health outcomes for YTK on soybean meal diets supplemented with either of these two bioactives (yeast or Pro(N8)ure®).

The global level microbiome results support the nutritional findings (with respect to SGR, relative feed intake, FCR, condition factor, PER, HIS and plasma biochemistry) of no significant differences among the five soy-based diets. In general there is no significant benefit of adding spent brewer's yeast, inulin powder, Protexin® powder or Pro(N8)ure®-IFS powder to a soy-based control diet for YTK, at least at the inclusion levels used in this study. However at the lower taxa level, varying abundances of certain OTUs may indicate health benefits to YTK with the addition of yeast or Pro(N8)ure® into a soybean meal diet. Elucidation of key taxa (e.g. OTU 2, OTU 3 and OTU 4), including definitive identification and pathogenicity tests, would be required to explore this notion further.

Conclusions and Recommendations

Four commercially available bioactive products were added to a soy-based control diet to examine whether they could improve the weight gain, feed intake and FCR of juvenile YTK. The products were spent brewer's yeast (2.0% diet), inulin powder (1.0% diet), Protexin® powder (0.1% diet) and Pro(N8)ure®-IFS powder (0.1% diet). A positive control diet composed of prime fishmeal (55.0%) and fish oil (15.9% diet) was used for comparative purposes. The digestibility of diets, plasma biochemistry and impacts on the gut microbiome were also examined. After 70 days there were no significant differences among soy-based diets with respect to SGR, relative fed intake, FCR, condition factor, PER or HSI. There were also no significant differences among diets with respect to levels of plasma cholesterol, triglycerides, total protein, glucose, lactate or aspartate aminotransferase (AST). Based on production responses and results on plasma biochemistry, diet digestibility and micobiome analyses the results indicate there was no benefit of adding small amounts of spent brewer's yeast, inulin powder, Protexin® powder or Pro(N8)ure®-IFS powder to a soy-based control diet for YTK.

Findings

- Adding small amounts of spent brewer's yeast, inulin powder, Protexin® powder or Pro(N8)ure®-IFS powder to a soy-based control diet did not improve specific growth rate (SGR), relative feed intake or FCR in juvenile YTK.
- Adding small amounts of spent brewer's yeast, inulin powder, Protexin® powder or Pro(N8)ure®-IFS powder to a soy-based control diet did not alter concentrations of cholesterol, triglycerides, total protein, glucose, lactate or aspartate aminotransferase (AST) the plasma of juvenile YTK.
- Juvenile YTK reared at 20 °C can be fed carefully formulated diets containing 25% and 5% of soybean meal and SPC, respectively, without incurring any loss in short term production.
- There results of this study indicate that there is no major benefit in adding any of the selected bioactives into diets for juvenile YTK, at least at the levels tested.
- Multiple bioactive supplements (prebiotics, probiotics etc.) are available and there may be merit in evaluating others.
- Diets for YTK that contain soybean meal and SPC and optimal levels of methionine, such as the soy-based formulation used in this trial, are worthy of further investigation.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, New South Wales Department of Primary Industries (NSW DPI) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

We would also like to acknowledge the technical assistance of Ian Russell, Dylan Nash, Marcus Armstrong and Steve Gamble (NSW DPI). We thank Cedric Simon, Barney Hines and staff at CSIRO for conducting the chemical analyses on samples generated from this experiment. Dr Michael Salini and Simon Tabrett were actively involved in supplying many of the raw materials used to manufacture the experimental feeds (Ridley).

References

ACEC, 2015. A Guide to Acceptable Procedures and Practices For Aquaculture and Fisheries Research - 4th Edition, 4th ed. NSW Department of Primary Industries (Fisheries) for NSW Fisheries Animal Care and Ethics Committee, Nelson Bay, NSW, Australia.

Akhter, N., Wu, B., Memon, A.M., Mohsin, M., 2015. Probiotics and prebiotics associated with aquaculture: A review. Fish Shellfish Immunol. 45, 733-741.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology 26, 32-46.

Booth, M., Pirozzi, I., 2017. Making sense of nonsense: using regression analysis to deal with highly variable data collected from a yellowtail kingfish (Seriola lalandi) digestibility experiment. Aquaculture 485, 39-48.

Bowyer, J.N., Qin, J.G., Smullen, R.P., Adams, L.R., Thomson, M.J.S., Stone, D.A.J., 2013a. The use of a soy product in juvenile yellowtail kingfish (Seriola lalandi) feeds at different water temperatures 1. Solvent extracted soybean meal. Aquaculture 384–387, 35-45.

Bowyer, J.N., Qin, J.G., Smullen, R.P., Adams, L.R., Thomson, M.J.S., Stone, D.A.J., 2013b. The use of a soy product in juvenile yellowtail kingfish (Seriola lalandi) feeds at different water temperatures: 2. Soy protein concentrate. Aquaculture 410–411, 1-10.

Bowyer, J.N., Booth, M.A., Qin, J.G., D'Antignana, T., Thomson, M.J.S., Stone, D.A.J., 2014. Temperature and dissolved oxygen influence growth and digestive enzyme activities of yellowtail kingfish Seriola lalandi (Valenciennes, 1833). Aquacult. Res. 45, 2010-2020.

Buentello, A., Jirsa, D., Barrows, F.T., Drawbridge, M., 2015. Minimizing fishmeal use in juvenile California yellowtail, *Seriola lalandi*, diets using non-GM soybeans selectively bred for aquafeeds. Aquaculture 435, 403-411.

Burr, G., Hume, M., Neill, W.H., Delbert M. Gatlin, I., 2008. Effects of prebiotics on nutrient digestibility of a soybean-meal-based diet by red drum Sciaenops ocellatus (Linnaeus). Aquacult. Res. 39, 1680-1686.

Camarinha-Silva, A., Jauregui, R., Chaves-Moreno, D., Oxley, A.P., Schaumburg, F., Becker, K., Wos-Oxley, M.L., Pieper, D.H., 2014. Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. Environ Microbiol. 16, 2939-2952.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7, 335-336.

Cerezuela, R., Meseguer, J., Esteban, M.A., 2011. Current Knowledge in Synbiotic Use for Fish Aquaculture: A Review. Journal of Aquaculture Research & Development, DOI: 10.4172/2155-9546.S4171-4008.

Chaves-Moreno, D., Wos-Oxley, M.L., Jáuregui, R., Medina, E., Oxley, A.P.A., Pieper, D.H., 2016. Application of a Novel "Pan-Genome"-Based Strategy for Assigning RNAseq Transcript Reads to Staphylococcus aureus Strains. PLoS One 10, e0145861.

Clarke, K.R., Warwick, R.M.R.M., Laboratory, P.M., 2001. Change in marine communities: an approach to statistical analysis and interpretation: Plymouth, U.K.: PRIMER-E Ltd.

Cho, C.Y., Slinger, S.J., Bayley, H.S., 1982. Bioenergetics of salmonid fishes: energy intake, expenditure and productivity. Comparative Biochemistry and Physiology 73B, 25-41.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research* 42 (Database issue), D633-D642.

Dawood, M.A.O., Koshio, S., 2016. Recent advances in the role of probiotics and prebiotics in carp aquaculture: A review. Aquaculture 454, 243-251.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460-2461.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nature Methods 10, 996-998.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194-2200.

Grisdale-Helland, B., Helland, S.J., Gatlin, D.M., 2008. The effects of dietary supplementation with mannanoligosaccharide, fructooligosaccharide or galactooligosaccharide on the growth and feed utilization of Atlantic salmon (Salmo salar). Aquaculture 283, 163-167.

Legrand, T.P.R.A., Catalano, S.R., Stephens, F., Oxley, A.P.A., Landos, M., Bansemer, M.S., Wos-Oxley, M.L., Stone, D.A.J., Qin, J.G., 2018. The Inner Workings of the Outer Surface: Skin and Gill Microbiota as Indicators of Changing Gut Health in Yellowtail Kingfish. Frontiers in Microbiology, doi: 10.3389/fmicb.2017.02664.

Li, P., Gatlin, D.M., 2003. Evaluation of brewers yeast (*Saccharomyces cerevisiae*) as a feed supplement for hybrid striped bass (*Morone chrysops* × *M. saxatilis*). Aquaculture 219, 681-692.

Pirozzi, I., Booth, M.A., 2009. The routine metabolic rate of mulloway (Argyrosomus japonicus: Sciaenidae) and yellowtail kingfish (Seriola lalandi: Carangidae) acclimated to six different temperatures. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 152, 586-592.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41, D590-D596.

RingØ, E., Olsen, R.E., Gifstad, T.Ø., Dalmo, R.A., Amlund, H., Hemre, G.-I., Bakke, A.M., 2010. Prebiotics in aquaculture: a review. Aquacult. Nutr. 16, 117-136.

Rutherfurd, S.M., Moughan, P.J., 2018. The chemical analysis of protein and amino acids. in: Moughan, P.J., Hendriks, W.H. (Eds.), Feed Evaluation Science. Waeningen Academic Publishers, Wageningen, The Netherlands, pp. 39-112.

Stone, D.A.J., Bellgrove, E.J., Forder, R.E.A., Howarth, G.S., Bansemer, M.S., 2018. Inducing subacute enteritis in Yellowtail kingfish Seriola lalandi: The effect of dietary inclusion of soybean meal and grape seed extract on hindgut morphology and inflammation. N. Am. J. Aquacult. 80, 59-68.

Szafranska, A.K., Oxley, A.P., Chaves-Moreno, D., Horst, S.A., Rosslenbroich, S., Peters, G., Goldmann, O., Rohde, M., Sinha, B., Pieper, D.H., Loffler, B., Jauregui, R., Wos-Oxley, M.L., Medina, E., 2014. High-resolution transcriptomic analysis of the adaptive response of Staphylococcus aureus during acute and chronic phases of osteomyelitis. mBio. 5.

Takagi, S., Murata, H., Goto, T., Hatate, H., Yamashita, H., Takano, A., Ukawa, M., 2013. Long-term feeding of the Yellowtail Seriola quinqueradiata with soy protein concentrate-based non-fishmeal diet supplemented with taurine. Aqauculture Science 61, 349-358.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology 73, 5261-5267.

Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30, 614-20.

Zhang, M., Sun, Y., Liu, Y., Qiao, F., Chen, L., Liu, W-T, Du, Z., Li, E., 2016. Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference. Aquaculture 454, 72.

Zhou, M., Liang, R., Mo, J., Yang, S., Gu, N., Wu, Z., Sarath Babu, V., Li, J., Huang, Y., Lin, L., 2018. Effects of brewer's yeast hydrolysate on the growth performance and the intestinal bacterial diversity of largemouth bass (*Micropterus salmoides*). Aquaculture 484, 139-144.

Table 3.1.6.1.1. Measured chemical composition or major raw materials used in experimental diets (dry mater basis).

Parameter	Prime fishmeal	Soybean meal	SPC	Feather meal	Poultry meal	Blood meal	Corn gluten	Maize starch	Wheat flour	Brewer's yeast	Inulin	Protexin®	Pro(N8)ure®
Ash (%)	12.38	6.38	6.40	2.02	13.67	1.59	1.61	0.06	0.46	6.83	0.00	90.40	77.69
Total lipid (%)	12.40	9.52	1.63	8.01	13.67	1.73	7.13	0.19	1.23	2.38	0.15	0.05	1.02
Total nitrogen (%)	12.42	8.65	11.16	14.80	12.30	15.36	11.06	0.11	2.36	8.68	0.08	0.06	0.18
Crude protein (%)	77.62	54.04	69.77	92.50	76.85	95.97	69.13	0.69	14.73	54.25	0.51	0.35	1.09
Estimated NFE (%)	-2.40	30.06	22.20	-2.54	-4.20	0.71	22.13	99.06	83.58	36.54	99.34	9.20	20.20
Gross energy (MJ kg^{-1})	22.31	21.46	20.72	24.69	21.87	24.61	23.78	17.42	18.28	19.78	17.19	0.03	1.59
Alanine	4.30	2.32	2.81	3.92	4.29	7.36	5.41	0.10	0.36	2.72	0.00	0.00	0.00
Arginine	4.05	3.22	4.33	5.51	4.23	3.13	1.88	0.03	0.38	2.31	0.02	0.00	0.00
Aspartic acid	6.66	6.18	7.56	6.03	5.76	10.35	4.28	0.14	0.48	4.61	0.00	0.00	0.00
Cysteine	0.81	0.85	0.88	4.27	0.82	1.15	0.84	0.00	0.22	0.26	0.01	0.00	0.00
Glutamic acid	9.23	9.51	11.59	9.63	9.09	9.12	13.94	0.05	4.62	7.45	0.00	0.00	0.03
Glycine	4.62	2.18	2.78	6.58	6.41	4.04	2.08	0.14	0.46	2.25	0.00	0.00	0.00
Histidine	2.93	1.17	1.57	0.57	1.37	5.30	1.21	0.01	0.22	0.88	0.00	0.00	0.00
Isoleucine	3.23	2.30	3.01	4.25	2.68	0.78	2.56	0.04	0.44	2.27	0.00	0.00	0.00
Leucine	5.31	3.86	4.94	6.90	4.61	11.38	10.20	0.06	0.84	3.27	0.00	0.00	0.01
Lysine	5.50	3.01	3.74	1.68	4.55	9.67	1.26	0.01	0.24	3.01	0.00	0.00	0.00
Methionine	2.16	0.46	0.52	0.53	1.47	1.56	1.38	0.00	0.16	0.60	0.00	0.00	0.05
Phenylalanine	3.05	2.63	3.44	4.21	2.64	7.16	4.09	0.05	0.62	2.08	0.00	0.00	0.00
Proline	3.12	2.52	3.27	8.86	4.27	3.89	6.18	0.04	1.50	2.20	0.00	0.00	0.01
Serine	3.02	2.63	3.28	9.73	2.66	5.28	3.71	0.36	0.50	2.21	0.00	0.00	0.00
Taurine	0.65	0.00	0.00	0.03	0.35	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00
Threonine	3.32	2.00	2.58	4.28	2.73	5.07	2.37	0.10	0.34	2.48	0.00	0.00	0.00
Tyrosine	2.72	1.72	2.11	2.68	2.17	3.25	3.53	0.12	0.32	1.32	0.00	0.00	0.00
Valine	3.66	2.35	3.05	6.28	3.10	7.71	3.01	0.06	0.50	2.58	0.00	0.00	0.00
∑ Amino acids	**68.35**	**48.91**	**61.47**	**85.94**	**63.19**	**96.20**	**67.91**	**1.31**	**12.21**	**42.55**	**0.03**	**-0.01**	**0.10**

Table 3.1.6.1.2. The raw material and nutrient composition of the experimental diets (dry matter basis).

Raw material (%)	**Fishmeal control**	**Soybean meal control**	**Brewer's yeast**	**Inulin**	**Protexin®**	**Pro(N8)ure®**
Prime fishmeal	55.0	25.0	25.0	25.0	25.0	25.0
Soybean meal	-	25.0	25.0	25.0	25.0	25.0
Soy protein conc.[#]	-	5.0	5.0	5.0	5.0	5.0
Blood meal	2.9	2.8	2.8	2.8	2.8	2.8
Corn gluten	1.0	3.5	3.5	3.5	3.5	3.5
Feather meal	8.7	9.4	9.4	9.4	9.4	9.4
Poultry meal	1.5	5.3	5.3	5.3	5.3	5.3
Maize starch	10.0	3.0	1.0	2.0	2.9	2.9
Wheat flour	2.4	-	-	-	-	-
Fish oil	15.9	17.0	17.0	17.0	17.0	17.0
Choline chl. (70%)	0.3	0.3	0.3	0.3	0.3	0.3
Lysine	-	0.5	0.5	0.5	0.5	0.5
Methionine	0.7	1.0	1.0	1.0	1.0	1.0
NaH_2PO_4	0.5	0.8	0.8	0.8	0.8	0.8
Stay-C® (35)	0.3	0.3	0.3	0.3	0.3	0.3
Taurine	0.3	0.6	0.6	0.6	0.6	0.6
Vit-min premix	0.5	0.5	0.5	0.5	0.5	0.5
Y_2O_3	0.1	0.1	0.1	0.1	0.1	0.1
Brewer's yeast	-	-	**2.0**	-	-	-
Inulin	-	-	-	**1.0**	-	-
Protexin®	-	-	-	-	**0.1**	-
Pro(N8)ure®	-	-	-	-	-	**0.1**
Nutrient or energy						
Ash (%)	8.3	7.2	7.0	7.1	7.3	7.1
Total lipid (%)	22.2	23.2	23.3	23.3	24.2	24.7
Nitrogen (%)	9.9	9.2	9.1	9.7	9.7	9.8
Crude protein (%)	**61.8**	**57.3**	**56.6**	**60.6**	**60.6**	**61.0**
Gross energy (MJ kg^{-1})	24.5	25.1	24.8	24.7	24.3	24.7
NFE* (%)	6.9	11.5	12.4	8.2	7.1	6.5
Ytt (mg kg^{-1})	786.6	782.3	801.5	766.3	793.7	752.8
CP:GE ratio	25.2	22.9	22.8	24.5	24.9	24.7
Apparent Digestibility Coefficient						
Dry Matter	0.739	0.709	0.705	0.725	0.733	0.732
Protein	0.848	0.844	0.830	0.858	0.859	0.858
Fat	0.884	0.918	0.879	0.914	0.883	0.848
Energy	0.863	0.873	0.881	0.887	0.879	0.871
FAME (mg g^{-1} lipid)						
Saturated	284.0	286.0	317.3	300.3	304.1	301.4
Mono-unsaturated	223.3	226.9	232.6	218.8	-	219.0
n-3	195.8	200.0	205.2	193.5	-	194.8
n-6	118.5	120.2	127.0	118.8	-	119.5
Total	821.6	833.1	882.1	831.3	-	834.6
n3:n6 ratio	1.7	1.7	1.6	1.6	-	1.6

#Soy protein concentrate = Selecta; *NFE by difference.

Table 3.1.6.1.3. Estimated amino acid composition of diets based on raw material chemistry (dry basis).

Amino acid (%)	Fishmeal control	Soybean meal control	Brewer's yeast	Inulin	Protexin®	Pro(N8)ure®
Alanine	3.06	2.79	2.84	2.79	2.79	2.79
Arginine	2.89	2.93	2.98	2.93	2.93	2.93
Aspartic acid	4.64	4.91	5.00	4.91	4.91	4.91
Cysteine	0.88	0.97	0.97	0.97	0.97	0.97
Glutamic acid	6.57	7.40	7.55	7.40	7.40	7.40
Glycine	3.38	2.99	3.03	2.99	2.99	2.99
Histidine	1.86	1.42	1.44	1.42	1.42	1.42
Isoleucine	2.25	2.19	2.23	2.19	2.19	2.19
Leucine	4.04	4.11	4.17	4.11	4.11	4.11
Lysine	3.54	3.29	3.35	3.29	3.29	3.29
Methionine	2.00	1.90	1.91	1.90	1.90	1.90
Phenylalanine	2.35	2.47	2.51	2.47	2.47	2.47
Proline	2.77	2.96	3.00	2.96	2.96	2.96
Serine	2.79	2.93	2.96	2.92	2.93	2.93
Taurine	0.63	0.69	0.70	0.69	0.69	0.69
Threonine	2.43	2.24	2.28	2.24	2.24	2.24
Tyrosine	1.91	1.80	1.83	1.80	1.80	1.80
Valine	2.88	2.74	2.79	2.74	2.74	2.74
∑ Amino acids	**50.86**	**50.72**	**51.54**	**50.71**	**50.72**	**50.72**

Table 3.1.6.1.4. Biometric performance criteria of Yellowtail Kingfish measured at stocking and harvest (70 day experiment).

Treatment	Stock wt (g)	Stock FL (mm)	*K*-factor stock	Harvest wt* (g)	Harvest FL* (mm)	*K*-factor harvest	SGR* (% d^{-1})	Relative feed intake (g $kgBW^{-1}$ d^{-1})	Relative gain (g $kgBW^{-1}$ d^{-1})	FCR	HSI (%)	PER
Fishmeal control	81.2	185.3	1.28	383.8	302.6	1.37	2.22	27.6	24.5	1.13 ^{b}	0.76	1.74^{a}
Soybean meal control	81.3	185.6	1.28	400.9	309.2	1.35	2.28	25.1	25.3	0.99 ^{a}	0.78	1.99^{b}
Brewer's yeast	80.8	186.8	1.24	403.8	310.9	1.32	2.29	25.8	24.5	1.02 ^{ab}	0.82	1.91^{ab}
Inulin	81.7	185.7	1.28	418.3	315.8	1.31	2.33	26.2	26.0	1.01 ^{a}	0.77	1.96^{b}
Protexin®	81.3	187.0	1.25	387.9	308.6	1.30	2.23	26.2	24.6	1.07 ^{ab}	0.77	1.85^{ab}
Pro(N8)ure®	81.6	185.8	1.28	410.7	310.9	1.34	2.30	26.6	25.6	1.04 ^{ab}	0.69	1.90^{ab}
SEM	*0.68*	*1.59*	*0.03*	*19.64*	*5.13*	*0.02*	*0.07*	*0.56*	*0.94*	*0.03*	*0.03*	*0.04*
F-value	*0.23*	*0.20*	*0.30*	*0.45*	*0.70*	*2.29*	*0.39*	*2.21*	*0.39*	*3.87*	*2.07*	*4.02*
P value	*0.94*	*0.96*	*0.90*	*0.80*	*0.63*	*0.11*	*0.84*	*0.12*	*0.84*	*0.025*	*0.14*	*0.023*

*Based on surviving fish.

Table 3.1.6.1.5. Plasma biochemistry of fasted Yellowtail Kingfish recorded at harvest (70 day experiment).

Treatment	Cholesterol (mmol L^{-1})	Triglycerides (mmol L^{-1})	HDL cholesterol (mmol L^{-1})	Non HDL cholesterol (mmol L^{-1})	Total cholesterol / HDL ratio	Total protein (g L^{-1})	AST (U L^{-1})	LD (U L^{-1})	Glucose (mmol L^{-1})	Lactate (mmol L^{-1})
Fishmeal control	6.97	1.67	1.40	5.60	5.10	37.77	96.67	274.37	5.10	3.23
Soybean meal control	6.03	1.17	1.37	4.70	4.50	36.67	160.57	442.90	5.40	3.23
Brewer's yeast	6.47	1.47	1.43	5.03	4.67	38.13	155.53	372.6	5.70	2.33
Inulin	6.33	1.43	1.43	4.93	4.47	37.20	97.13	233.50	5.77	3.83
Protexin®	6.40	1.50	1.37	5.07	4.73	35.60	135.0	447.47	5.27	3.37
Pro(N8)ure®	6.30	1.37	1.37	4.90	4.60	39.97	130.23	368.43	5.20	3.47
SEM	*0.28*	*0.19*	*0.05*	*0.27*	*0.21*	*1.37*	*32.31*	*84.53*	*0.32*	*0.61*
F-value	*1.21*	*0.71*	*0.53*	*1.27*	*1.22*	*0.53*	*0.73*	*1.06*	*0.71*	*0.66*
P value	*0.36*	*0.63*	*0.75*	*0.34*	*0.36*	*0.75*	*0.62*	*0.43*	*0.63*	*0.66*

Table 3.1.6.1.6. Sample information pertaining to the bioactives trial microbiome component with rectal swab samples collected from Yellowtail Kingfish across six diets.

Sample type	Fork length (cm)	Weight (g)	Location	Site	Tank ID	Date sample collected	library size	# bacterial OTUs
FMC	31.1	356	NSW	PSFI	Tank 2	16/10/18	83016	443
FMC	28.2	309	NSW	PSFI	Tank 2	16/10/18	98694	354
FMC	32.1	418	NSW	PSFI	Tank 2	16/10/18	19062	294
FMC	31.5	381	NSW	PSFI	Tank 7	16/10/18	32988	239
FMC	31.2	467	NSW	PSFI	Tank 7	16/10/18	74332	271
FMC	32.9	474	NSW	PSFI	Tank 7	16/10/18	38782	328
FMC	33.3	512	NSW	PSFI	Tank 13	16/10/18	27190	268
FMC	29.4	328	NSW	PSFI	Tank 13	16/10/18	42803	221
FMC	29.5	333	NSW	PSFI	Tank 13	16/10/18	33149	273
SBMC	28.4	341	NSW	PSFI	Tank 5	16/10/18	39349	148
SBMC	27.2	296	NSW	PSFI	Tank 5	16/10/18	46219	339
SBMC	32.4	385	NSW	PSFI	Tank 5	16/10/18	82333	173
SBMC	28.4	330	NSW	PSFI	Tank 6	16/10/18	97713	233
SBMC	33.1	500	NSW	PSFI	Tank 6	16/10/18	219525	180
SBMC	32.2	471	NSW	PSFI	Tank 14	16/10/18	65006	404
SBMC	32.3	457	NSW	PSFI	Tank 14	16/10/18	51338	368
SBMC	32.6	496	NSW	PSFI	Tank 14	16/10/18	17243	374
Yeast	31.9	470	NSW	PSFI	Tank 1	16/10/18	89203	258
Yeast	32.2	430	NSW	PSFI	Tank 1	16/10/18	250644	356
Yeast	31.1	393	NSW	PSFI	Tank 1	16/10/18	187465	304
Yeast	32.9	417	NSW	PSFI	Tank 4	16/10/18	92996	188
Yeast	30.9	393	NSW	PSFI	Tank 4	16/10/18	31380	224
Yeast	32.1	396	NSW	PSFI	Tank 4	16/10/18	98144	296
Yeast	33.0	488	NSW	PSFI	Tank 11	16/10/18	29502	312
Yeast	30.1	382	NSW	PSFI	Tank 11	16/10/18	4183	170
Yeast	30.0	481	NSW	PSFI	Tank 11	16/10/18	9711	310
Inulin	29.2	284	NSW	PSFI	Tank 10	16/10/18	29442	168
Inulin	30.3	318	NSW	PSFI	Tank 10	16/10/18	55274	202
Inulin	32.1	426	NSW	PSFI	Tank 10	16/10/18	143498	339
Inulin	34.3	513	NSW	PSFI	Tank 16	16/10/18	37103	306
Inulin	34.0	55	NSW	PSFI	Tank 16	16/10/18	34185	366
Inulin	31.6	440	NSW	PSFI	Tank 16	16/10/18	19903	190
Inulin	32.1	433	NSW	PSFI	Tank 18	16/10/18	32990	249
Inulin	30.3	368	NSW	PSFI	Tank 18	16/10/18	47550	380
Inulin	33.2	467	NSW	PSFI	Tank 18	16/10/18	58729	275
Protexin®	30.1	336	NSW	PSFI	Tank 3	16/10/18	88079	241
Protexin®	27.1	273	NSW	PSFI	Tank 3	16/10/18	81637	387
Protexin®	28.6	325	NSW	PSFI	Tank 3	16/10/18	49804	219
Protexin®	33.4	443	NSW	PSFI	Tank 9	16/10/18	23318	232
Protexin®	31.3	417	NSW	PSFI	Tank 9	16/10/18	21441	282
Protexin®	29.3	360	NSW	PSFI	Tank 9	16/10/18	3237	119
Protexin®	33.2	539	NSW	PSFI	Tank 12	16/10/18	60604	391
Protexin®	35.2	580	NSW	PSFI	Tank 12	16/10/18	30001	448
Protexin®	30.8	406	NSW	PSFI	Tank 12	16/10/18	41845	336
Pro(N8)ure®	31.1	381	NSW	PSFI	Tank 8	16/10/18	59452	180
Pro(N8)ure®	31.1	504	NSW	PSFI	Tank 8	16/10/18	32488	453
Pro(N8)ure®	33.9	552	NSW	PSFI	Tank 15	16/10/18	40282	375
Pro(N8)ure®	34.3	596	NSW	PSFI	Tank 15	16/10/18	107974	377
Pro(N8)ure®	33.4	468	NSW	PSFI	Tank 15	16/10/18	58239	478

Sample type	Fork length (cm)	Weight (g)	Location	Site	Tank ID	Date sample collected	library size	# bacterial OTUs
Pro(N8)ure®	34.8	552	NSW	PSFI	Tank 17	16/10/18	46473	364
Pro(N8)ure®	30.4	382	NSW	PSFI	Tank 17	16/10/18	77022	165
Pro(N8)ure®	33.4	407	NSW	PSFI	Tank 17	16/10/18	22521	274

Abbreviations: FMC, fishmeal (+ve) control; NSW, New South Wales; PSFI, Port Stephens Fisheries Institute; SBMC, soybean meal (-ve) control.

Table 3.1.6.1.7. Summary of sequenced sample parameters.

Data-set	No. of samples	Total reads	Median library size	Range	Bacterial OTUs post- filtering
Bioactives NSW	52	3,334,090	49,434	3,269-266,328	611

Table 3.1.6.1.8. One-way PERMANOVA: Pairwise test between the bioactives trial A) two control diets, B) five soybean meal diets including the negative control diet compared to the four treatment diets and C) four soybean meal bioactives diets with Yellowtail Kingfish.[1,2]

Sample type	P	Significant?
A) control diets, no bioactives		
FMC, SBMC	0.6130	No
B) soybean meal diets		
SBMC, yeast	0.4286	No
SBMC, inulin	0.9593	No
SBMC, PRTX	0.8084	No
SBMC, PRN8	0.7565	No
C) bioactives diets		
yeast, inulin	0.3680	No
yeast, PRTX	0.1649	No
yeast, PRN8	0.4230	No
inulin, PRTX	0.5866	No
inulin, PRN8	0.9105	No
PRTX, PRN8	0.4689	No

Abbreviations: FMC, fishmeal (+ve) control; PRN8, Pro(N8)ure®; PRTX, Protexin®; SBMC, soybean meal (-ve) control.
[1]Control diets: fishmeal (+ve) and soybean meal (-ve); treatment diets: brewer's yeast, inulin, Protexin® and Pro(N8)ure®.
[2]Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.1.6.1.9. ANOVA results for diversity indices comparing NSW bioactives trial samples from two control and four trial diets.[1,2]

Diversity measure	ANOVA summary[3]	Tukey's posthoc test[4]	Adjusted P-value
Species richness (S)	F=0.5797 *P*=0.7152		
Pielou's evenness (J')	F=0.6147 *P*=0.6891		
Shannon's diversity (H')	F=0.5962 *P*=0.7029		
Simpson's diversity (1-λ)	F=0.6193 *P*=0.6857		
Delta+ (Δ+)	F=3.931 ***P*=0.0047**	FMC vs inulin yeast vs inulin PRTX vs inulin	**0.0166** **0.0172** **0.0061**
Lambda+ (λ+)	F=2.641 *P*=0.5673		

Abbreviations: FMC, fishmeal (+ve) control; PRN8, Pro(N8)ure®; PRTX, Protexin®; SBMC, soybean meal (-ve) control.
[1] Control diets: fishmeal (+ve) and soybean meal (-ve); treatment diets: brewer's yeast, inulin, Protexin® and Pro(N8)ure®.
[2] Significant difference denoted by $P < 0.05$, bolded if significant.
[3] Where a significant difference ($P < 0.05$) is observed then the Tukey's pairwise test was performed.
[4] Only significant pairwise comparisons are shown.

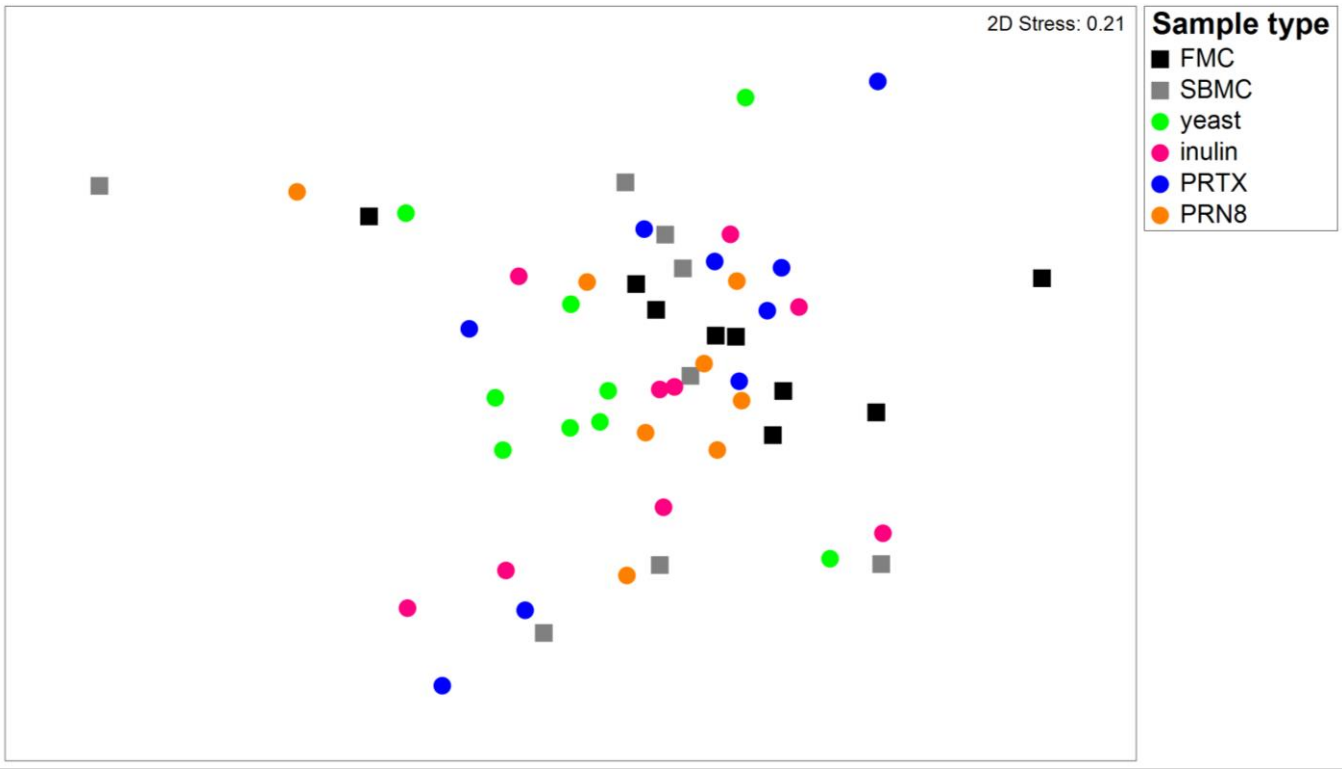


Figure 3.1.6.1.1. Difference between the global community structure of all 52 samples from the bioactives trial control and treatment diets as analysed by non-metric multidimensional scaling (nMDS).[1,2]
Abbreviations: FMC, fishmeal (+ve) control; PRN8, Pro(N8)ure®; PRTX, Protexin®; SBMC, soybean meal (-ve) control
[1]Rectal swab samples from nine YTK on control and treatment diets, including fishmeal (+ve) control, soybean meal (-ve) control, brewer's yeast, inulin, Protexin® and Pro(N8)ure®.

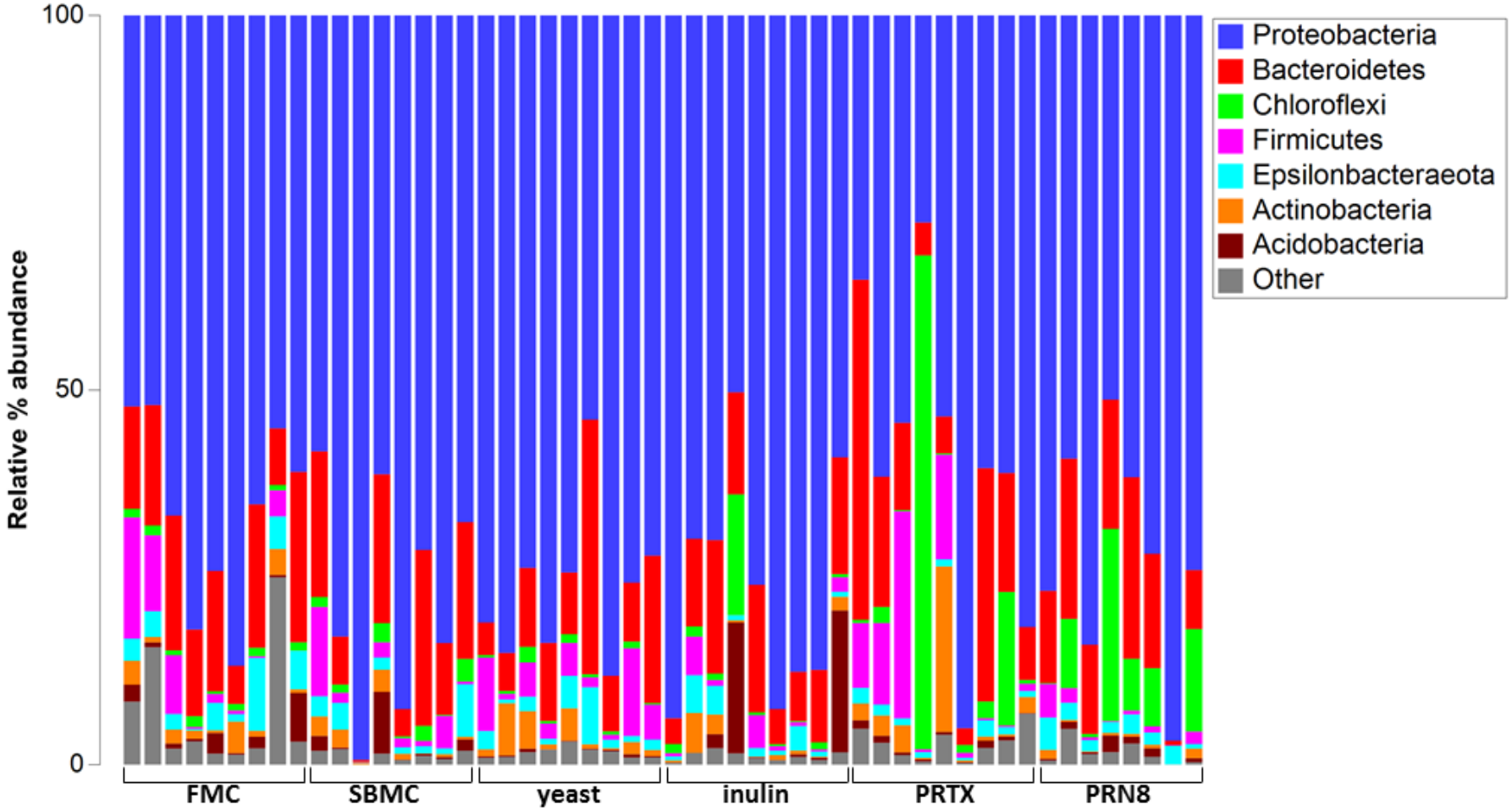


Figure 3.1.6.1.2. Relative percent abundance of bacterial phyla associated with rectal swab samples from Yellowtail Kingfish from the bioactives trial control and treatment diets.[1]

Abbreviations: FMC, fishmeal (+ve) control; PRN8, Pro(N8)ure®; PRTX, Protexin®; SBMC, soybean meal (-ve) control

[1]Control diets: fishmeal (+ve) and soybean meal (-ve); treatment diets: brewer's yeast, inulin, Protexin® and Pro(N8)ure®.

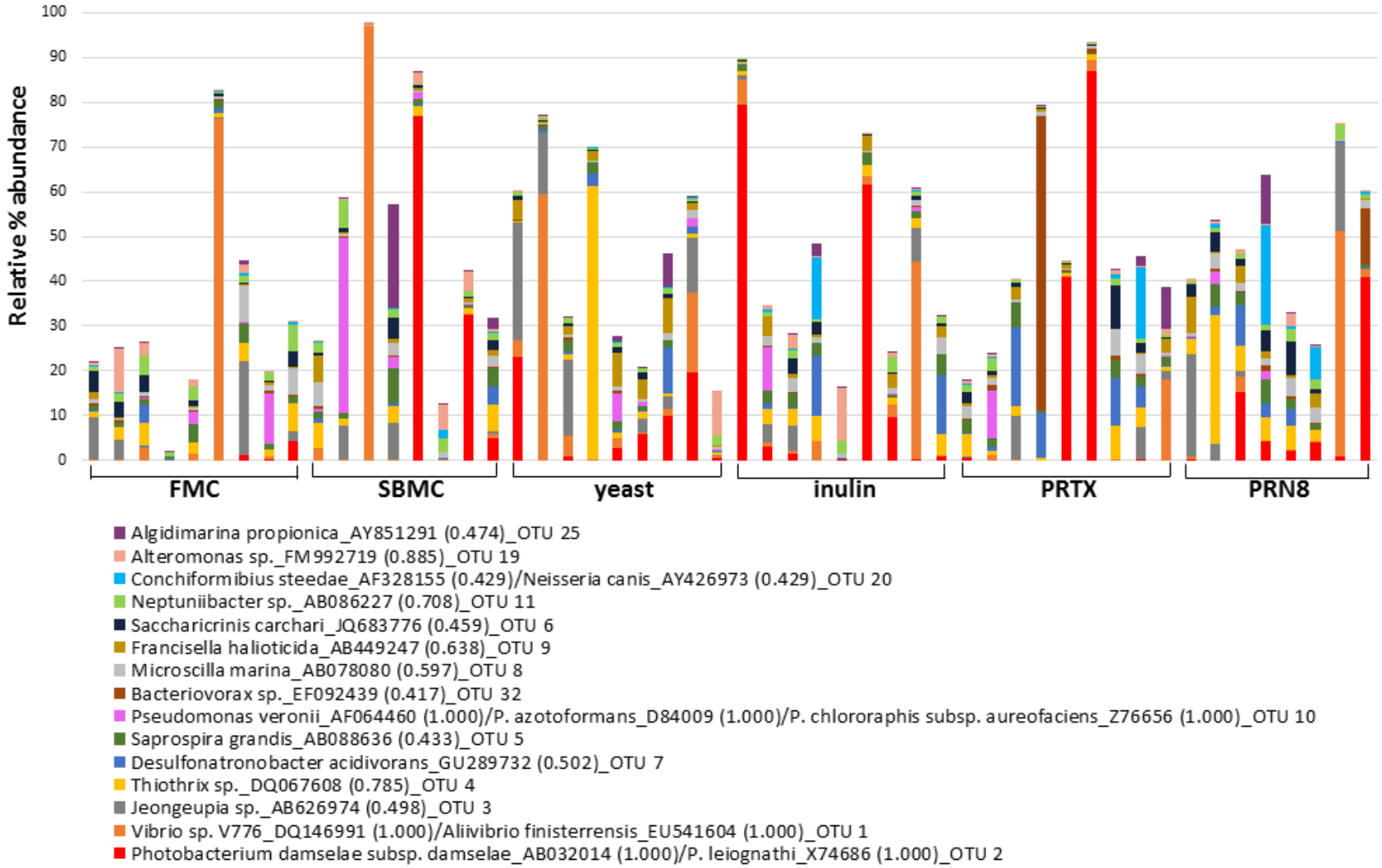


Figure 3.1.6.1.3. Relative percent abundance of the 15 most abundant bacterial OTUs in the rectal swab samples from Yellowtail Kingfish from the bioactives trial control and treatment diets.[1]

Abbreviations: FMC, fishmeal (+ve) control; PRN8, Pro(N8)ure®; PRTX, Protexin®; SBMC, soybean meal (-ve) control.

[1]Control diets: fishmeal (+ve) and soybean meal (-ve); treatment diets: brewer's yeast, inulin, Protexin® and Pro(N8)ure®.

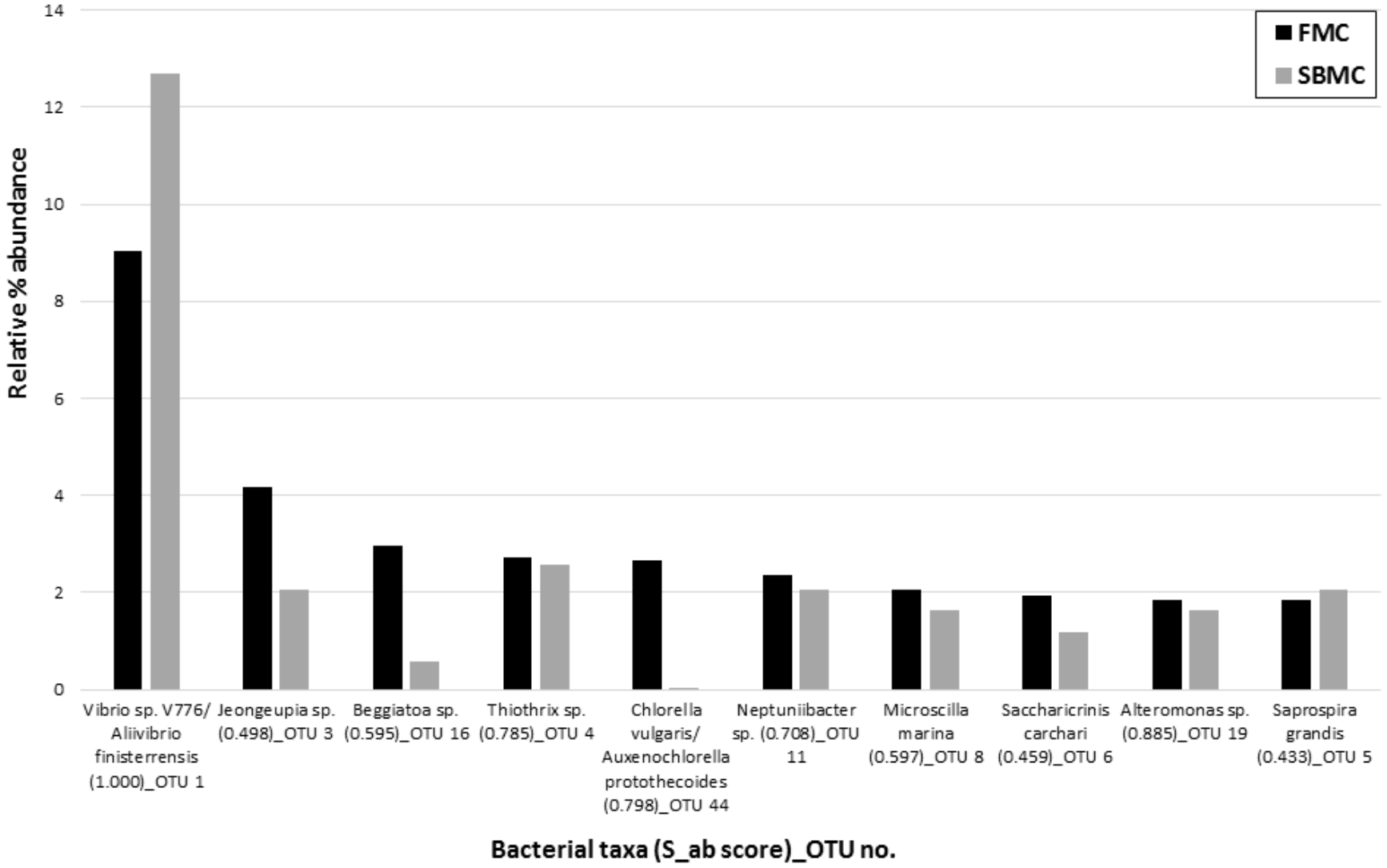


Figure 3.1.6.1.4. Relative percent abundance of the 10 most abundant bacterial OTUs in the fishmeal positive control diet samples in comparison to the corresponding abundances for these OTUs in the soybean meal negative control diet samples.[1]
Abbreviations: FMC, fishmeal (+ve) control; SBMC, soybean meal (-ve) control.

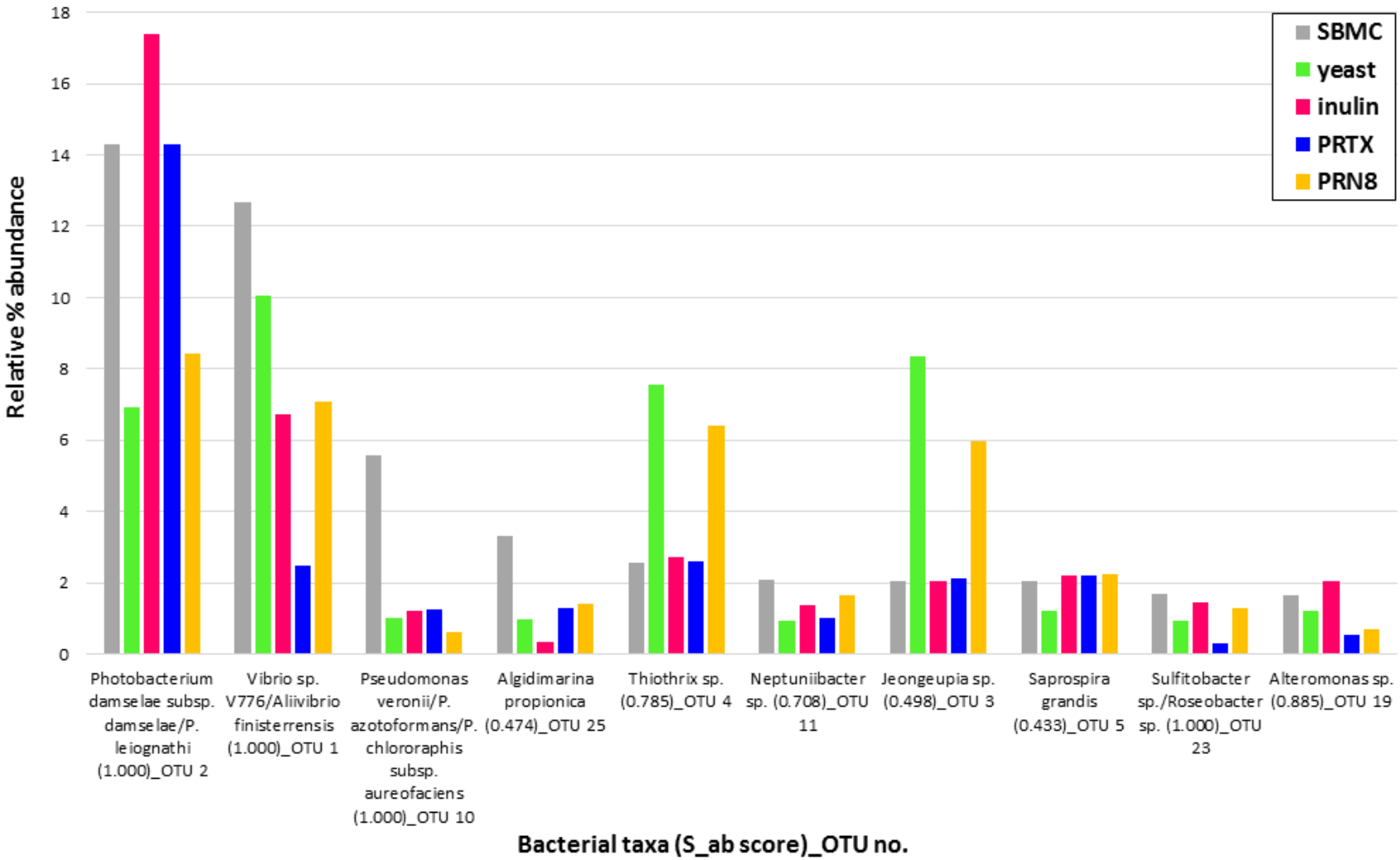


Figure 3.1.6.1.5. Relative percent abundance of the 10 most abundant bacterial OTUs in the soybean meal negative control diet samples in comparison to the corresponding abundances for these OTUs in the four soybean meal bioactives diets.[1]

Abbreviations: PRN8, Pro(N8)ure®; PRTX, Protexin®; SBMC, soybean meal (-ve) control.

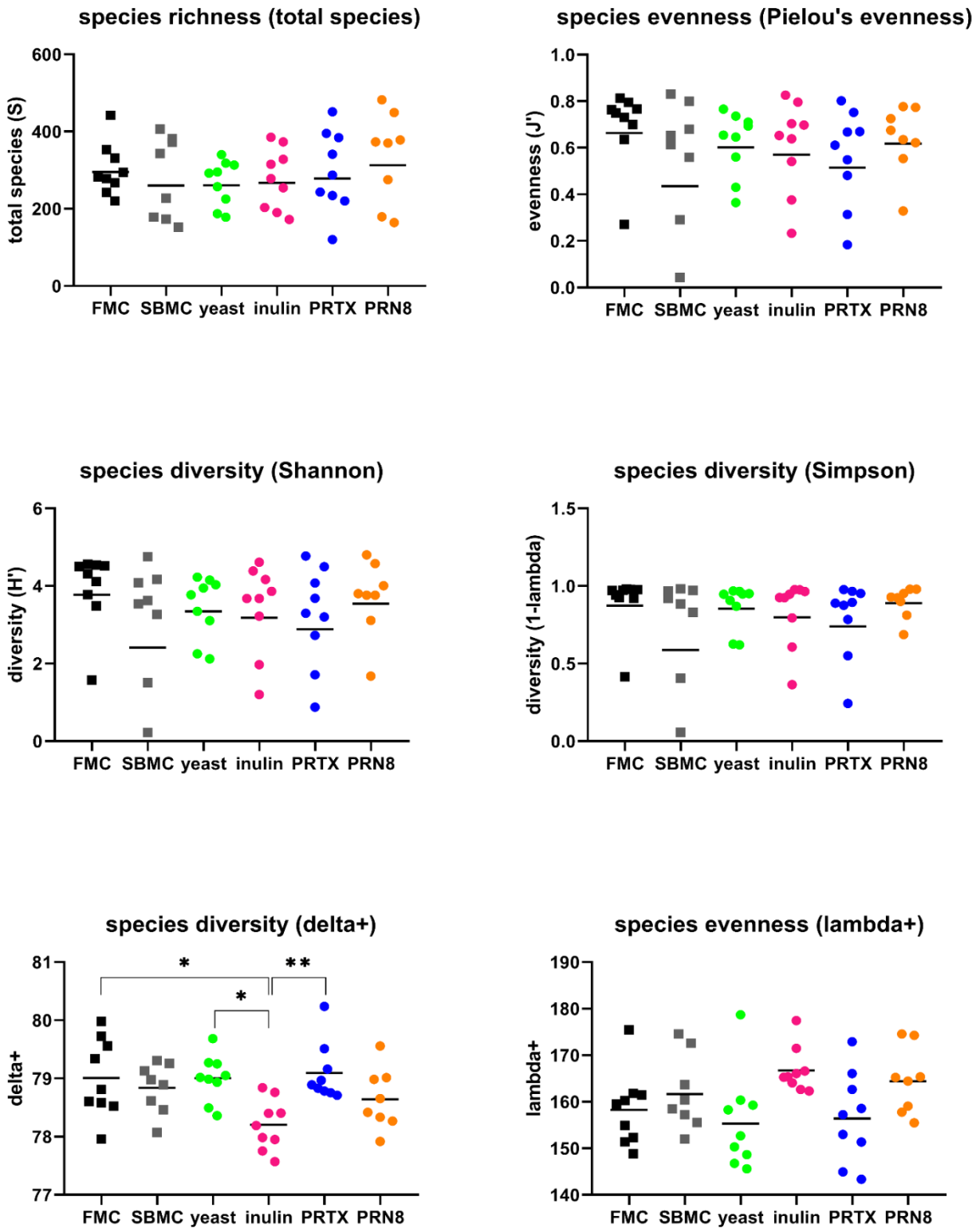
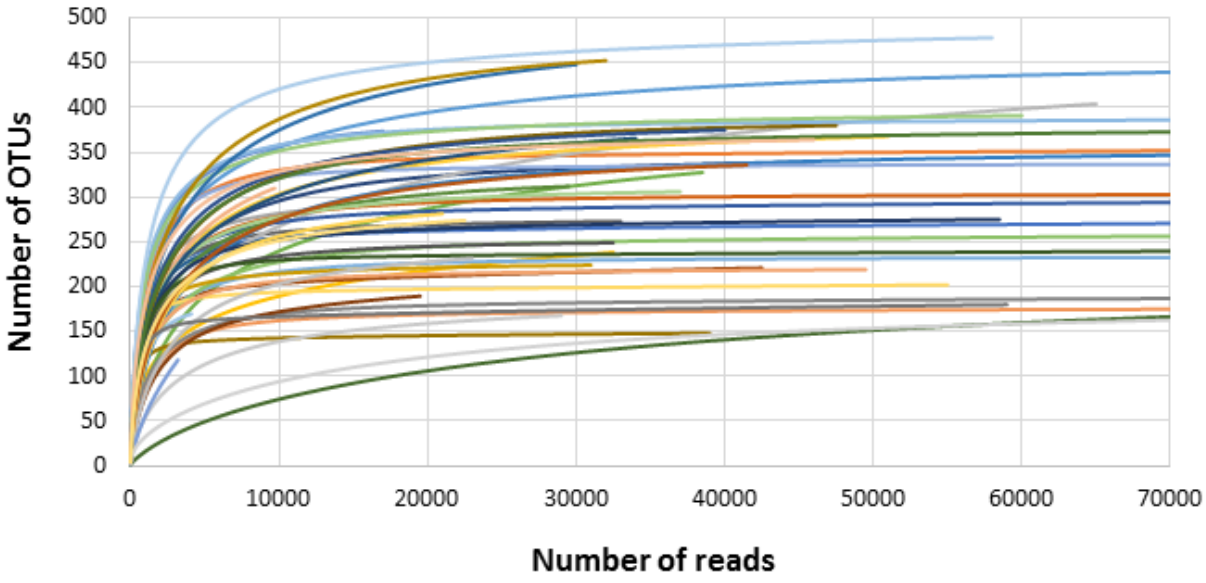


Figure 3.1.6.1.6. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for the bioactives trial control and treatment diets.[1]

Abbreviations: FMC, fishmeal (+ve) control; PRN8, Pro(N8)ure®; PRTX, Protexin®; SBMC, soybean meal (-ve) control.

[1]Mean values are plotted for each of the groups of interest (FMC vs SBMC vs yeast vs inulin vs Protexin® vs Pro(N8)ure®).

Appendix 3.1.6.1.1. Rarefaction curves portraying the number of resolved OTUs against sequencing depth of each sample collected from the bioactives trial.



3.2. Theme - Feeding Strategies

3.2.1. Chapter - Effects of oxygenation for large Yellowtail Kingfish.

3.2.1.1. Manuscript - Intermittent feed-induced hypoxia effects the growth and feed utilisation of large Yellowtail Kingfish (Seriola lalandi) at summer water temperatures.

Matthew S. Bansemer [a], David A.J. Stone [a, b, c, d], Aaron Y.L. Teoh[b], Paul Skordas [a]

[a] South Australian Research and Development Institute, South Australian Aquatic Sciences Centre, West Beach, SA 5024, Australia

[b] Flinders University, College of Science and Engineering, GPO Box 2100, Adelaide, SA 5001, Australia

[c] University of Adelaide, School of Animal and Veterinary Sciences, Roseworthy, SA 5371, Australia

[d] Marine Innovation Southern Australia

This manuscript may be referenced as: Bansemer, M.S., Stone, D.A.J., Teoh, A.Y.L. and Skordas, P. (2019). 3.2.1.1. Manuscript - Intermittent feed-induced hypoxia effects the growth and feed utilisation of large Yellowtail Kingfish (*Seriola lalandi*) at summer water temperatures (FS2, Output 3a). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.261-280.

Abstract

Hypoxic conditions during feeding and post-feeding are problematic for the Yellowtail Kingfish (*Seriola lalandi*; YTK) industry, particularly during periods of low tidal movement. In this 85 day study, the effect of dissolved oxygen saturation level and intermittent feed-induced hypoxia (during feeding and post-feeding) on the growth, feed utilisation and health of large YTK (2.15 kg) were investigated. Hypoxia may be defined as any level of dissolved oxygen low enough to negatively impact the behaviour and physiology of an organism. Fish were exposed to four dissolved oxygen saturation treatments, (1) 100% constant; (2) 85% constant; (3) Held at 85%, with a 3 h hypoxic event (60%) implemented daily post-feeding to simulate a daily feed induced-hypoxic event; and (4) Held at 85%, with a 3 h hypoxic event (60%) post-feeding implemented twice fortnightly (day 13 and 14 of each fortnight) to simulate feed induced-hypoxic event during twice fortnightly periods of low tidal flow (dodge/neap tide simulation). Fish were fed a commercial extruded diet to apparent satiation once daily at 09:00 h. YTK exposed to intermittent feed induced-hypoxia (Treatment 4) exhibited significantly reduced specific growth rate (SGR) and feed conversion ratio (FCR), compared to fish exposed to other oxygen treatments, which did not differ. However, there was a tendency for SGR to decrease from 100% constant to 85% constant to 85% (when dropped to 60% daily). Results from a linked Honours project also indicated that YTK exposed to the dodge tide simulation treatment tended to have reduced dietary dry matter, protein and energy digestibility and were under increased oxidative stress. This study suggests that YTK may be able to adapt to consistent environmental conditions, including consistent daily hypoxic events, while fish exposed to intermittent hypoxic events exhibit inferior growth and feed utilisation. In terms of feed management, commercial producers of YTK may be able to mitigate exposure to intermittent feed-induced hypoxic events by selecting sites with adequate water flow or by utilising nets with larger mesh sizes to allow high water exchange. Consideration also needs to be given to feeding practices in relation to infrequent farm management practices including disease treatments and weight checks. Further research in pilot scale commercial on-farm trials are needed to validate potential feed management solutions.

Introduction

Yellowtail Kingfish (*Seriola lalandi*; YTK) aquaculture is a rapidly developing global industry. YTK have a higher metabolic rate and energy and oxygen demand than other aquaculture species, including Mulloway (*Argyrosomus japonicas*) and Barramundi (*Lates calcarifer*) (Partridge et al., 2003; Pirozzi and Booth, 2009; Gamble et al., 2014) and as a result typically consume more feed and oxygen. In Australia, YTK are primarily cultured in sea-cage systems, which experience fluctuating dissolved oxygen levels that are influenced by a number of factors including water temperature, tidal flow, stocking densities and feed rate (Boywer et al., 2014).

Hypoxic conditions are problematic for the YTK industry, particularly during dodge tides, high stocking densities or if fish are overfed. Hypoxia may be defined as any level of dissolved oxygen low enough to negatively impact the behaviour and physiology of an organism (Pollock et al., 2007). Hypoxic conditions can become critical, especially during periods of high summer water temperatures (> 24 °C) when combined with hyersaline conditions (Stone et al., 2014), due to the temperature-dependent solubility of oxygen (Barnes et al., 2011; Bowyer et al., 2014). Some fish species are able to maintain a constant metabolic rate during hypoxic events by increasing respiratory volume or down regulating aerobic or anaerobic metabolic pathways. In contrast, YTK have a predominately aerobic metabolic scope (Clark and Seymour, 2006; Nilsson and Ostlund-Nilsson, 2008; Dong et al., 2011) and as a results, when exposed to chronic hypoxic conditions exhibit reduced survival, growth, feed intake and efficiency, compared to fish at normoxic dissolved oxygen levels (Bowyer et al., 2014). While the effect of chronic hypoxia on the growth performance, feed utilisation and digestive physiology of small YTK (< 100 g) has been evaluated (Bowyer et al., 2014), acute hypoxia during and post-feeding is a major concern for the production of large YTK (> 2 kg).

Aim

The aim of the current study was to determine the effect of low oxygen saturation levels during feeding and post-feeding on the growth, feed utilisation and health of large YTK (> 2 kg) at summer water temperatures. In this study, YTK were exposed to four oxygen saturation treatments, (1) 100% constant; (2) 85% constant; (3) Held at 85%, with a 3 h hypoxic event (60%) implemented daily post-feeding to simulate a daily feed induced-hypoxic event; and (4) Held at 85%, with a 3 h hypoxic event (60%) post-feeding implemented twice fortnightly (day 13 and 14 of each fortnight) to simulate feed induced-hypoxic event during twice fortnightly periods of low tidal flow (simulate dodge/neap tide). Dodge/neep tides typically occur on two consecutive days each fortnight in the Spencer Gulf, South Australia (Bureau of Meteorology, 2016).

Methods

Experimental treatments and feeding

In the current study, four oxygen supplementation treatments were investigated (Figure 3.2.1.1.1):

- Treatment 1: 100% saturation constant;
- Treatment 2: 85% saturation constant;
- Treatment 3: Held at 85% saturation, but before feeding the oxygen set point was manually changed to 60% saturation daily and held for 3 h before the oxygen set point was manually changed back to 85% saturation; and
- Treatment 4: Held at 85% saturation, but twice fortnightly (Day 13 and 14), oxygen set point was manually changed to 60% saturation before feeding and held for 3 h before manually set back to 85% saturation.

Oxygen levels were controlled using an OxyGuard® Pacific Commander system (OxyGuard International A/S, Birkerød, Denmark), 2 × Power Unit (C11PWS5) and 2 × Pacific Combi units (C11PCO). Specifically, a dissolved oxygen probe, which read the dissolved oxygen saturation levels every 20 secs, was immersed adjacent to the overflow point of the tank. When dissolved oxygen levels

dropped below or rose above the set-point (treatment dependent), the OxyGuard system opened or closed a solenoid valve, respectively, which supplied industrial grade oxygen (BOC Pty. Ltd., South Australia, Australia) through a fine air-bubble diffuser (100-500 µm) to the tanks. Tanks were supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated sea water at ambient temperature. All tanks were supplied with aeration and oxygenation throughout the study.
Fish were fed a commercial extruded diet (Ridley Cleanseas 2014 Pelagic Sink 9 mm pellet diameter [Product code: 107595; Run: B629452 and B614279]) for 85 days (Table 3.2.1.1.1; Table 3.2.1.1.2). Fish were fed to apparent satiation at 09:00 h daily, which involved feeding fish for four minutes $tank^{-1}$ or until a feed refusal response was observed. Feed input was recorded daily. Tanks were cleaned daily.

Experimental fish

Experimental work was conducted in the pool-farm facility at the South Australian Research and Development Institute, South Australia Aquatic Science Centre (SARDI SAASC; West Beach, South Australia, Australia). YTK were obtained from Clean Seas Seafood (Port Lincoln, South Australia, Australia). Upon arrival at the SARDI SAASC facility, YTK were transferred to 5000 L tanks supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated, aerated sea water at ambient temperature and held for ~3 months and fed the Ridley Cleanseas 2014 Pelagic Sink 9 mm pellet diameter diet.

Skin and gill fluke treatment

Upon arrival at SARDI SAASC, YTK were inspected, and were observed to have a low burden of skin flukes (*Benedenia seriola*) and gill flukes (*Zeuxapta seriola*). Treatment was necessary, and was prescribed by Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd.). Prior to the commencement of the experiment, fish were exposed to two treatments (16/11/15 and 30/11/15) of formalin (250 ppm for 30 min) at 19-22 °C.

Experimental Stocking

At the commencement of the experiment (February 2016), YTK (n = 300; initial weight 2.15 ± 0.17 kg; initial fork length 514 ± 14 mm; mean ± standard deviation) were anaesthetised in 5000 L tanks (total water volume 2500 L) using AQUI-S® (AQUI-S® New Zealand Ltd., Lower Hutt, New Zealand) at a concentration of 14 mg L^{-1} of seawater. Twenty five fish were removed from their holding tank, measured, weighed and stocked into one of the three replicate 5000 L tanks $treatment\ combination^{-1}$ (n = 4 treatments; n = 12 tanks).

Intermediate weight checks

At four weeks and eight weeks post-stocking, all fish were anaesthetised using AQUI-S® at a concentration of 14 mg L^{-1} of seawater. YTK were measured, weighed and visually inspected for skin and gill flukes, before fish were returned back to their respective tanks.

Water quality analyses

Water quality parameters were measured daily at 14:30 h and maintained at appropriate levels for acceptable growth of YTK throughout the study (Table 3.2.1.1.3). Water temperature was measured using a thermometer. Dissolved oxygen (mg L^{-1} and % saturation) was measured using a dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark). The pH was measured daily using a meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, Illinois, United States of America). Salinity (g L^{-1}) was measured weekly using a portable salinity refractometer (model RF20, Extech Instruments, Nashua, New Hampshire, United States of America).Ammonia was measured daily using an Aquarium test kit (Ammonia NH_3/NH_4^+ test kit, product #LR8600; Aquarium Pharmaceuticals,

Chalfont, Pennsylvania, United States of America).

Performance indices

All data reported for each treatment for animal performance were based on the mean of the replicate tanks. All calculations using fish weight and diets were based on wet or as fed values, respectively:

- Weight gain = final weight - initial weight
- Biomass gain (kg $tank^{-1}$) = (final weight + ∑mortality weight) - (initial weight + ∑replacement weight)
- Specific growth rate (SGR, % d^{-1}) = ([ln final weight - ln initial weight] / d) × 100
- Length growth rate (mm d^{-1}) = (final fish length - initial fish length) / d
- Condition factor = (fish weight [g] / fish length $[cm]^3$) × 100
- Apparent feed conversion ratio (FCR) = feed consumed / fish weight gain
- Apparent protein deposition = ([final whole protein - initial whole protein] / protein intake) × 100
- Apparent energy deposition = ([final whole energy - initial whole energy] / energy intake) × 100
- Visceral index (VSI; %) = wet visceral wt × 100 / final wet fish wt
- Hepatosomatic index (HSI; %) = wet liver wt × 100 / final wet fish wt
- Haematocrit count = red blood cell (mm) / total blood (red blood cell and plasma [mm]) 100 × 100

Biochemical and histological analyses

The proximate composition analyses of whole body tissue were conducted according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardization (DIN) (2000). One kg sample of the commercial diet was collected, homogenised and stored frozen at -20 °C prior to analysis. The diet was analysed for proximate composition (moisture, protein, fat, ash, carbohydrate and energy), amino acid profile, taurine level, mineral composition, fatty acid profile and cholesterol level. A total of twelve fish (n = 12 fish) at the start of the experiment, and four fish from each tank (n = 4 fish $tank^{-1}$; n = 12 tanks; n = 48 fish) at the conclusion of the experiment were collected and stored frozen at -20 °C prior to analysis. Whole fish samples were partially thawed, homogenised and analysed for proximate composition (moisture, protein, fat, ash, carbohydrate and energy).

Blood samples from three fish per tank (n = 3 fish $tank^{-1}$; n = 12 tanks; n = 36 fish) were collected in Vacuette® tubes (lithium heparin) for haematology and biochemistry analysis conducted by IDEXX (Unley, South Australia, Australia). Unfortunately, despite coating needles with lithium heparin and collecting in suitable tubes, there were blood clots present in all samples. Serum biochemistry was able to be analysed; however, blood haematology was not. After discussions with IDEXX, this resulted in us changing our blood collection methodology, where we split blood samples into two separate Vacuette® or BD vacutainer ® tubes (EDTA and Z serum clot activator tubes). For histology, a 1 cm section of the second gill arch, and $1cm^3$ spleen and hindgut samples were collected from the blood sampled fish. In brief, samples were fixed in 10% seawater formalin for > 48 h, processed and embedded in paraffin wax. Tissue sections were cut using a microtome and floated onto Starfrost® glass slides and dried for > 24 h at room temperature before being stained. Gill sections were stained with hematoxylin and eosin (H and E) and were subjectively scored for telangiectasis, hyperplasic nodules, hyperplastic lamellar tips (clubbing), filament tip inflammation by Dr Fran Stephens (Aquatilia Healthcare, Western Australia, Australia). Blood fluke egg and epitheliocystis counts were also conducted. Spleen sections were also stained with hematoxylin and eosin subjectively scored for depleted haematopoietic tissue and congestion by Dr Fran Stephens. Melanomacrophages centres (per 10 fields of view) were also counted. Subjective scores for gill and spleen sections ranged from: 0, not observed; 1, rare; 2, mild; 3, moderate; to 4, severe. Hindgut sections were stained with both hematoxylin and eosin and high iron diamine/alcian blue pH 2.5 (HID/AB pH 2.5). Villus height, width, perimeter, area and branching, and total goblet cell number were measured.

Statistical analyses

IBM SPSS (version 24 for Windows; IBM SPSS Inc., USA) was used for all statistical analyses. Homogeneity of variances and normality among mean values were assessed using Levene's test for equality of variance errors and Shapiro-Wilk test, respectively. Data were compared across all treatments using a one-factor ANOVA. When significant effects were observed, post-hoc tests were used to detect significant differences between all treatments (Student-Newman-Keuls test). A significance level of $P < 0.05$ was used for all statistical tests. All values are presented as means ± standard error (SE) of the mean unless otherwise stated.

Results

General observations

In the current experiment, there were no significant differences in the initial weight and length of YTK between treatments ($P > 0.05$). The average initial weight and fork length were 2.15 ± 0.17 kg; 514 ± 14 mm (mean ± standard deviation). Fish fed actively during the experiment with no apparent differences observed between treatments. There were no mortalities and no apparent signs of diseases in the current experiment. Apart from the initial presence of gill and skin flukes, there were negligible gill and skin flukes burdens observed throughout the experiment.

Growth performance

Specific growth rate and biomass gain of YTK were significantly affected by oxygen treatment ($P < 0.05$; one-factor ANOVA; Table 3.2.1.1.4; Figure 3.2.1.1.2). Fish exposed to intermittent feed induced-hypoxia twice fortnightly (Treatment 4) had significantly reduced SGR and biomass gain compared to fish in Treatment 1, 2 and 3 (Table 3.2.1.1.4). While there were no significant differences for SGR and biomass gain for YTK in Treatment 1, 2, and 3 (100% to 85%, and 85%, dropped to 60% daily for 3 h), there was a tendency for SGR and biomass gain to decrease in fish at 100% to 85% to 85% (dropped to 60% daily) (Figure 3.2.1.1.2). Final weight and final fork length, length growth rate and final condition factor were not significantly affected by oxygen treatments ($P > 0.05$; one-factor ANOVA; Table 3.2.1.1.4).

Feed utilisation

Feed conversion ratio (FCR) was significantly affected by oxygen treatment (Treatment 4 > 3 = 2 = 1; $P = 0.015$; one-factor ANOVA; Table 3.2.1.1.3; Figure 3.2.1.1.3). The apparent feed consumption (kg tank^{-1}) and apparent feed intake rate (% BW d^{-1}) of YTK were not significantly influenced by oxygen treatments ($P > 0.05$; Table 3.2.1.1.3)

Whole fish proximate and energy composition

The tissue moisture (57.7-59.8% wet), protein (18.5-19.9% wet), lipid (18.8-20.7% wet) ash (2.1-2.6% wet), carbohydrate (< 1.5% wet) and energy contents (10.23-11.07 MJ kg^{-1} wet) of fish were not significantly influenced by oxygen treatments ($P > 0.05$; one-factor ANOVA; Table 3.2.1.1.4).

Nutrient utilisation

Apparent protein deposition (18.51-22.95%) and apparent energy deposition (23.20-32.05%) were not significantly affected by oxygen treatments ($P > 0.05$; one-factor ANOVA; Table 3.2.1.1.4). There was a tendency for the apparent protein deposition and apparent energy deposition of fish to be lower when

exposed to the intermittent feed induced-hypoxia twice fortnightly (Treatment 4) than those held at 100% constant oxygen saturation (Treatment 1).

Blood haematocrit and biochemistry

Blood triglyceride levels were significantly higher for YTK in Treatment 1 (100% saturation) than fish in Treatment 2, 3 and 4 ($P = 0.001$), while there was no significant different between Treatment 2, 3 and 4 ($P > 0.05$; one-factor ANOVA; Table 3.2.1.1.5). Blood bile acids were significantly higher for fish in Treatment 1 than in Treatment 4 ($P < 0.05$). There were no statistical differences for blood bile acids observed between treatments (Treatment 1= 2 = 3; Treatment 2 = 3 = 4; $P > 0.05$; Table 3.2.1.1.5). Blood hematocrit and other biochemistry factors were not significantly influenced by treatments ($P > 0.05$; Table 3.2.1.1.5).

Visceral somatic parameters, and spleen, gill and hindgut morhology

Oxygen treatment did not significantly influence viscerosomatic index (6.25-6.48%) and hepatosomatic index (0.97-1.11%; $P > 0.05$; one-factor ANOVA; Table 3.2.1.1.6).

Spleen morphology scores for depleted haematopoietic tissue and congestion were not significantly affected by oxygen treatment ($P > 0.05$; one-factor ANOVA; Table 3.2.1.1.6). Scores for both parameters were low (0-1), but variable. Oxygen treatment did not significant effect, spleen melanomacrophages centres ($P > 0.05$; one-factor ANOVA; Table 3.2.1.1.6). Spleen melanomacrophage centers appeared normal for the size of YTK tested (Dr Fran Stephens, Aquatilia Healthcare, Morangup, Western Australia; *personal communication*; Figure 3.2.1.1.4a and b).

Gill histology scores for telangiectasis, hyperplasic nodules, hyperplastic lamellar tips (clubbing) and filament tip inflammation were not significantly affected by oxygen treatment ($P > 0.05$; one-factor ANOVA; Table 3.2.1.1.6; Figure 3.2.1.1.5a and b). Scores for all of these parameters were low to moderate (0-2). The number of blood fluke eggs and epitheliocystis were not significantly influenced by oxygen treatment ($P > 0.05$).

Hindgut morphology was variable. Oxygen treatment did not significantly affect villus area, perimeter, length, breadth and branching ($P > 0.05$; one-factor ANOVA; Table 3.2.1.1.6). Goblet cell number per millimetre villi length were also not significantly affected by oxygen treatment ($P > 0.05$; one-factor ANOVA).

Discussion

Dissolved oxygen saturation level is a key environmental parameter that influences numerous aspect of fish production including, feed intake and efficiency, innate immune system and ultimately growth (Burt et al., 2014). Hypoxic conditions are problematic for the YTK industry, particularly during dodge tides, high stocking densities or if fish are overfed. Hypoxic conditions can become critical, especially during periods of high summer water temperatures (can exceed 24 °C) when combined with hyersaline conditions (Stone et al., 2014), due to the temperature-dependent solubility of oxygen (Barnes et al., 2011; Bowyer et al., 2014). In this 85 day study, we investigated the effect of dissolved oxygen saturation level and low oxygen saturation levels during feeding and post-feeding on the growth, feed utilisation and health of large YTK (2.15 kg).

YTK held constantly at levels of 100% and 85% dissolved oxygen saturation, and fish held at 85% dissolved oxygen saturation and exposed to a daily hypoxic event where oxygen saturation dropped to 60% for 3 h during feeding and post-feeding exhibited similar SGR and FCR. However, there was a tendency for SGR to decrease as the dissolved oxygen levels decreased from 100% to 85% (constant) to 85% (dropped to 60% daily) (Treatments 1 > 2 > 3). However, fish exposed to intermittent feed induced-hypoxia (Treatment 4; two days per fortnight) exhibited significantly reduced specific growth rate (SGR) and feed conversion ratio (FCR), compared to fish exposed to other oxygen treatments investigated in the current study. This suggests, at least to a point, that YTK are be able to strive to adapt to consistent daily patterns of reductions in dissolved oxygen levels, however, they lack the capacity to

adapt to less frequent and intermittent reductions. This may have severe implications for YTK production in sites exposed to dodge or neap tides.

Previous studies have observed a number of fish species are able to maintain a constant metabolic rate during hypoxic events by increasing respiratory volume or down regulating aerobic or anaerobic metabolic pathways (Clark and Seymour, 2006; Nilsson and Ostlund-Nilsson, 2008; Dong et al., 2011). This implies that some species have the capability to adapt and maintain normal growth when challenged with a hypoxic event. Although no attempt was made to measure metabolic rate in the current study, this does not appear to be the case for YTK. Specific growth rate and FCR tended to deteriorate once saturation levels of oxygen departed from 100%, and were exacerbated when fish were exposed to intermittent as opposed to regular hypoxic events. In the presence of sufficient oxygen availability, YTK have been reported to exhibit normal patterns of oxygen consumption during feeding and digestion. Partrgidge et al. (2003) reported prior to feeding, the oxygen consumption of YTK is low, but increases during feeding and throughout the day due to increased oxygen demands associated with digestive processes. However, results in the current study suggest in periods of low oxygen availability, metabolic processes related to feed intake and growth may be limited. Prior to the current study, Pirozzi and Booth (2009) hypothesised that YTK may not possess mechanisms to increase respiratory volume or regulate metabolic pathways, given the predominately aerobic metabolic scope of this fish species. It appears the responses observed in the current study support the hypothesis of Pirozzi and Booth (2009).

Teoh (2016), undertook an Honours project linked directly to the current study that investigated mitochondrial abundance (citrate synthase activity [CS]) in red muscle, liver oxidative stress and nutrient digestibility in response to oxygen saturation levels. Oxidative stress was assessed in the liver by measuring thiobarbituric acid reactive substances (TBARS) and the enzymatic activities levels of glutathione peroxidase (GPx). Teoh (2016) reported tendencies for dietary nutrient digestibility for dry matter, protein and energy to be reduced in fish exposed to Treatment 4 (dodge tide simulation) (Table 3.2.1.1.7). When exposed to dodge tide simulation (Treatment 4) YTK had a significant increase in TBARs values in the liver, indicating the fish were under increased oxidative stress (Table 3.2.1.1.7). Teoh (2016) also reported a tendency for GPx activity to decline with decreasing oxygen saturation levels, which also became significant in fish exposed to intermittent oxygen saturation levels of treatment 4 (dodge tide simulation) compared to fish kept at constant 100 or 85% oxygen saturation levels (Table 3.2.1.1.7). The reduction in GPx activity was likely a result of an imbalance in reactive oxygen species (ROS) and antioxidant defences where the antioxidant capacity was exceeded, which resulted in oxidative stress observed in fish exposed to intermittent hypoxia. The purpose of assessing CS activity in red muscle of YTK was to determine if there was any physiological response on long term intermittent hypoxia, indicated by alteration of mitochondrial abundance. Teoh (2016) found that there was a trend, although not statistically significant, of a reduction in CS activity in response to long term intermittent hypoxia dodge tide simulation treatment (Treatment 4) compared to other treatments (Table 3.2.1.1.7). This suggests that intermittent hypoxia may have compromised metabolic processes within the cells and caused oxidative stress, leading to a physiological response of decreasing mitochondrial abundance to reduce oxidative capacity. The effect of intermittent hypoxia is consistent with findings by Onukwufor et al. (2016) on hypoxia intolerant Rainbow Trout (*Oncorhynchus mykiss*) where hypoxia/reoxygenation stimulated increased ROS production and damaged mitochondrial structure. The response to the possible increased ROS production seen in this current study are also supported by studies with aquatic and terrestrial vertebrates on hypoxia inducible factors that regulate mitochondrial abundance, where mitochondrial autophagy was stimulated to reduce mitochondrial abundance and overall muscle oxidative capacity as a means of hypoxia acclimatisation (Johnston and Bernard, 1982; Zhang et al., 2008). Further research in relation to hypoxic stress is warranted for harvest sized YTK.

Conclusions and Recommendations

As hypoxia may be defined as any level of dissolved oxygen low enough to negatively impact the behaviour and physiology, results from this study indicate YTK are relatively susceptible to this condition. Feed utilisation, oxidative stress and ultimately growth of large YTK were negatively impacted by reductions in dissolved oxygen saturation levels. More so when exposed to hypoxic events on an irregular basis. This has important implications for site selection and farm management practices such as feeding leading up to weight checks, disease treatments and feeding during periods of low water

movement (dodge/neap tides), especially as water temperatures exceed 24 ºC during summer. Prior to the current study there was interest to oxygenate sea-cages by Clean Seas Seafood to improve production over summer (Dr T. D'Antignana; Clean Seas Seafood; *personal communication*). Subsequently, based on results from the current study, combined with an in-depth economic evaluation conducted by Clean Seas Seafood management, it was decided this practice was cost prohibitive and logistically impractical (Dr T. D'Antignana; Clean Seas Seafood; *personal communication*). In terms of feed management however, commercial producers of YTK may be able to mitigate exposure to feed-induced hypoxic events by adopting a number of different feeding strategies. For example, commercial producers may be able to monitor tidal movements and dissolved oxygen levels prior to feeding, and withhold feed or reduce feed rates during periods of low water movement (dodge/neap tides). However, careful consideration needs to be given to this approach, as restricting feed rates also results in reduction in YTK growth rate (Stone et al., 2016). Other strategies that may be adopted to improve dissolved oxygen levels in sea-cages include careful site selection, utilising computer modelling, such as those recently developed by the Oceanography group at SARDI SAASC, to ensure adequate water flow, or by utilising nets with larger mesh sizes to reduce fouling effects and allow higher water exchange. Further research in pilot scale commercial trials are needed to validate these hypotheses before implementing these strategies on-farm.

Findings

- The feed utilisation, oxidative stress and ultimately growth of large YTK were impacted by reductions in dissolved oxygen saturation levels. More so, when fish were exposed to irregular hypoxic events.
- This information has led to alterations in the criteria for site selection processes for YTK production in Australia. Results may also be used to adapt new improved feeding strategies to maximise YTK production.
- An overarching goal of the K4P project was to provide information to assist fish producers and feed companies to develop feeding strategies and formulate commercial diets for large YTK that would result in FCRs of < 2.2 for fish between 1.5-3.5 kg. The Manuscript provided information to develop management strategies that may aid in achieving this goal.
    - All FCRs in the current study were ≤ 1.73.
    - FCRs tended to increase (worsen) as dissolved oxygen levels decreased.
    - FCRs were significantly increased (worsened) for large YTK exposed infrequent hypoxic events, such as those observed during periods of dodge/neap tides in SA waters.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The authors would also like to acknowledge the support of the South Australian Research and Development Institute (SARDI) and Marine Innovation Southern Australia for their financial support of Assoc. Prof. David Stone and the provision of the SARDI SAASC experimental facilities at West Beach, South Australia. We would also like to thank Dr Richard Smullen and Dr Michael Salini of Ridley for

experimental diet formulation and manufacture. We would also like to thank Dr Trent D'Antignana Clean Seas Seafood for his input into experimental design. Thanks also to Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd., Ballina, New South Wales, Australia) for the diagnosis and prescription of skin and gill fluke treatments. We also thank Leigh Kuerschner, Krishna-Lee Currie, Jessica Buss, Nicole Thompson, Aaron Teoh for their technical assistance during the experiment.

References

Barnes, R., King, H., Carter, C.G., 2011. Hypoxia tolerance and oxygen regulation in Atlantic salmon, *Salmo salar* from a Tasmanian population. Aquaculture 318, 397-401.

Bowyer, J. N., Booth, M.A., Qin, J.G., D'Antignana, T., Thomson, M.J., Stone, D.A., 2014. Temperature and dissolved oxygen influence growth and digestive enzyme activities of yellowtail kingfish *Seriola lalandi* (Valenciennes, 1833). Aquaculture Research 45, 2010-2020.

Bureau of Meteorology, 2016. Dodge tide. [ONLINE] Accessed 21 March 2016. Available at: http://www.bom.gov.au/oceanography/projects/ntc/dodge/dodge.shtml.

Burt, K., Hamoutene, D., Perez-Casanova, J., Gamper, A.K., Volkoff, H., 2014. The effect of intermittent hypoxia on growth, appetite and some aspects of the immune response of Atlantic salmon (*Salmo salar*). Aquaculture Research 45, 124-137.

Clark, T.D., Seymour, R.S., 2006 Cardiorespiratory physiology and swimming energetics of a high-energy-demand teleost, the yellowtail kingfish (*Seriola lalandi*). Journal of Experimental Biology 209, 3940-3951.

Dong, X.Y., Qin, J.G., Zhang, X.M., 2011. Fish adaptation to oxygen variations in aquaculture: from hypoxia to hyperoxia. Journal of Fisheries and Aquaculture 2, 23-28.

Gamble, S., Carton, A.G., Pirozzi, I., 2014. Open-top static respirometry is a reliable method to determine the routine metabolic rate of barramundi, *Lates calcarifer*. Marine and Freshwater Behaviour and Physiology 47, 19-28.

Johnston, I.A., Bernard, L.M., 1982. Routine oxygen consumption and characteristics of the myotomal muscle in tench: effects of long-term acclimation to hypoxia. Cell Tissue Research 227, 161-177.

Nilsson, G, E., Ostlund-Nilsson, S., 2008. Does size matter for hypoxia tolerance in fish. Biological Reviews 83, 173-189.

Onukwufor, J.O., Kibenge, F., Stevens, D., Kamunde, C., 2016. Hypoxia-reoxygenation differentially alters the thermal sensitivity of complex I basal and maximal mitochondrial oxidative capacity. Comparative Biochemistry Physiology. Part A, Molecular and Integrative Physiology 201, 87-94.

Partridge, G., Walsh, D., Bradshaw, B., Kolkovski, S., 2003. Water flow and metabolism-based inter-relationsihps in land-based finfish culture: studies at Challenger TAFE's Aquaculture Development Unit. Austasia Aquaculture, 54-58.

Pirozzi, I., Booth, M.A., 2009. The routine metabolic rate of mulloway (*Argyrosomus japonicus*: Sciaenidae) and yellowtail kingfish (*Seriola lalandi*: Carangidae) acclimated to six different temperatures. Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology 152, 586-592.

Pollock, M.S., Clarke, L.M.J., Dube, M.G., 2007).The effects of hypoxia on fishes: from ecological relevance to physiological effects. Environmental Reviews, NRC Research Press 15, 1-14.

Teoh, A.Y.L., 2016. Effects of feed induced intermittent hypoxia on digestive physiology and stress response in yellowtail kingfish, *Seriola lalandi*. Honours thesis. Flinders University, South Australia, Australia. 86 pp.

Stone, D.A.J., Roberts, S., Currie, K-L. 2014. Hyper-Saline Conditions Affect Growth, Osmoregulation and Survival of Fingerling and Juvenile Yellowtail Kingfish, *Seriola lalandi*. Jounral of Aquaulcture and Marine Biology 1, 1-11.

Stone, D.A.J., D'Antignana, T.D. and Bansemer, M.S. 2016. Final Report. Refining Yellowtail Kingfish feeds and feed management. Prepared by the South Australian Research and Development Institute (Aquatic Sciences), Adelaide. CRC Project No. 2013/730.

Zhang, H., Bosch-Marce, M., Shimoda, L.A., Tan, Y.S., Baek, J.H., Wesley, J.B., Gonzalez, F.J., Semenza, G.L., 2008. Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. Journal of Biological Chemistry 283, 10892-10903.

Table 3.2.1.1.1. The proximate, mineral and amino acid composition of the Ridley Pelagica diet fed to Yellowtail Kingfish for 85 days.

Item (as fed)[1]	Ridley Pelagica diet
Analysed proximate composition (g 100 g^{-1})	
Moisture	7.8
Crude protein	43.79
Crude lipid	25.4
Ash	8.6
Carbohydrate[2]	14.4
Gross energy (MJ kg^{-1})	19.30
Rancidity test	
p-Anisidine Value	16.9
Peroxide Value	1.6
Analysed minerals (mg kg^{-1})	
Calcium	25500
Copper	9.4
Iodine	1.5
Iron	305
Magnesium	2000
Manganese	45
Phosphorus	16500
Potassium	4850
Selenium	2.2
Zinc	145
Analysed amino acids (g 100 g^{-1})	
Alanine	2.551
Arginine	2.573
Aspartic acid	3.256
Glutamic acid	6.490
Glycine	2.804
Histidine	1.184
Isoleucine	1.648
Leucine	3.326
Lysine	3.016
Methionine	1.158
Phenylalanine	1.979
Proline	2.487
Hyroxy Proline	0.669
Serine	1.688
Threonine	1.704
Tyrosine	1.294
Valine	2.369
Total amino acids	40.20
Other (mg 100 g^{-1})	
Cholesterol	234
Taurine	1261
Choline (Hydroxide)	296.25

[1] Diets supplied by Ridley (Narangba, Queensland, Australia).
[2] Carbohydrate = 100 - (moisture + lipid + protein + ash).

Table 3.2.1.1.2. The fatty acid composition of the Ridley Pelagica diet fed to Yellowtail Kingfish for 85 days.

Item (as fed)[1]	Ridley Pelagica diet
Analysed fatty acids (mg 100 g^{-1})	
Saturated Fatty Acids	
C4:0 Butyric	<10
C6:0 Caproic	<10
C8:0 Caprylic	<10
C10:0 Capric	<10
C12:0 Lauric	<10
C13:0 Trisdecanoic	<10
C14:0 Myristic	860
C15:0 Pentadecanoic	105
C16:0 Palmitic	5505
C17:0 Margaric	130
C18:0 Stearic	1615
C20:0 Arachidic	68
C22:0 Docosanoic	16
C24:0 Tetracosanoic	32
Mono-unsaturated Fatty Acids	
C10:1 Decenoic	<10
C14:1 Myristoleic	55
C15:1 Pentadecenoic	<10
C16:1 Palmitoleic	1425
C17:1 Heptadecenoic	56
C18:1n-6 Octadecenoic	<10
C18:1n-7 Octadecenoic	670
C18:1n-9 Oleic	7235
C20:1n-9 Eicosenoic	275
C20:1n-11,13 Eicosenoic	47
C20:1 Eicosenoic (total)	320
C22:1n-9 Docosenoic	42
C22:1n-11,13 Docosenoic	77
C24:1 Tetracosenoic	71
Poly-unsaturated Fatty Acids	
C18:2n-6 Linoleic	2215
C18:2 Conjugated 9c 11t Octadecadienoic	<10
C18:3n-6 Gamma Linolenic	36
C20:2n-6 Eicosadienoic	<10
C20:3n-6 Dihomo-gamma-linoleic	35
C20:4n-6 Arachidonic	265
C22:4n-6 Docosatetraenoic	72
C22:5n-6 Docosapentaenoic	42
C18:3n-4 Octadectrenoic acid	<10
C18:3n-3 Alpha Linolenic	380
C18:4n-3 Steridonic	165
C20:3n-3 Eicosatrienoic	<10
C20:4n-3 Eicosatetraenoic	108
C20:5n-3 Eicosapentanaeoic	1255
C21:5n-3 Heneicosapentaenoic acid	<10
C22:5n-3 Docosapentaenoic	275
C22:6n-3 Docosahexaenoic	1690
∑LC n3 PUFA	3220
n-3 FA:n-6 FA	1.45

1 Diets supplied by Ridley (Narangba, Queensland, Australia).

Table 3.2.1.1.3. Summary of water quality parameters.

Item[1]	Temperature (°C)	pH	Salinity (mg L^{-1})	Ammonia (ppm)	CO_2 (mg L^{-1})
Mean	21.8 ± 1.5	7.71 ± 0.08	36 ± 0	0.4 ± 0.3	3 ± 1
Range	19.0 - 24.5	7.27 - 7.97	36 - 36	0.0 - 2.0	2 - 5

[1] Values means ± standard deviation.

Table 3.2.1.1.4. Growth performance, feed utilisation, whole body composition and nutrient retention of Yellowtail Kingfish exposed to different dissolved oxygen treatments for 85 days.

Treatment[1,2]	1	2	3	4	ANOVA[3]
Growth performance					
Initial weight (kg)	2.14±0.01	2.15±0.01	2.16±0.02	2.15±0.01	$P = 0.776$
Final weight (kg)	3.92±0.05	3.89±0.02	3.89±0.04	3.77±0.02	$P = 0.064$
Biomass gain (kg tank^{-1})	44.50±0.93^{a}	43.51±0.36^{a}	43.21±0.60^{a}	40.48±0.48^{b}	$P = 0.010$
SGR (% d^{-1})	0.71±0.01^{a}	0.70±0.01^{a}	0.69±0.01^{a}	0.66±0.01^{b}	$P = 0.005$
Initial fork length (mm)	513.80±1.04	515.00±0.76	513.87±1.18	513.93±0.35	$P = 0.751$
Final fork length (mm)	605.20±2.16	606.67±1.64	602.80±2.00	598.67±1.57	$P = 0.068$
Length growth rate (mm d^{-1})	1.08±0.03	1.08±0.02	1.05±0.02	1.00±0.02	$P = 0.055$
Final Condition factor	1.77±0.01	1.74±0.01	1.78±0.00	1.76±0.01	$P = 0.080$
Feed utilisation (as fed)					
Apparent feed consumption (kg tank$^{-1)}$	72.29±0.57	70.85±0.54	70.96±0.85	69.94±1.39	$P = 0.390$
Apparent feed intake (% BW d^{-1})	1.16±0.00	1.14±0.01	1.14±0.01	1.15±0.02	$P = 0.525$
Apparent FCR	1.63±0.02^{b}	1.63±0.00^{b}	1.64±0.00^{b}	1.73±0.03^{a}	$P = 0.015$
Proximate composition (wet basis)					
Moisture (%)	57.7±0.8	59.6±0.4	59.2±0.3	59.8±0.9	$P = 0.184$
Protein (%)	19.9±0.5	19.4±0.7	19.0±0.2	18.5±0.8	$P = 0.445$
Lipid (%)	20.7±1.0	18.8±0.4	19.6±0.3	18.9±1.2	$P = 0.387$
Ash (%)	2.2±0.2	2.6±0.3	2.1±0.1	2.1±0.1	$P = 0.446$
Carbohydrate (%)	<1.5	<1.5	<1.5	<1.5	$P = 1.000$
Energy (MJ kg^{-1})	11.07±0.28	10.23±0.09	10.50±0.12	10.30±0.42	$P = 0.181$
Nutrient retention[4]					
Apparent PD	28.39±1.37	26.78±2.10	25.21±0.57	22.40±2.24	$P = 0.161$
Apparent ED	46.19±2.40	40.44±0.63	42.10±0.81	39.08±2.37	$P = 0.092$

[1] Values are mean ± SE; $n = 3$.

[2] Treatment 1, 100% saturation constant; Treatment 2, 85% saturation constant; Treatment 3, Held at 85% saturation, but before feeding the oxygen set point was manually changed to 60% saturation daily and held for 3 h before the oxygen set point was manually changed back to 85% saturation; and Treatment 4, Held at 85% saturation, but twice fortnightly (Day 13 and 14), oxygen set point was manually changed to 60% saturation before feeding and held for 3 h before manually set back to 85% saturation.

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

[4] ED = energy deposition; PD = protein deposition

Table 3.2.1.1.5. Blood haematocrit and biochemistry of Yellowtail Kingfish exposed to different dissolved oxygen treatments for 85 days.

Treatment [1,2]	1	2	3	4	ANOVA[3]
Hematology					
Haematocrit (L L^{-1})	44.45±3.92	48.59±1.91	47.59±0.97	46.33±1.03	$P = 0.627$
Biochemistry[4]					
Sodium (mmol L^{-1})	202±2	201±0	200±3	200±1	$P = 0.885$
Potassium (mmol L^{-1})	11.8±1.8	15.8±2.6	11.7±2.1	11.1±2.3	$P = 0.462$
Urea (mmol L^{-1})	4.2±0.1	4.0±0.4	4.5±0.8	5.4±0.8	$P = 0.380$
Creatinine (mmol L^{-1})	0.033±0.003	0.027±0.004	0.027±0.001	0.026±0.002	$P = 0.299$
Calcium (mmol L^{-1})	3.40±0.17	3.31±0.03	3.35±0.08	3.35±0.01	$P = 0.914$
Protein (g L^{-1})	40±2	41±1	42±2	41±0	$P = 0.923$
Albumin (g L^{-1})	21±1	18±1	21±2	19±1	$P = 0.169$
Globulin (g L^{-1})	19±3	23±1	22±1	22±1	$P = 0.618$
Total Bilirubin (mmol L^{-1})	0±0	0±0	1±0	0±0	$P = 0.487$
ALT (IU L^{-1})	9±1	8±0	12±3	10±0	$P = 0.356$
ALP (IU L^{-1})	36±5	24±2	26±3	25±2	$P = 0.066$
Magnesium (mmol L^{-1})	2.10±0.13	1.95±0.05	2.09±0.15	2.03±0.12	$P = 0.789$
Cholesterol (mmol L^{-1})	4.6±0.1	4.5±0.2	4.9±0.6	5.0±0.3	$P = 0.661$
Triglyceride (mmol L^{-1})	$2.46±0.11^{a}$	$1.59±0.18^{b}$	$1.80±0.04^{b}$	$1.55±0.04^{b}$	$P = 0.001$
Bile Acids (mmol L^{-1})	$35.37±12.57^{a}$	$12.27±3.28^{ab}$	$8.88±4.10^{ab}$	$4.13±0.89^{b}$	$P = 0.048$

[1] Values are mean ± SE; $n = 3$.

[2] Treatment 1, 100% saturation constant; Treatment 2, 85% saturation constant; Treatment 3, Held at 85% saturation, but before feeding the oxygen set point was manually changed to 60% saturation daily and held for 3 h before the oxygen set point was manually changed back to 85% saturation; and Treatment 4, Held at 85% saturation, but twice fortnightly (Day 13 and 14), oxygen set point was manually changed to 60% saturation before feeding and held for 3 h before manually set back to 85% saturation.

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

[4] ALT = alanine aminotransferase; ALP = alkaline phosphatase.

Table 3.2.1.1.6. Visceral somatic indices, spleen, gill and hind gut histological measurements of Yellowtail Kingfish exposed to different dissolved oxygen treatments for 85 days.

Treatment [1,2]	1	2	3	4	ANOVA[3]
Visceral somatic indices					
Viscerosomatic index (VSI; %)	6.34±0.18	6.25±0.28	6.48±0.51	6.28±0.18	$P = 0.954$
Hepatosomatic index (HSI; %)	1.11±0.02	0.97±0.01	1.06±0.04	1.10±0.05	$P = 0.091$
Spleen					
Depleted haematopoietic tissue	0.67±0.38	1.11±0.59	0.78±0.48	1.67±0.33	$P = 0.457$
Congested	0.67±0.19	0.78±0.11	1.00±0.38	0.78±0.44	$P = 0.895$
Melanomacrophages centres (per ×10 field)	16±1	14±1	15±2	11±1	$P = 0.173$
Gill					
Telangiectasis[4]	1.11±0.80	0.22±0.22	0.44±0.44	0.67±0.19	$P = 0.620$
Hyperplasic nodules[4]	1.11±0.22	1.44±0.68	2.33±0.33	1.89±0.40	$P = 0.298$
Number of blood fluke eggs	14±10	12±11	5±4	7±7	$P = 0.830$
Number of epitheliocystis	0.1±0.1	0.0±0.0	1.1±0.4	0.9±0.7	$P = 0.234$
Hyperplastic lamellar tips (clubbing)[4]	0.22±0.11	0.44±0.11	0.33±0.19	0.94±0.78	$P = 0.632$
Filament tip inflammation	0.22±0.22	0.22±0.22	0.33±0.19	0.22±0.22	$P = 0.976$
Hind gut					
Villus area (μm^2)	79234±29995	95066±29372	104534±57213	47840±25108	$P = 0.660$
Villus perimeter (μm)	3235±456	2476±107	2455±187	2007±371	$P = 0.148$
Villus length (μm)	817±237	655±113	821±281	550±150	$P = 0.711$
Villus breadth (μm)	197±57	273±59	230±50	174±65	$P = 0.672$
Villus branching	2±0	2±0	2±1	2±0	$P = 0.886$
Goblet cells per villi length (mm)	125±63	119±12	183±100	205±64	$P = 0.679$

[1] Values are mean ± SE; $n = 3$.

[2] Treatment 1, 100% saturation constant; Treatment 2, 85% saturation constant; Treatment 3, Held at 85% saturation, but before feeding the oxygen set point was manually changed to 60% saturation daily and held for 3 h before the oxygen set point was manually changed back to 85% saturation; and Treatment 4, Held at 85% saturation, but twice fortnightly (Day 13 and 14), oxygen set point was manually changed to 60% saturation before feeding and held for 3 h before manually set back to 85% saturation.

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

[4] Scores were based on subjective blind readings: Not observed, 0; Rare, 1; Mild, 2; Moderate, 3; Severe, 4.

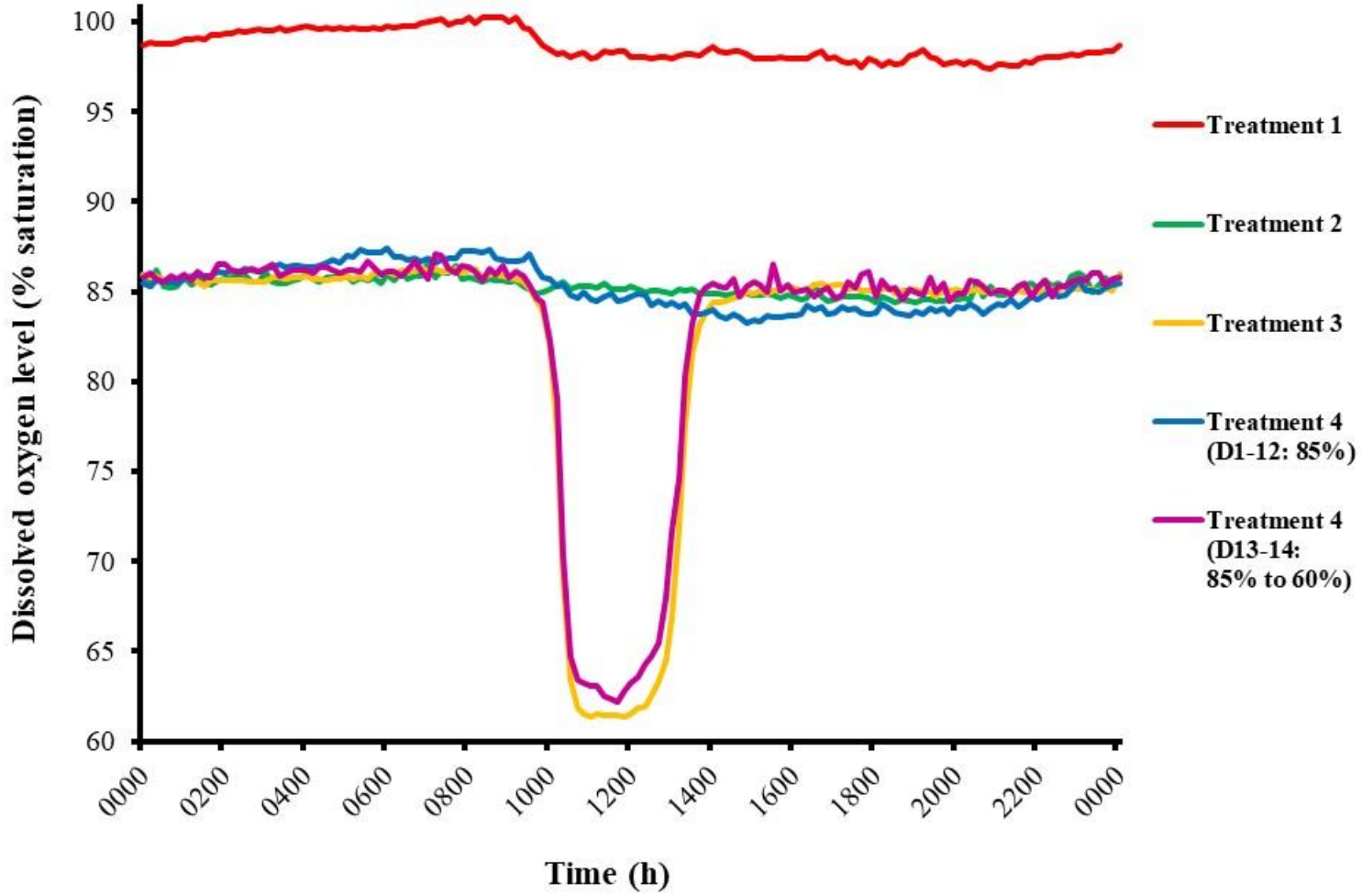


Figure 3.2.1.1.1. Average daily dissolved oxygen levels for each experimental treatment.
Treatment 1, 100% saturation constant; Treatment 2, 85% saturation constant; Treatment 3, Held at 85% saturation, but before feeding the oxygen set point was manually changed to 60% saturation daily and held for 3 h before the oxygen set point was manually changed back to 85% saturation; and Treatment 4, Held at 85% saturation, but twice fortnightly (Day 13 and 14), oxygen set point was manually changed to 60% saturation before feeding and held for 3 h before manually set back to 85% saturation.

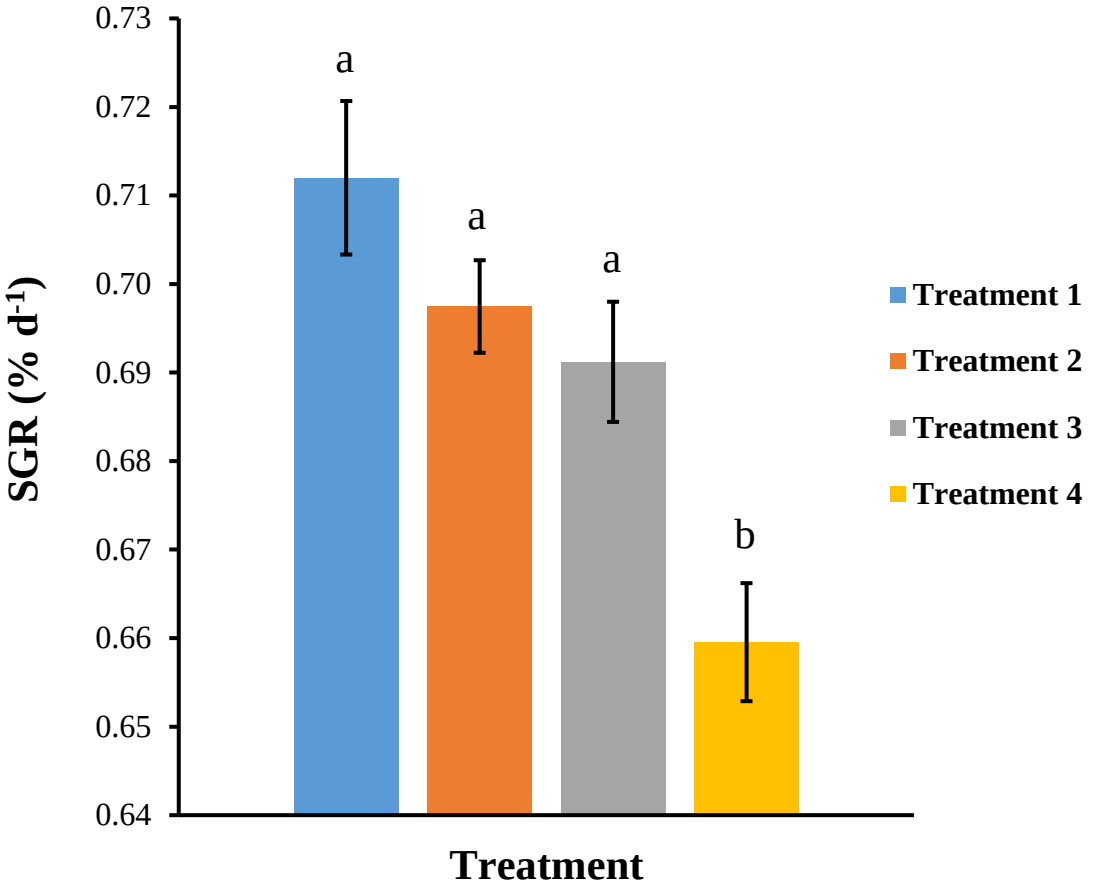


Figure 3.2.1.1.2. Specific growth rate of Yellowtail Kingfish exposed to different dissolved oxygen treatments for 85 days.

Values are mean ± SE; *n* = 3; Treatment 1, 100% saturation constant; Treatment 2, 85% saturation constant; Treatment 3, Held at 85% saturation, but before feeding the oxygen set point was manually changed to 60% saturation daily and held for 3 h before the oxygen set point was manually changed back to 85% saturation; and Treatment 4, Held at 85% saturation, but twice fortnightly (Day 13 and 14), oxygen set point was manually changed to 60% saturation before feeding and held for 3 h before manually set back to 85% saturation; A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

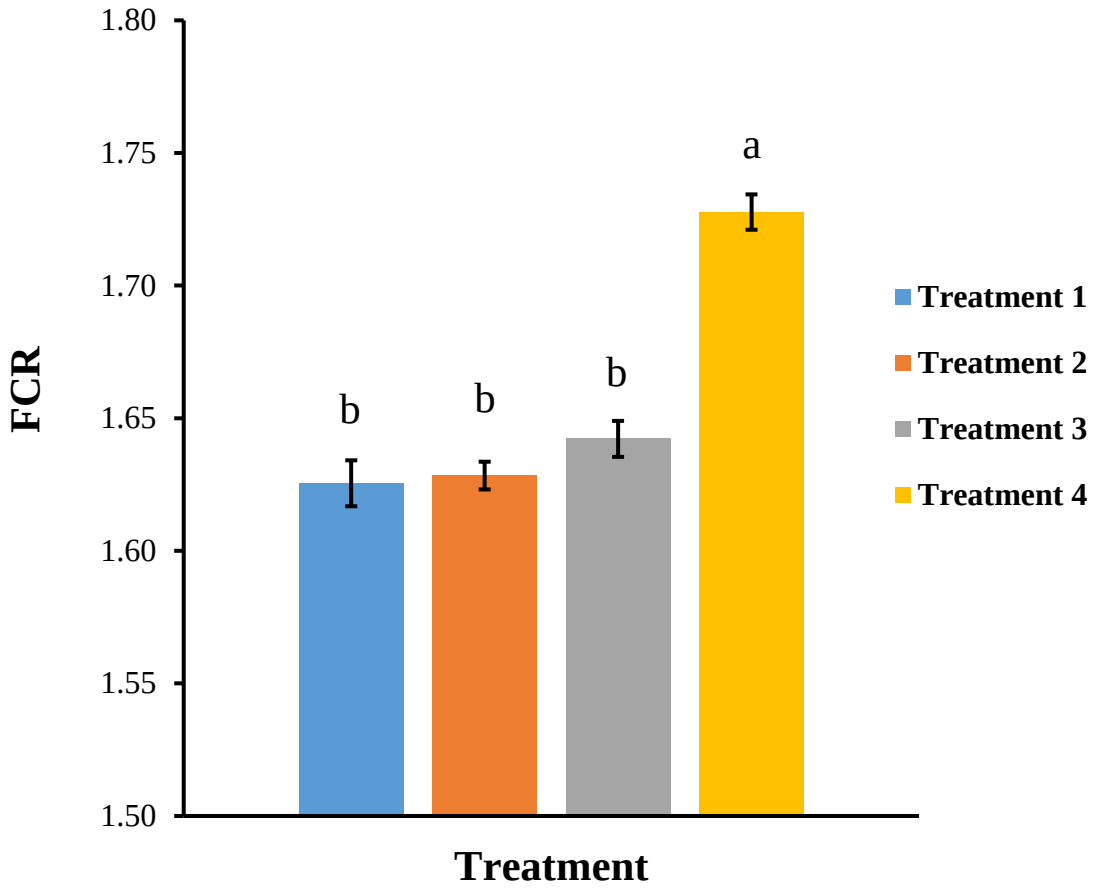


Figure 3.2.1.1.3. Apparent feed conversion ratio of Yellowtail Kingfish exposed to different dissolved oxygen treatments for 85 days.

Values are mean ± SE; $n = 3$; Treatment 1, 100% saturation constant; Treatment 2, 85% saturation constant; Treatment 3, Held at 85% saturation, but before feeding the oxygen set point was manually changed to 60% saturation daily and held for 3 h before the oxygen set point was manually changed back to 85% saturation; and Treatment 4, Held at 85% saturation, but twice fortnightly (Day 13 and 14), oxygen set point was manually changed to 60% saturation before feeding and held for 3 h before manually set back to 85% saturation; A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

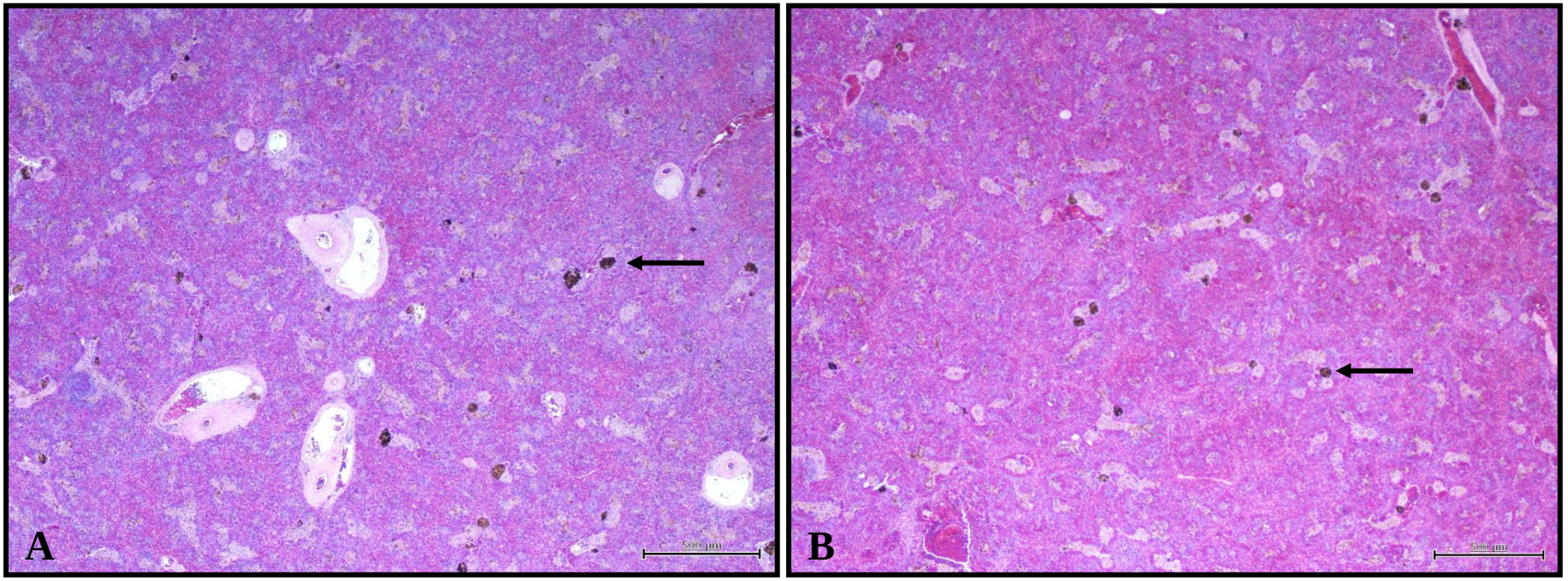


Figure 3.2.1.1.4. Spleen histology from Treatment 1 (A) and Treatment 4 (B), showing normal melanomacrophage centres (arrow) for Yellowtail Kingfish (Dr. Fran Stephens, Aquatilia Healthcare, Morangup, WA). Spleen histological samples were stained with hematoxylin and eosin (H and E), scale bar denotes 500 µm.
Treatment 1, 100% saturation constant; and Treatment 4, Held at 85% saturation, but twice fortnightly (Day 13 and 14), oxygen set point was manually changed to 60% saturation before feeding and held for 3 h before manually set back to 85% saturation.

3.2.2. Chapter - Development of bioenergetics models for sub-adult Yellowtail Kingfish.

3.2.2.1. Manuscript - Protein, amino acid and energy utilisation and maintenance requirements of juvenile Yellowtail Kingfish (Seriola lalandi): quantifying abiotic influences.

Igor Pirozzi[a b], Marina Benito Rubio[c] and Mark Booth[a]

[a] New South Wales Department of Primary Industries, Port Stephens Fisheries Research Institute, Taylors Beach 2316, NSW, Australia.

[b] College of Science and Engineering and Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville 4801, QLD, Australia.

[c]Wageningen University and Research. 6700 HB Wageningen, The Netherlands.

This manuscript may be referenced as: Pirozzi, I., Rubio, M.B. and Booth, M. (2019). 3.2.2.1. Manuscript - Protein, amino acid and energy utilisation and maintenance requirements of juvenile Yellowtail Kingfish (*Seriola lalandi*): quantifying abiotic influences. In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.281-304.

Abstract

Two experiments were conducted to evaluate the effect of abiotic factors on the digestible nutrient and energy utilisation of sub-adult Yellowtail Kingfish (*Seriola lalandi*; YTK). Experiment 1 considered the effect of temperature at 15 ºC or 24 ºC. Experiment 2 considered the effect of dissolved oxygen (DO) at 60% or 100% saturation. Duplicate groups of YTK per feed treatment (initial body weight Experiment 1 = 187.2 ± 0.4 g fish^{-1}; Experiment 2 = 220.9 ± 2.35 g fish^{-1}) were fed a commercial diet at one of five ration levels. Feeding levels ranged from a restricted ration, equivalent to approximately 10% of satiation feeding, through to maximal satiation feeding. Feed trials were run over 35-38 days. Growth and nutrient deposition, feed intake and diet digestibility were assessed to establish nutrient retention efficiencies and maintenance requirements. Water temperature was shown to have a varying effect on utilisation responses in YTK with the magnitude of the response dependent on the nutrient examined; for example there was little influence of temperature on the utilisation response of most essential amino acids with the exception of arginine and taurine utilisation, which varied significantly between temperatures. Maintenance requirements of all nutrients generally increased with increasing water temperature. Low DO saturation of 60% negatively affected the nutrient and energy utilisation efficiencies in YTK, with this response tending to be more pronounced with increasing nutrient and energy intake. However DO saturation did not significantly affect feed intake. This study provides insight into the effects of water temperature and dissolved oxygen on the nutritional physiology of YTK. Data generated from this study will be used to improve bioenergetic feed models for YTK, facilitating better feed management and feed formulation through a better understanding of the influence of abiotic conditions on nutrient demand and utilisation.

Introduction

Bioenergetic models (BEMs) have proved very useful for predicting the temperature dependent growth rate and digestible protein (DP) and energy (DE) demands of fish (Cho and Bureau, 1998; Lupatsch and Kissil, 2005; Glencross, 2008). Integrating this information with the known DP and DE content of aquafeeds allows development of management tools that can predict growth rate, feed demand and feed

conversion ratio. Models can be extended to estimate the flow of nutrients into and out of the animal and, therefore, used to model nutrient fluxes at the wider scale, such as the flux of carbon, nitrogen and phosphorus from fish farms to the environment. Fundamental to the development of these models is a need to understand the efficiency with which nutrients are utilised. Total nutrient requirement of an animal can be described as:

$$a \times BW(kg)^b + c \times Growth$$

Where; a = maintenance requirement; b = weight exponent; c = utilisation coefficient.

A preliminary factorial model has been published for Yellowtail Kingfish (*Seriola lalandi;* YTK) (Booth et al., 2010) based on nutrient utilisation efficiencies established at 23.1 ± 1.4 °C and under normoxic conditions. As an ectotherm, coefficient values derived for YTK may be affected by water temperature which will in turn influence the predictive capacity of the model. BEMs are sensitive to small variations of key input parameters including nutrient utilisation coefficients (Pirozzi et al., 2010a), therefore, it is necessary to quantify the influence of different abiotic conditions on these parameters to improve the robustness and accuracy of the preliminary YTK model. Pirozzi et al. (2010b) found that maintenance requirements for energy, but not protein, increased with increasing water temperature from 20°C to 26°C in Mulloway (*Argyrosomus japonicus*) while nutrient utilisation coefficients remained relatively constant at these temperatures. These temperature ranges can be considered within the species thermal optima (Pirozzi and Booth, 2009), hence utilisation efficiencies may remain relatively conserved. Glencross and Bermudes (2010) found similar influences of water temperature on the utilisation efficiencies in Barramundi (*Lates calcarifer*) held within thermal optima (25 °C-32 °C); however, at a supra-optimal temperature (36 °C) utilisation efficiencies tended to decline.

Significant periodic or chronic variation in dissolved oxygen (DO) in aquaculture systems due to natural environmental perturbations, stocking density, feeding regimes or biofouling will affect the metabolic rate of aquaculture species and this can impact on the energy available for growth and the conversion efficiency of feed ingested (Bejda et al., 1992; Yang et al., 2015). The impact of low DO may therefore reduce the accuracy of growth and nutritional models, but this is rarely considered when applying these models in real world situations. There are conflicting reports in the literature on the impact of low DO environments on the mechanisms driving nutrient utilisation in finfish. For example, reduced DO has been documented to affect maximum feed intake in Rainbow Trout (*Oncorhynchus mykiss*), but not protein or energy utilization (Glencross, 2009). However studies on YTK have shown a general tendency for feed utilisation efficiencies to decrease in low oxygen environments (Bowyer et al., 2014).

The aim of this study was to evaluate the effect of the abiotic factors water temperature and dissolved oxygen saturation on the nutrient and energy utilisation of sub-adult YTK.

Methods

This study was performed under the NSW DPI Fisheries Animal Care and Ethics (ACEC) Research Authority known as 'Aquaculture Nutrition ACEC 93/5–Port Stephens'. Care, husbandry and termination of fish was carried out according to methods outlined in 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2015).

Two experiments were conducted to determine the influence of abiotic factors on the dietary protein and energy utilisation efficiencies of YTK; the first experiment considered the effect of two water temperatures (15 °C or 24 °C) and the second experiment considered the effect of two oxygen saturation levels (60% or 100% saturation).

Juvenile YTK used in each experiment were progeny of wild caught broodstock held at NSW Port Stephens Fisheries Institute (PSFI). Prior to the experiment juveniles were reared at low densities in a recirculating aquaculture system (RAS) comprising 10 kL tanks at an ambient temperature of approximately 20 °C to 22 °C and fed daily a commercial marine finfish feed. Prior to stocking YTK were sedated with Aqui-S™, individually weighed and measured (fork length). Fish were stocked at ambient temperature and each RAS temperature adjusted up or down by 1 °C per day until the target experiment temperature was reached. A representative sample of five fish were also collected at the beginning of each experiment, euthanized with an overdose of benzocaine (ethyl-*p*-aminobenzoate) and stored frozen (-20 °C) until processing for chemical compositional analyses. At the conclusion of the

growing period of each experiment all fish were re-weighed and measured. Five fish were then subsampled from each replicate unit and killed for chemical compositional analyses.

Experiment design

The protein and energy utilization efficiency of YTK at different water temperatures (Experiment 1; 15 °C or 24 °C) or DO saturation levels (Experiment 2; 60 or 100% saturation) was determined by feeding one of five feeding levels (L1-L5) ranging from a low feeding level (near maintenance; L1) to a high feeding level (apparent satiation, L5). There were $n = 2$ replicate experiment units per feed treatment. A commercial YTK diet (Ridley, Narangba, QLD) was used for each experiment. The proximate compositions of the diets are presented in Table 3.2.2.1.1.

Experiment 1: Effects of water temperature

A 35 day growth experiment was carried out in two separate recirculating aquaculture systems (RAS's). One RAS was allocated as cool temperature (15 ºC) and the other as warm temperature (24 ºC). Each experiment system consisted of 10 × 1 kL square intermediate bulk containers (IBC) that had been modified to grow fish. Each tank was connected to a sump tank collecting effluent that was continuously pumped through a sand filter and a fluidised-bed biological filter and temperature controlled by reverse cycle refrigeration units. Influent rates at each tank were set at 6 L min^{-1}. Approximately 15% of the RAS water was exchanged daily and topped up with fresh, sand filtered and chlorinated / dechlorinated estuarine water pumped from the Tilligerry Creek adjacent the Port Stephens Fisheries Institute (PSFI). Twenty fish were randomly distributed into each duplicate tank per treatment. The YTK used in this experiment were on average 187.2 g ± 0.4 g SEM at stocking.

Experiment 2: Effects of dissolved oxygen

A 38 day growth experiment was carried out in two separate RAS's held at 20 °C. One was allocated as low DO saturation at approximately 60% (S60) and the other as normal DO saturation at approximately 100% (S100). The S60 groups of YTK were acclimated from 100% saturation down to 60% saturation over several days following initial stocking procedures. Each experiment system consisted of a 10 kL tank (3.4 m diameter × 1.2 m depth) containing 10 × 200 L HDPE mesh cages (10 mm oyster mesh) with ten fish randomly distributed into each duplicate cage per treatment. Each main tank was connected to a sump tank collecting effluent that was continuously pumped through a sand filter and a fluidised-bed biological filter. Influent rates at each 10 kL tank were set at 2 L min^{-1}. Approximately 15% of the RAS water was exchanged daily and topped up with fresh, sand filtered and chlorinated / dechlorinated estuarine water pumped from the Tilligerry Creek adjacent PSFI. Ten fish were randomly distributed into each duplicate cage per treatment. YTK used in this experiment were on average 220.9 ± 2.35 g SEM at stocking.

Water quality

With the exception of the S60 RAS, the DO in each experiment system was kept at approximately 100% saturation by supplying industrial oxygen gas (BOC) at a flow rate of 1L min^{-1} as well as normal aeration using submerged air stone diffusers. Oxygen saturation (%) and concentration (mg L^{-1}) were measured using a Hach luminescent dissolved oxygen meter. pH and salinity were measured using a Horiba Water Checker (model U-10). Total ammonia nitrogen (TAN) was measured using a quick test kit (Aquamerck).

The DO level for the S60 system was controlled by N_2 gas injection at a flow rate of ~3 L min^{-1} and normal aeration using a high-volume low-pressure air blower (Robuschi). Both gasses were injected into the holding tank system through submerged air stone diffusers. This controlled, antagonistic approach (N_2 gas and aeration) created a stable low DO saturation environment in the 10 kL tank regardless of the fluctuating O_2 demand due to the biomass of YTK. Carbon dioxide (CO_2) levels were also measured for

Experiment 2 using an OxyGuard CO_2 Analyser. Average water quality values for each experiment system are presented in Table 3.2.2.1.2.

Establishing satiation and restricted feeding rations – levels L1 to L5

Apparent satiety was determined by carefully handfeeding YTK until a loss in feeding activity was observed. In addition, any uneaten pellets were removed from each cage or IBC tank approximately one hour after feeding and counted. Feed intake was adjusted using a correction factor based on average pellet weight. The L1 feeding level was calculated as 10% of the average satiated feeding ration observed during the first week, and set at 11.8 g day^{-1} for the cool water system and 26.9 g day^{-1} for the warm water system in Experiment 1 (i.e. the temperature experiment) and 11.1 g day^{-1} for Experiment 2 (i.e. the DO experiment). The three intermediate feeding levels were determined daily at approximately 25% (L2), 50% (L3) and 75% (L4) of satiation. Fish were fed once or twice daily, depending on ration size, to allow maximum voluntarily feed intake in the higher ration treatments. Paired feeding between temperature treatments in Experiment 1 was not possible at the higher ration levels as the difference in voluntary feed intake between cool and warm water groups was too great. At the conclusion of the trial all fish were fasted for 24-48 h prior to final sampling for whole body compositional analyses.

Sample processing and compositional analyses

Chemical changes in whole body composition of YTK were determined by comparing the initial carcass samples from Experiments 1 or 2 with the final carcass samples from respective experiments (i.e. comparative slaughter technique). Sample processing for whole carcass composition involved placing weighed fish from each replicate unit into a single autoclave bag and then autoclaving the animals for 99 min at 121 °C. After cooling to room temperature any changes in weight were accounted for and the moisture value of carcass adjusted accordingly. The samples were then transferred to a Robot-Coupe Blixer® 5 food processor and homogenised. A subsample (approximately 2 g) of the homogenate was used to determine dry matter composition. A further subsample (approximately 20 g) of the wet homogenate was then stored frozen pending chemical analyses. Analyses was conducted in accordance with AOAC (2005). Protein was calculated from total nitrogen based on N × 6.25 using the Dumas method. Dry matter was calculated gravimetrically after oven drying at 105 °C. Ash was calculated gravimetrically after incineration at 550 °C for 2 h. Gross energy was determined by adiabatic bomb calorimetry. Lipid was measured gravimetrically after chloroform-methanol extraction. Diet and carcass samples were analysed for dry matter, nitrogen, gross energy, fat, ash and amino acids by CSIRO Agriculture and Food (St Lucia, QLD 4067, Australia).

Performance indices

The following performance indices were calculated using all fish in each replicate to derive an average value per tank:

- Daily weight gain (g $fish^{-1}$ day^{-1}) = final body weight – initial body weight / number of days
- Specific growth rate (% d^{-1}) = [Ln(final weight) - Ln(initial weight)] / days × 100
- Condition factor *K* = [individual weight of fish (g) / fork length of fish $(mm)^3$] × 10^5
- Daily nutrient gain (g $fish^{-1}$ day^{-1}) = final carcass nutrient content – initial carcass nutrient content / number of days
- Daily energy gain (kJ $fish^{-1}$ day^{-1}) = final carcass energy content – initial carcass energy content / number of days
- Feed conversion ratio (FCR) = total feed intake / weight gain

Digestibility of diets used in experiments

A digestibility assay was carried out at the end of each trial using fish remaining from the L5 feed treatment. This part of the trial continued for a further two weeks. Yttrium oxide (Yttrium III oxide, Sigma-Aldrich) was used as an inert marker in the diets. The marker was added at 1 g kg^{-1} of diet and thoroughly mixed. The mixture was then re-pelleted and dried in an air drying oven at 40 °C for 8-9 h.

Prior to stripping, fish were anaesthetised using 5-25 mg L^{-1} Aqui-S^{TM}. Faeces were collected from the posterior intestine by applying gentle abdominal pressure using the thumb and forefinger. Contamination with urine or mucous were minimized and samples were immediately stored at -20 °C. This procedure was repeated twice a week until approximately 3g dry faecal matter was obtained. Faecal samples were also analysed for dry matter, nitrogen, gross energy, fat, ash and amino acids (CSIRO Agriculture and Food, St Lucia, QLD 4067, Australia).

Apparent digestibility of the diet was calculated using the formula:

$$ADC(\%) = 100 \times [1 - \left(\frac{F}{D} \times \frac{D_{marker}}{F_{marker}}\right)]$$

where F = % nutrient in faeces; D = % nutrient in diet; D_{marker} = % marker in diet; F_{marker} = % marker in faeces.

Data analyses

Regression analyses was applied across the data sets to determine utilisation co-efficients (nutrient efficiencies), nutrient cost of growth and maintenance requirements for digestible protein, energy and key amino acids. All results were regarded as significant at $P < 0.05$. All data were analysed using NCSS8 and Graphpad Prism statistical software. Data are also expressed as geometric mean body weights (GMBW) and scaled using the metabolic body weight exponent value of 0.7 for protein and amino acid retention data and 0.8 for energy retention data (Booth et al., 2010) and 0.9 for fat retention (Salini et al., 2016). Protein and energy utilisation coefficient data from the two YTK studies in the current trial and data published by Booth et al. (2010) were combined and regressed over water temperature. The experiments were designed to establish key coefficients and *x*-intercepts using regression analyses, therefore, treatment number ($k = 5$) was increased at the expense of replication ($n = 2$). A two-way ANOVA was applied irrespective of low power to ascertain significant interactions between feeding level and abiotic treatment. The Tukey-Kramer test was used for *a posteriori* multiple comparisons of means on significant terms.

Results

Experiment 1: Effects of water temperature

Survival was 98% across both RAS systems and there were no significant differences in survival when considering water temperature or feeding level. Growth data are presented in Table 3.2.2.1.3. Weight gain of YTK responded systematically to feed intake relative to each cool and warm water treatment. After 35 days YTK fed to apparent satiation (L5) in the warm water RAS had approximately doubled in body weight while YTK in the cool water system had only increased in weigh by approximately 33% (Table 3.2.2.1.3).

Digestibility of protein, fat and energy from the test diet was generally poor. There were no significant differences between nutrient ADCs at different water temperatures with the exception of taurine, glycine and serine which all increased in warm water (Table 3.2.2.1.1).

Feed intake data are presented in Tables 3.2.2.1.3. FCRs ranged between 1.4 to 5.7 for the cool water group and 1.4 to 4.4 for the warm water group, with the better FCRs at intermediate rations for both temperature treatments. Nutrient intake data are presented in Table 3.2.2.1.4.

Non-linear regression analyses indicated that there were significant differences in the relative growth and nutrient deposition responses across the feed regimes for crude feed (DM) and digestible protein and energy intake ($P < 0.05$) between temperatures (Figure 3.2.2.1.1). However, there were no significant differences in the relative fat deposition response across the feed regimes between temperatures (Figure 3.2.2.1.1). The rate of amino acid deposition relative to digestible amino acid intake was significantly different between temperature treatments for all essential amino acids except for methionine, lysine and threonine (Figure 3.2.2.1.2). There was a highly significant effect of temperature on arginine deposition (Figure 3.2.2.1.2).

Utilisation coefficients and maintenance values are presented in Tables 3.2.2.1.6 and 3.2.2.1.7, respectively. Water temperature significantly affected the utilisation efficiency of arginine and taurine, while no differences were found between all other nutrients. The nutrient cost of gain significantly increased (almost six fold), for arginine with increasing temperature (Table 3.2.2.1.6). Taurine utilisation efficiency also significantly increased with increased water temperature; however, in absolute terms the magnitude of change was relatively small (Table 3.2.2.1.6). Maintenance requirements for all nutrients increased with increasing water temperature; however, the magnitude of change varied depending on the nutrient.

Experiment 2: Effects of dissolved oxygen

Survival was 100% during the trial. Growth data are presented in Table 3.2.2.1.3. Weight gain of YTK responded systematically to feed intake in both the normoxic and low DO treatments. After 38 days fish fed to apparent satiation (L5) had approximately doubled in body weight (Table 3.2.2.1.3).

Overall digestibility of the test diet was generally poor. Digestibility of dietary fat was significantly greater in 100S compared to 60S for L5 fish. While not statistically significant there was also a marked difference in histidine digestibility between oxygen environments with histidine ADCs in 60S much higher than for 100S L5 fish (Table 3.2.2.1.1).

Interaction terms were significant ($P < 0.05$) when considering growth and FCR (Table 3.2.2.1.3). With the exception of feeding L1, SGR of YTK was higher at all feeding levels under the normoxic regime. This response was partly responsible for the interaction of main effects. A one-way ANOVA on SGR indicated there was no significant difference between saturation regimes at each feeding level (all $P > 0.05$).

There was no significant difference in feed intake or crude nutrient and energy intake between each of the paired ration levels at either DO saturation (Table 3.2.2.1.3). However, on a digestible basis, fat intake was significantly greater in 100S L5 while histidine intake was significantly greater in S60 L5. This is reflected in the different ADCs of these nutrients in different oxygen environments (Table 3.2.2.1.2). Digestible intake was similar for all other nutrients and dietary energy among fish fed 100S L5 and 60S L5. FCRs ranged between 1.14 to 2.21 for the S100 group and 1.21 to 1.51 for the S60 group, with the better FCRs at intermediate rations for both DO treatments (Table 3.2.2.1.3).

Non-linear regression analyses indicated that there were no significant differences in growth responses across the feed regimes for crude feed (DM) and digestible fat intake ($P > 0.05$). However, both protein and energy utilisation efficiency for the S100 treatment improved significantly ($P < 0.05$) with increasing intake relative to the S60 group (Figure 3.2.2.1.3). Further, the rate of amino acid deposition relative to digestible amino acid intake is significantly different between DO treatments for all essential amino acids except for threonine and phenylalanine (Figure 3.2.2.1.4).

Utilisation coefficients and maintenance values are presented in Table 3.2.2.1.8. Where significant differences were found between DO treatments the amino acid utilisation efficiency decreased in at low saturation. Different DO saturation significantly affected all amino acid utilisation efficiencies except for phenylalanine and threonine (Table 3.2.2.1.8). DO saturation did not significantly affect protein and energy maintenance requirements. The effect of DO saturation on amino acid maintenance requirements varied depending on the amino acid (Table 3.2.2.1.8).

Discussion

The aim of this study was to better understand the impact of water temperature and dissolved oxygen saturation on nutrient and energy utilisation in order to make improvements to the preliminary BEM for YTK (Booth et al., 2010). Utilisation coefficient values in particular have a large influence on output data generated in BEMs (Pirozzi et al., 2010a), therefore, integrating significant abiotic factors into BEMs is important for predictive accuracy. Utilisation coefficient values for YTK determined in the present study were similar to those determined by Booth et al. (2010), who found coefficients of 0.41 and 0.55 for protein and energy utilisation, respectively for fish reared at 23 °C. When considering these data globally (Figure 3.2.2.1.5), protein utilisation efficiency increases with temperature to a vertex at

20.9 ºC while energy utilisation coefficient values continue to increase linearly with temperature. This response coincides with improved FCR observed in YTK grown in relatively warmer water to an upper limit, after which FCR deteriorates (Abbink et al., 2012; Bowyer et al., 2014) and reinforces a thermal optimal range for YTK of between 20 °C and 24 °C (Pirozzi and Booth, 2009; Bowyer et al., 2014).

Utilisation coefficients and growth responses

Utilisation coefficient values are calculated using linear regression analyses. This method has been applied in numerous bioenergetics studies to describe the nutrient utilisation efficiencies over the range of feed intake levels (Lupatsch and Kissil, 2005; Glencross, 2008; Pirozzi et al., 2010b; Trung et al., 2011; Chowdhury et al., 2013). This is generally considered appropriate because voluntary feed intake in fish can be highly variable meaning they are not always eating a constant or predictable amount of feed each day during experiments. However, linear regression is less appropriate in accurately describing the growth response relative to nutrient intake. This can be seen in Figure 3.2.2.1.1 and Figure 3.2.2.1.3 where growth responses are clearly non-linear. Statistical comparisons of non-linear models show significant differences for protein, energy and essential amino acid (except PHE and THR) and taurine utilisation when comparing DO treatments. In all cases, nutrient deposition (growth) responses were reduced with increasing nutrient intake in low DO compared to a normoxic environment.

Amino acid utilisation efficiencies have not typically been considered within the development of factorial BEMs with most published studies only describing growth responses and requirement for digestible protein (Lupatsch and Kissil, 2005; Glencross, 2008; Pirozzi et al., 2010a; Chowdhury et al., 2013). The impact of DO saturation was more profound than water temperature on amino acid utilisation efficiencies (Tables 3.2.2.1.6 and Table 3.2.2.1.8), consistently improving with increasing DO saturation. This occurred despite there being no statistical difference between protein utilisation efficiencies in different DO saturation environments. This may be partly explained by the difference in magnitude of DO effect on individual amino acids (with data only shown for essential amino acids) and also by the relative difference between nutrient digestibilities (Table 3.2.2.1.1), resulting in no significant difference between protein i.e. being the sum of responses of constituent amino acids.

The effect of temperature on arginine utilisation was highly significant with utilisation coefficients ranging from 0.92 in cool water to 0.16 in warm water. Combined with Experiment 2 data (Figure 3.2.2.1.6) it is clear that increasing water temperature has a negative impact on arginine utilisation efficiencies. While arginine requirements have been quantified for many aquaculture species (e.g Klein and Halver, 1970;. Ball et al., 2007; Ren et al., 2014) there is surprisingly very little work looking at temperature effects on requirement for arginine in aquaculture species; however, rearing temperature and amino acid interactions have been demonstrated to influence arginine requirement in poultry (Chamruspollert et al., 2004; Kodambashi Emami et al., 2017). This is an area that warrants further investigation for YTK and finfish species in general.

Unlike protein and energy utilisation, fat deposition responded linearly with digestible fat intake (Figure 3.2.2.1.1 and Figure 3.2.2.1.3). The mode of absorption varies depending on the macronutrient and the products of lipid digestion, including fatty acids, can enter the enterocyte by simple diffusion across the plasma membrane or are facilitated via transporter proteins in the membrane. In comparison, amino acids and monosaccharides, the products of protein and carbohydrate digestion respectively, are predominantly absorbed across cell membranes either through facilitated diffusion or by active transport. This differential mode of absorption is likely partly responsible for the comparative macronutrient responses seen in Figure 3.2.2.1.1 and Figure 3.2.2.1.3.

Maintenance requirements

Overall, the magnitude of the effect of water temperature was far more profound than the effect of DO saturation on maintenance requirements when comparing the two studies. While Experiments 1 and 2 studied the effects of temperature and DO in isolation, an orthogonal design considering *Temperature* × *DO* interactions may find these effects exacerbated (e.g. Bowyer et al., 2014). Maintenance requirements for energy were 47.2 and 64.7 DE $kJ^{-0.8}$ day^{-1} at 15 °C and 24 °C, respectively, which bracket that determined in Experiment 2 (20 ºC; 100% DO saturation) at 52.9 DE $kJ^{-0.8}$ day^{-1}. The maintenance

requirements for protein at 24 ºC (0.66 g DP $kg^{-0.7}$ day^{-1}) in this study were also very similar to that at 20 ºC in Experiment 2 (0.68); however, maintenance requirements for protein decreased to 0.28 g DP $kg^{-0.7}$ day^{-1} at 15 ºC. Small or non-significant relative changes in maintenance requirement for protein with temperature may indicate culture conditions around thermal optima (e.g. Pirozzi et al., 2010b), while in contrast, significant differences in protein maintenance requirement may be seen when culture conditions are outside of optimal temperatures (e.g. Amin et al., 2016).

Maintenance requirements for protein (0.68 g DP $kg^{-0.7}$ day^{-1}) and energy (52.9 DE $kJ^{-0.8}$ day^{-1}) in the current study were comparatively lower than those determined by Booth et al. (2010) at 1.70 g DP $kg^{-0.7}$ day^{-1} and 64.9 DE $kJ^{-0.8}$ day^{-1}. This is likely due to a combination of lower temperature in the current study (20 ºC *cf.* 23 ºC), the use of a fasted group by Booth et al. (2010) and different models used to estimate the x-intercept; the theoretical maintenance requirement.

Although taurine is a classed as a β-amino, as it does not contain the carboxyl group of α-amino acids it is recognised as an important essential nutrient for finfish (Salze and Davis, 2015). Maintenance requirements for taurine could not be determined at temperatures < 24 °C as the x-intercept calculated from the regression was < 0. A low maintenance requirement for taurine may indicate some capacity in YTK for *de novo* synthesis of this nutrient, but presently there is no research to confirm this. Alternatively, the results of Manuscript 3.1.5.3 demonstrated a sparing effect of added dietary methionine on taurine in juvenile YTK reared at 23 °C and this may partly explain this response. Identification and quantification of key enzyme activities including cysteine dioxygenase (CDO) and cysteine sulfinate decarboxylase (CSD) may indicate a capacity for taurine biosynthesis (Wang et al., 2016) and studies such as these should be undertaken with YTK.

Feed intake

Feed intake predictably increased with increasing water temperature when considering the satiated groups. Intake was only slightly depressed in the low DO satiated group compared to the normoxic treatment, however this was not statistically different. Glencross (2009) found no effect of DO on the utilisation efficiency of digestible protein and energy in Rainbow Trout, but did find that feed intake was significantly reduced in low DO. However, DO levels in that study were not constant across all feeding levels, decreasing significantly from 78% and 97% saturation for the fasted groups to 42% and 87% saturation for the satiated group in the low and normoxic DO treatments, respectively. Furthermore, regression analyses was done using the combined data from all groups, irrespective of shifts in abiotic factors. In Experiment 2 of the current study the lowest DO saturation was maintained consistently at 60%. Saturation levels below this may induce poor intake responses (Bowyer et al., 2014) and it seems that based on the results of the current study and other published data, that 60% saturation may represent an important physiological threshold for YTK.

Apparent digestibility

While there was no statistically significant effect of DO on ADCs for all nutrients except for fat, it is likely that utilisation coefficient values are nonetheless influenced by the variation in ADCs, especially as there were numerically large differences between some nutrients (e.g. histidine). As growth is regressed against digestible intake, efficiencies will, therefore, decrease or increase relative to the digestibility of a feed and its nutrients. There are very few studies that have looked at both the effect of DO on ADCs and utilisation efficiencies over a range of feed ration levels. Glencross (2009) employed a similar method; however, ADCs were determined in a separate study and it was not stated if these were established in different DO environments with only single ADC values presented for protein and energy therefore direct interpretation is difficult. Similarly, ADCs did not vary greatly between temperature treatments. Similar results have been seen in studies on other species such as salmon (e.g. Ng et al., 2004). The exception in the current study was for taurine, with ADCs significantly reduced in cooler water. Numerical differences in ADCs applied to respective treatment groups will have an influence on relative digestible nutrient intake; in turn influencing maintenance requirements, but not utilisation efficiencies, determined using the factorial method in this study.

Conclusions and Recommendations

This study quantified the effect of temperature and dissolved oxygen on key bioenergetic modelling parameters; i.e. nutrient utilisation coefficients and maintenance requirements. The results of this study will facilitate the integration of abiotic parameters into existing bioenergetic models for YTK resulting in more accurate predictive tools for nutrient requirements and feed management with changing aquaculture conditions.

Temperature had a significant effect on some of the parameters tested in this study; however, the magnitude of the effect varied depending on the nutrient assessed. For example, the relative decrease in utilisation efficiency of arginine at 24 °C compared to 15 °C was particularly profound and warrants further investigation. Protein and energy utilisation efficiencies were not statistically different at different temperatures as determined by linear regression analyses; however, it is nonetheless pertinent to consider integrating a temperature function (Figure 3.2.2.1.5) when incorporating this data into predictive models to ensure predictive accuracy.

Low DO at 60% saturation negatively affected the nutrient and energy utilisation response in YTK with this response tending to be more pronounced with increasing nutrient and energy intake. As feed intake was not significantly different between DO levels within each paired feed ration in Experiment 2 these responses are likely due to a combination of the effect of DO on nutrient digestibility and the differential rate limited response of nutrient deposition in a low DO environment. The underlying mechanisms for this are unknown and warrant further investigation to provide greater insight on the nutritional physiology of YTK.

While the negative effects of a low DO environment are broadly understood in terms of general finfish husbandry, this study has quantified the negative impacts on nutrient utilisation in YTK in a low DO environment, with these effects exacerbated at higher feeding levels implying that a restricted feed ration may be more appropriate in a low DO environment. Experiment 2 was conducted at 20 °C with a DO concentration of 5.4 mg L^{-1}, which might be considered acceptable by some aquaculturists; however, the saturation was only 60%. In comparison DO saturation of 100% in saltwater at 20 °C is equivalent to a concentration of approximately 8.0 mg L^{-1}. This highlights the importance of considering both DO saturation and concentration when determining YTK welfare in aquaculture systems.

Findings

- Water temperature has a predictable effect on protein and energy utilisation.
- The magnitude of the effect of water temperature on amino acid utilization varies depending on the specific the amino acid.
- Low DO saturation of 60% did not negatively affect feed intake.
- Low DO saturation of 60% negatively affects amino acid utilisation.
- Understanding the influence of abiotic factors on YTK nutritional physiology will enable the development of more accurate nutrient and feed demand models.
- In turn this will facilitate better feed management and feed formulation through a better understanding of nutrient requirements and dietary specifications for YTK.

Publications

Pirozzi, I., Benito, M.R., Booth, M. n.d. Protein, amino acid and energy utilisation and maintenance requirements of juvenile Yellowtail Kingfish (*Seriola lalandi*): quantifying abiotic influences. Aquaculture (in preparation).

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, New South Wales Department of Primary Industries (NSW DPI) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

We would also like to acknowledge the technical assistance of Dr Basseer Codabaccus, Ian Russell, Justin Tierney, Marcus Armstrong and Steve Gamble (NSW DPI). We thank Cedric Simon, Barney Hines and staff at CSIRO for conducting the chemical analyses on samples generated from this experiment. Dr Michael Salini and Simon Tabrett were actively involved in supplying many of the raw materials used to manufacture the experimental feeds (Ridley).

References

Abbink, W., Blanco Garcia, A., Roques, J.A.C., Partridge, G.J., Kloet, K., Schneider, O., 2012. The effect of temperature and pH on the growth and physiological response of juvenile yellowtail kingfish *Seriola lalandi* in recirculating aquaculture systems. Aquaculture 330-333, 130-135.

ACEC, 2015. A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research, 4th Edition, NSW DPI Animal Care and Ethics Committee, pp. 56.

Amin, M.N., Carter, C.G., Katersky Barnes, R.S., Adams, L.R., 2016. Protein and energy nutrition of brook trout (*Salvelinus fontinalis*) at optimal and elevated temperatures. Aquac. Nutr. 22, 527-540.

AOAC Association of the Official Analytical Chemists, 2005. Official methods of analysis of the AOAC International, 18th ed. AOAC International, Gaithersburg, MD, USA.

Ball, R.O., Urschel, K.L., Pencharz, P.B., 2007. Nutritional consequences of interspecies differences in arginine and lysine metabolism. J. Nutr. 137, 1626S-1641S.

Bejda, A.J., Phelan, B.A., Studholme, A.L., 1992. The effect of dissolved-oxygen on the growth of young-of-the-year winter flounder, *Pseudopleuronectes americanus*. Environ. Biol. Fishes 34, 321-327.

Booth, M.A., Allan, G.L., Pirozzi, I., 2010. Estimation of digestible protein and energy requirements of yellowtail kingfish *Seriola lalandi* using a factorial approach. Aquaculture 307, 247-259.

Bowyer, J.N., Booth, M.A., Qin, J.G., D'Antignana, T., Thomson, M.J.S., Stone, D.A.J., 2014. Temperature and dissolved oxygen influence growth and digestive enzyme activities of yellowtail kingfish *Seriola lalandi* (Valenciennes, 1833). Aquac. Res. 45, 2010-2020.

Chamruspollert, M., Pesti, G., Bakalli, R., 2004. Influence of temperature on the arginine and methionine requirements of young broiler chicks. Journal of Applied Poultry Research 13, 628-638.

Cho, C.Y., Bureau, D.P., 1998. Development of bioenergetic models and the Fish-PrFEQ software to estimate production, feeding ration and waste output in aquaculture. Aquat. Living Resour. 11, 199-210.

Chowdhury, M.A.K., Siddiqui, S., Hua, K., Bureau, D.P., 2013. Bioenergetics-based factorial model to determine feed requirement and waste output of tilapia produced under commercial conditions. Aquaculture 410, 138-147.

Glencross, B., 2008. A factorial growth and feed utilization model for barramundi, *Lates calcarifer* based on Australian production conditions. Aquac. Nutr. 14, 360-373.

Glencross, B., Bermudes, M., 2010. Effect of high water temperatures on the utilisation efficiencies of energy and protein by juvenile barramundi, *Lates calcarifer*. Fisheries and Aquaculture Journal. FAJ-14.

Glencross, B.D., 2009. Reduced water oxygen levels affect maximal feed intake, but not protein or energy utilization efficiency of rainbow trout (*Oncorhynchus mykiss*). Aquacult. Nutr. 15, 1-8.

Klein, R.G., Halver, J.E., 1970. Nutrition of salmonoid fishes: Arginine and histidine requirements of Chinook and coho salmon. J. Nutr. 100, 1105-1109.

Kodambashi Emami, N., Golian, A., Rhoads, D., Danesh Mesgaran, M., 2017. Interactive effects of temperature and dietary supplementation of arginine or guanidinoacetic acid on nutritional and physiological responses in male broiler chickens. Br. Poult. Sci. 58, 87-94.

Lupatsch, I., Kissil, G.W., 2005. Feed formulations based on energy and protein demands in white grouper (Epinephelus aeneus). Aquaculture 248, 83-95.

Ng, W.-K., Sigholt, T., Gordon Bell, J., 2004. The influence of environmental temperature on the apparent nutrient and fatty acid digestibility in Atlantic salmon (*Salmo salar* L.) fed finishing diets containing different blends of fish oil, rapeseed oil and palm oil. Aquac. Res. 35, 1228-1237.

Pirozzi, I., Booth, M.A., 2009. The routine metabolic rate of mulloway (*Argyrosomus japonicus*: Sciaenidae) and yellowtail kingfish (*Seriola lalandi*: Carangidae) acclimated to six different temperatures. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 152, 586-592.

Pirozzi, I., Booth, M.A., Allan, G.L., 2010a. A factorial approach to deriving dietary specifications and daily feed intake for mulloway, *Argyrosomus japonicus*, based on the requirements for digestible protein and energy. Aquaculture 302, 235-242.

Pirozzi, I., Booth, M.A., Allan, G.L., 2010b. Protein and energy utilization and the requirements for maintenance in juvenile mulloway (*Argyrosomus japonicus*). Fish Physiol. Biochem. 36, 109-121.

Ren, M., Ai, Q., Mai, K., 2014. Dietary arginine requirement of juvenile cobia (*Rachycentron canadum*). Aquac. Res. 45, 225-233.

Salini, M.J., Poppi, D., Turchini, G.M., Glencross, B.D., 2016. Defining the allometric relationship between size and individual fatty acid turnover in barramundi *Lates calcarifer*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology. 201, 79-86.

Salze, G.P., Davis, D.A., 2015. Taurine: a critical nutrient for future fish feeds. Aquaculture 437, 215-229.

Trung, D.V., Diu, N.T., Hao, N.T., Glencross, B., 2011. Development of a nutritional model to define the energy and protein requirements of tilapia, *Oreochromis niloticus*. Aquaculture 320, 69-75.

Wang, X., He, G., Mai, K.S., Xu, W., Zhou, H.H., 2016. Differential regulation of taurine biosynthesis in rainbow trout and Japanese flounder. Scientific Reports. 6.

Yang, K., Fan, Q., Zhang, L., Li, B., Gao, Y., Zeng, K., Wang, Q., Zhu, S., Fang, G., 2015. Effect of dissolved oxygen levels on growth performance, energy budget and antioxidant responses of yellow catfish, *Pelteobagrus fulvidraco* (Richardson). Aquac. Res. 46, 2025-2033.

Table 3.2.2.1.1. Diet composition (dry matter basis; g $100g^{-1}$ unless otherwise indicated) and diet apparent digestibility coefficients (ADCs; +/- SD, *n* = 2) for Experiment 1 (Temperature; 15 °C or 24 °C) and Experiment 2 (Dissolved Oxygen; 60% or 100% saturation). Significant differences ($P < 0.05$) between ADC's within Experiments and nutrients are denoted with superscript letters. ADC data determined from L5 feed group only.

	Experiment 1: Water temperature			**Experiment 2: Dissolved oxygen**		
Nutrient	**Diet composition**	**15 °C ADC**	**24 °C ADC**	**Diet composition**	**60S ADC**	**100S ADC**
Dry matter	92.30	0.49±0.02	0.42±0.02	91.85	0.43±0.02	0.43±0.04
Crude protein	57.31	0.68±0.02	0.61±0.03	56.41	0.60±0.04	0.61±0.04
Fat	12.68	0.60±0.002	0.55±0.10	13.21	0.50±0.02[a]	0.63±0.03[b]
Ash	7.73	-	-	7.59	-	-
Organic matter	92.27	0.55±0.02	0.49±0.05	92.41	0.48±0.03	0.48±0.04
NFE	22.28	0.19±0.01	0.14±0.07	22.79	0.18±0.001	0.06±0.05
Energy (MJ kg^{-1})	22.52	0.58±0.02	0.52±0.05	23.32	0.51±0.03	0.53±0.04
DP:DE (g MJ^{-1})		29.8	29.9		28.5	27.8
Amino Acids						
Alanine	2.63	0.63±0.04	0.66±0.05	3.13	0.61±0.13	0.56±0.04
Arginine	2.59	0.86±0.04	0.88±0.05	3.48	0.84±0.01	0.83±0.08
Aspartic acid	4.64	0.46±0.01	0.55±0.01	5.29	0.42±0.07	0.52±0.07
Cystine	1.28	0.67±0.001	0.75±0.07	0.97	0.54±0.36	0.49±0.10
Glutamic acid	8.23	0.73±0.04	0.78±0.02	9.52	0.73±0.01	0.73±0.03
Glycine	4.04	0.64±0.01[a]	0.70±0.001[b]	3.97	0.53±0.01	0.50±0.10
Histidine	2.07	0.72±0.01	0.78±0.03	2.04	0.62±0.09	0.45±0.08
Isoleucine	1.36	0.77±0.01	0.74±0.06	1.40	0.67±0.03	0.64±0.03
Leucine	3.30	0.70±0.01	0.70±0.05	3.49	0.63±0.03	0.59±0.05
Lysine	2.85	0.65±0.07	0.66±0.007	3.25	0.46±0.02	0.44±0.14
Methionine	1.25	0.78±0.01	0.81±0.05	1.32	0.61±0.08	0.55±0.08
Phenylalanine	1.43	0.69±0.04	0.72±0.06	1.59	0.60±0.09	0.63±0.05
Proline	3.08	0.70±0.02	0.70±0.009	3.36	0.68±0.09	0.64±0.06
Serine	4.13	0.78±0.02[a]	0.81±0.005[b]	4.06	0.67±0.04	0.61±0.10
Threonine	2.77	0.69±0.02	0.72±0.004	2.71	0.52±0.03	0.52±0.06
Tyrosine	1.74	0.76±0.01	0.76±0.009	1.90	0.63±0.12	0.54±0.09
Valine	3.67	0.68±0.01	0.76±0.04	3.85	0.72±0.08	0.65±0.03
Taurine	1.38	0.60±0.001[a]	0.78±0.01[b]	1.56	0.81±0.01	0.85±0.12

Table 3.2.2.1.2. Mean water quality parameters (± SD) over the duration of each experiment.

	Experiment 1: Water temperature		**Experiment 2: Dissolved oxygen**	
Parameter	**15°C**	**24°C**	**60S**	**100S**
Dissolved oxygen (mg L^{-1})	9.1±1.51	8.4±1.47	5.4±0.7	7.9±0.6
Dissolved oxygen (%)	95.2±2.03	99.7±3.45	61.8±3.0	98.2±4.7
Temperature (°C)	15.0±0.33	23.8±1.84	19.3±0.6	19.6±0.9
pH	8.1±0.09	7.9±0.13	7.9±0.2	8.0±0.2
Salinity (‰)	33.2±2.4	33.2±2.4	33.3±0.1	33.0±0.2
TAN (mg L^{-1})	0.3±0.24	0.4±0.29	0.48±0.55	0.39±0.46
CO_2 (mg L^{-1})	-	-	4.4±1.3	4.0±1.2

Table 3.2.2.1.3. Growth and feed intake metrics (+/- SE; *n* = 2) for each feed level and abiotic treatment for Experiments 1 and 2. +/- SE are shown for L4 and L5 feed intake only (SE = 0 at low restricted rations). Interaction term (abiotic factor × feed level) on final performance variables; ns = not significant. Tukey-Kramer post-hoc analyses on significant main effects. Values within rows sharing superscript letters are not significantly different ($P > 0.05$). Statistical comparison of feed intake data applied to Experiment 2 L5 only; $P > 0.05$.

Experiment 1	**15 °C**					**24 °C**					**Interaction term**
Feed Level	**L1**	**L2**	**L3**	**L4**	**L5**	**L1**	**L2**	**L3**	**L4**	**L5**	
Initial Weight (g)	185.5±1.0	185.5±1.1	188.5±0.3	187.6±2.4	187.7±1.2	186.7±0.85	189.8±1.0	187.0±1.9	188.9±0.2	185.3±0.8	-
Final Weight (g)	191.1±4.4^{a}	209.8±1.1^{a}	238.3±1.0^{b}	243.2±4.7^{b}	253.0±2.2^{b}	198.4±5.9^{a}	250.7±9.4^{b}	298.9±7.6^{c}	333.6±4.3^{d}	364.6±3.4^{e}	$P < 0.0001$
Growth (g fish^{-1} d^{-1})	0.16±0.09^{a}	0.67±0.02^{a}	1.38±0.04^{b}	1.55±0.06^{b}	1.81±0.03^{b}	0.34±0.14^{a}	1.74±0.24^{b}	3.20±0.16^{c}	4.14±0.12^{c}	5.13±0.07^{c}	$P < 0.0001$
SGR (% d^{-1})	0.03±0.02^{a}	0.13±0.001^{b}	0.26±0.01^{c}	0.30±0.01^{c}	0.35±0.003^{c}	0.17±0.07^{a,b}	0.77±0.09^{c}	10.1±0.04^{d}	1.58±0.03^{e}	1.88±0.01^{f}	$P < 0.0001$
Initial Condition	1.36±0.01	1.36±0.02	1.35±0.01	1.36±0.02	1.38±0.01	1.38±0.01	1.37±0.02	1.34±0.005	1.38±0.001	1.38±0.01	-
Final Condition	1.28±0.02^{a}	1.37±0.05^{abc}	1.37±0.002^{abc}	1.40±0.01^{abc}	1.43±0.01^{bc}	1.32±0.01^{as}	1.39±0.02^{abc}	1.43±0.03^{bcd}	1.48±0.02^{cd}	1.56±0.0002^{d}	ns
Feed Intake (g fish^{-1} d^{-1})	0.57	1.24	1.91	2.58±0.001	3.32±0.18	1.20	2.88	4.54	6.20±0.00	8.05±0.27	-
FCR	5.69±3.43	1.84±0.002	1.38±0.04	1.68±0.07	1.83±0.07	4.39±1.89	1.69±0.23	1.42±0.07	1.50±0.04	1.57±0.03	ns
Experiment 2	**60% saturation**					**100% saturation**					
Feed Level	**L1**	**L2**	**L3**	**L4**	**L5**	**L1**	**L2**	**L3**	**L4**	**L5**	**Interaction term**
Initial Weight (g)	217.7±2.2	222.3±0.6	220.9±0.7	218.6±1	220.3±1.1	223.3±1.1	222.6±2.8	220.9±0.9	221.3±2.7	221.2±1.2	-
Final Weight (g)	244.6±6.9^{a}	313.3±3.1^{ab}	356.7±2.4^{cd}	387.8±6.3^{d}	433.7±12.9^{e}	241.8±1.9^{a}	323.2±3^{bc}	380.2±4.85^{d}	425.4±10.3^{e}	454.1±2.15^{e}	ns
Growth (g fish^{-1} d^{-1})	0.73±0.13^{a}	2.46±0.07^{b}	3.67±0.08^{c}	4.57±0.14^{de}	5.77±0.32^{e}	0.49±0.02^{a}	2.65±0.01^{b}	4.19±0.1^{cd}	5.37±0.2^{e}	6.13±0.09^{e}	$P < 0.05$
SGR (% d^{-1})	0.31±0.05^{a}	0.93±0.02^{b}	1.30±0.03^{c}	1.55±0.03^{d}	1.83±0.07^{e}	0.22±0.01^{a}	1.01±0.01^{b}	1.47±0.02^{cd}	1.77±0.03^{e}	1.94±0.03^{e}	$P < 0.01$
Initial Condition	1.36±0.003	1.37±0.03	1.39±0.002	1.36±0.02	1.36±0.01	1.37±0.002	1.39±0.02	1.41±0.02	1.37±0.04	1.36±0.003	-
Final Condition	1.29±0.04^{ab}	1.35±0.03^{abc}	1.37±0.001^{bcd}	1.44±0.01^{cd}	1.49±0.01^{d}	1.24±0.004^{a}	1.31±0^{ab}	1.36±0.005^{bc}	1.38±0.05^{bcd}	1.45±0.02^{cd}	ns
Feed Intake (g fish^{-1} d^{-1})	1.06	2.96	4.86	6.51±0.02	8.92±0.33	1.03	3.03	5.05	6.97±0.03	9.46±0.31	-
FCR	1.51±0.27^{a}	1.21±0.03^{a}	1.330.03^{a}	1.43±0.05^{a}	1.55±0.03^{a}	2.12±0.09^{b}	1.14±0.01^{a}	1.21±0.03^{a}	1.30±0.04^{a}	1.54±0.07^{a}	$P < 0.05$

Table 3.2.2.1.4. Experiment 1; Daily digestible nutrient and energy intake. +/- SE shown for L4 and L5 feed rations only (SE = 0 at low restricted rations).

Water Temperature	**15 °C**					**24 °C**				
Feed Level	**L1**	**L2**	**L3**	**L4**	**L5**	**L1**	**L2**	**L3**	**L4**	**L5**
DE Intake (kJ $fish^{-1}$ d^{-1})	7.37	16.08	24.83	33.56±0.11	43.11±2.33	14.04	33.62	52.98	72.34±0.001	93.95±3.16
DFat Intake (g $fish^{-1}$ d^{-1})	0.04	0.09	0.15	0.20±0.00004	0.25±0.01	0.08	0.20	0.32	0.440±0.000003	0.57±0.02
DP Intake (g $fish^{-1}$ d^{-1})	0.22	0.48	0.75	1.01±0.0002	1.30±0.09	0.42	1.01	1.59	2.17±0.00002	2.82±0.09
DArg Intake (mg $fish^{-1}$ d^{-1})	12.69	27.72	42.78	57.83±0.01	74.29±4.01	27.58	66.06	104.10	142.15±0.001	184.61±6.21
DHis Intake (mg $fish^{-1}$ d^{-1})	8.49	18.54	28.62	38.69±0.01	49.70±2.68	19.38	46.43	73.17	99.91±0.001	129.76±4.36
DIso Intake (mg $fish^{-1}$ d^{-1})	5.98	13.06	20.15	27.24±0.01	35.00±1.89	12.18	29.18	45.99	62.80±0.0005	81.56±2.74
DLeu Intake (mg $fish^{-1}$ d^{-1})	5.14	11.22	17.32	23.41±0.005	30.07±1.62	9.75	23.36	36.81	50.27±0.0004	65.28±2.19
DLys Intake (mg $fish^{-1}$ d^{-1})	10.51	22.95	35.43	47.89±0.01	61.52±3.32	22.68	54.32	85.60	116.89±0.001	151.81±5.10
DMet Intake (mg $fish^{-1}$ d^{-1})	5.51	12.02	18.56	25.08±0.01	32.23±1.74	12.17	29.16	45.95	62.75±0.0005	81.49±2.74
DPhe Intake (mg $fish^{-1}$ d^{-1})	5.58	12.19	18.81	25.43±0.01	32.67±1.76	12.35	29.59	46.63	63.68±0.0005	82.70±2.78
DThr Intake (mg $fish^{-1}$ d^{-1})	4.27	9.31	14.38	19.43±0.004	24.96±1.35	8.40	20.11	31.69	43.28±0.0003	56.20±1.89
DVal Intake (mg $fish^{-1}$ d^{-1})	14.25	31.11	48.03	64.92±0.01	83.40±4.50	33.59	80.45	126.79	173.13±0.001	224.85±7.56
DTau Intake (mg $fish^{-1}$ d^{-1})	4.67	10.20	15.74	21.28±0.005	27.34±1.48	12.96	31.03	48.91	66.78±0.001	86.73±2.92

Table 3.2.2.1.5. Experiment 2; Daily feed and digestible nutrient and energy intake. +/- SE are shown for L4 and L5 feed rations only (SE = 0 at low restricted rations). Statistical comparison between treatments was performed on L5 satiated group only. Data within rows denoted with different superscript letters are significantly different ($P > 0.05$).

DO saturation	**60%**					**100%**				
Feed Level	**L1**	**L2**	**L3**	**L4**	**L5**	**L1**	**L2**	**L3**	**L4**	**L5**
DE Intake (kJ fish^{-1} d^{-1})	12.62	35.18	57.72	77.32±0.22	105.95±3.92	12.65	37.18	61.98	85.46±0.37	116.01±3.80
DFat Intake (g fish^{-1} d^{-1})	0.07	0.20	0.32	0.43±0.001	0.59±0.04^{a}	0.86	0.25	0.42	0.58±0.003	0.79±0.03^{b}
DP Intake (g fish^{-1} d^{-1})	0.36	1.00	1.64	2.20±0.01	3.01±0.11	0.36	1.04	1.74	2.40±0.01	3.26±0.11
DArg Intake (mg fish^{-1} d^{-1})	30.90	86.11	141.31	189.28±0.53	259.36±9.59	29.62	87.05	145.12	200.12±0.53	271.65±8.90
DHis Intake (mg fish^{-1} d^{-1})	13.47	37.52	61.58	82.48±0.23	113.02±4.18^{b}	9.52	27.97	46.62	64.29±0.23	87.27±2.86^{a}
DIso Intake (mg fish^{-1} d^{-1})	9.98	27.82	45.65	61.15±0.17	83.78±3.10	9.29	27.31	45.53	62.79±0.17	85.23±2.79
DLeu Intake (mg fish^{-1} d^{-1})	7.91	22.03	36.15	48.42±0.14	66.35±2.45	7.37	21.67	36.12	49.82±0.14	67.62±2.21
DLys Intake (mg fish^{-1} d^{-1})	15.95	44.45	72.93	97.69±0.27	133.86±4.95	14.60	42.91	71.54	98.65±0.27	133.91±4.39
DMet Intake (mg fish^{-1} d^{-1})	8.51	23.72	38.92	52.13±0.15	71.43±2.64	7.44	21.86	36.45	50.26±0.15	68.23±2.23
DPhe Intake (mg fish^{-1} d^{-1})	10.08	28.10	46.11	61.77±0.17	84.63±3.13	10.36	30.46	50.78	70.03±0.17	95.06±3.11
DThr Intake (mg fish^{-1} d^{-1})	5.02	13.99	22.96	30.76±0.09	42.15±1.56	4.98	14.64	24.40	33.65±0.09	45.68±1.50
DVal Intake (mg fish^{-1} d^{-1})	29.56	82.36	135.15	181.03±0.51	248.05±9.17	25.74	75.64	126.10	173.88±0.51	236.04±7.73
DTau Intake (mg fish^{-1} d^{-1})	13.43	37.43	61.43	82.28±0.23	112.75±4.17	13.73	40.36	67.28	92.78±0.23	125.94±4.12

Table 3.2.2.1.6. Experiment 1; Digestible energy and nutrient utilisation coefficients values. Linear equations are presented to indicate utilisation efficiency across the different feed levels. Equations and equation parameters are pooled between temperature treatments unless significantly different ($P < 0.05$).

Nutrient	Linear equation	Nutrient cost of growth (unit gain^{-1})	R^2
Energy (kJ kg$^{-0.8}$ day^{-1})	0.605x-34.01	1.65	0.96
Protein (g kg$^{-0.7}$ day^{-1})	0.389x+0.05	2.57	0.96
Fat (g kg$^{-0.9}$ day^{-1})	0.970x-0.62	1.03	0.95
Arg 15°C (mg kg$^{-0.7}$ day^{-1})	0.923x-24.3	1.08	0.90
Arg 24°C (mg kg$^{-0.7}$ day^{-1})	0.156x-3.41	6.43	0.92
His (mg kg$^{-0.7}$ day^{-1})	0.434x-16.65	2.30	0.91
Iso (mg kg$^{-0.7}$ day^{-1})	0.620x-15.31	1.61	0.92
Leu (mg kg$^{-0.7}$ day^{-1})	1.299x-29.47	0.77	0.92
Lys (mg kg$^{-0.7}$ day^{-1})	0.592x-21.32	1.69	0.92
Met (mg kg$^{-0.7}$ day^{-1})	0.412x-7.45	2.73	0.92
Phe (mg kg$^{-0.7}$ day^{-1})	0.528x-10.44	1.89	0.92
Thr (mg kg$^{-0.7}$ day^{-1})	0.915x-14.15	1.09	0.93
Val (mg kg$^{-0.7}$ day^{-1})	0.242x-10.66	4.14	0.91
Tau 15°C (mg kg$^{-0.7}$ day^{-1})	0.166x+6.26	6.02	0.90
Tau 24°C (mg kg$^{-0.7}$ day^{-1})	0.180x-11.4	5.55	0.88

Table 3.2.2.1.7. Experiment 1; Digestible energy and nutrient maintenance requirements. Estimated from the x-intercept using non-linear regression (Figure 3.2.2.1.1 and Figure 3.2.2.1.2), except for fat where linear regression was the appropriate model. Maintenance values are not presented for taurine at 15 °C as the x-intercept was < 0.

	15 °C		24 °C	
Nutrient	Maintenance requirement	R^2	Maintenance requirement	R^2
Energy (kJ kg$^{-0.8}$ day^{-1})	47.20	0.95	64.74	0.98
Protein (g kg$^{-0.7}$ day^{-1})	0.28	0.97	0.66	0.98
Fat (g kg$^{-0.9}$ day^{-1})	0.64	0.90	0.68	0.95
Arg (mg kg$^{-0.7}$ day^{-1})	41.30	0.93	76.24	0.99
His (mg kg$^{-0.7}$ day^{-1})	38.62	0.96	71.55	0.95
Iso (mg kg$^{-0.7}$ day^{-1})	23.94	0.98	45.98	0.95
Leu (mg kg$^{-0.7}$ day^{-1})	20.47	0.98	36.64	0.94
Lys (mg kg$^{-0.7}$ day^{-1})	37.16	0.98	79.73	0.93
Met (mg kg$^{-0.7}$ day^{-1})	19.24	0.98	41.52	0.92
Phe (mg kg$^{-0.7}$ day^{-1})	20.90	0.98	45.89	0.95
Thr (mg kg$^{-0.7}$ day^{-1})	14.85	0.99	29.21	0.94
Val (mg kg$^{-0.7}$ day^{-1})	53.32	0.98	125.04	0.95
Tau (mg kg$^{-0.7}$ day^{-1})	-	-	64.35	0.89

Table 3.2.2.1.8. Experiment 2; Digestible energy (kJ $kg^{-0.8}$ day^{-1}), protein (g $kg^{-0.7}$ day^{-1}) and amino acid (mg $kg^{-0.7}$ day^{-1}) utilisation coefficients and maintenance values. Linear equations are presented to indicate utilisation efficiency across the different feed levels. Equations and equation parameters are pooled between DO treatments unless significantly different ($P < 0.05$). Maintenance values are estimated from the x-intercept using non-linear regression (Figure 3.2.2.1.3 and Figure 3.2.2.1.4) except for fat where linear regression was the appropriate model and therefore R^2 remains unchanged. Maintenance values are not presented for taurine as the x-intercept was < 0.

Nutrient	Linear equation	Nutrient cost of growth (unit gain^{-1})	R^2	Maintenance requirement	R^2
Energy	0.521x-21.690	1.92	0.91	52.94	0.94
Protein	0.411x+0.007	2.43	0.94	0.68	0.98
Fat	0.883-0.437	1.13	0.82	0.49	0.82
Arg S60	0.215x+6.999	4.65	0.95	38.38	0.98
Arg S100	0.257x+7.124	3.89	0.92	48.49	0.97
His S60	0.390x+1.580	2.56	0.92	26.00	0.97
His S100	0.631x+1.789	1.59	0.89	20.70	0.95
Iso S60	0.474x+0.980	2.11	0.94	19.55	0.98
Iso S100	0.580x+1.120	1.73	0.93	20.47	0.96
Leu S60	0.967x+1.369	1.03	0.93	15.60	0.98
Leu S100	1.185x+1.547	0.84	0.90	16.34	0.96
Lys S60	0.528x+1.200	1.89	0.94	30.07	0.98
Lys S100	0.661x+1.260	1.51	0.90	31.33	0.95
Met S60	0.363x+2.402	2.75	0.94	12.94	0.98
Met S100	0.478x+2.450	2.09	0.91	13.69	0.96
Phe	0.460x-0.921	2.18	0.91	21.43	0.95
Thr	1.009x+0.799	0.99	0.90	9.69	0.90
Val S60	0.184x-0.999	5.43	0.95	60.58	0.95
Val S100	0.240x-0.934	4.17	0.92	58.70	0.92
Tau S60	0.096x+8.811	10.42	0.95	-	
Tau S100	0.116x+8.80	8.63	0.92	-	

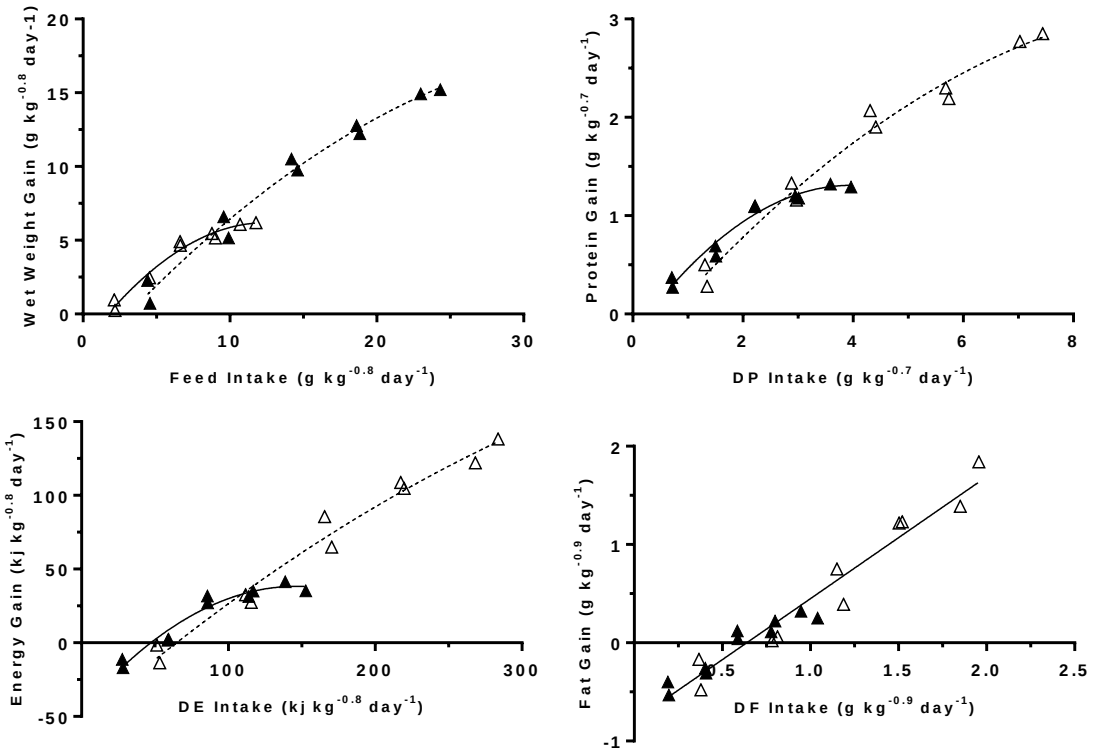


Figure 3.2.2.1.1. Experiment 1; Relationship between feed intake and wet weight gain, whole carcass compositional gain and digestible nutrient intake. Temperature significantly affects the rate of protein and energy deposition but not fat deposition relative to intake. Solid triangles and solid lines = 15 ^{o}C; Open triangles and dashed lines = 24 ^{o}C.

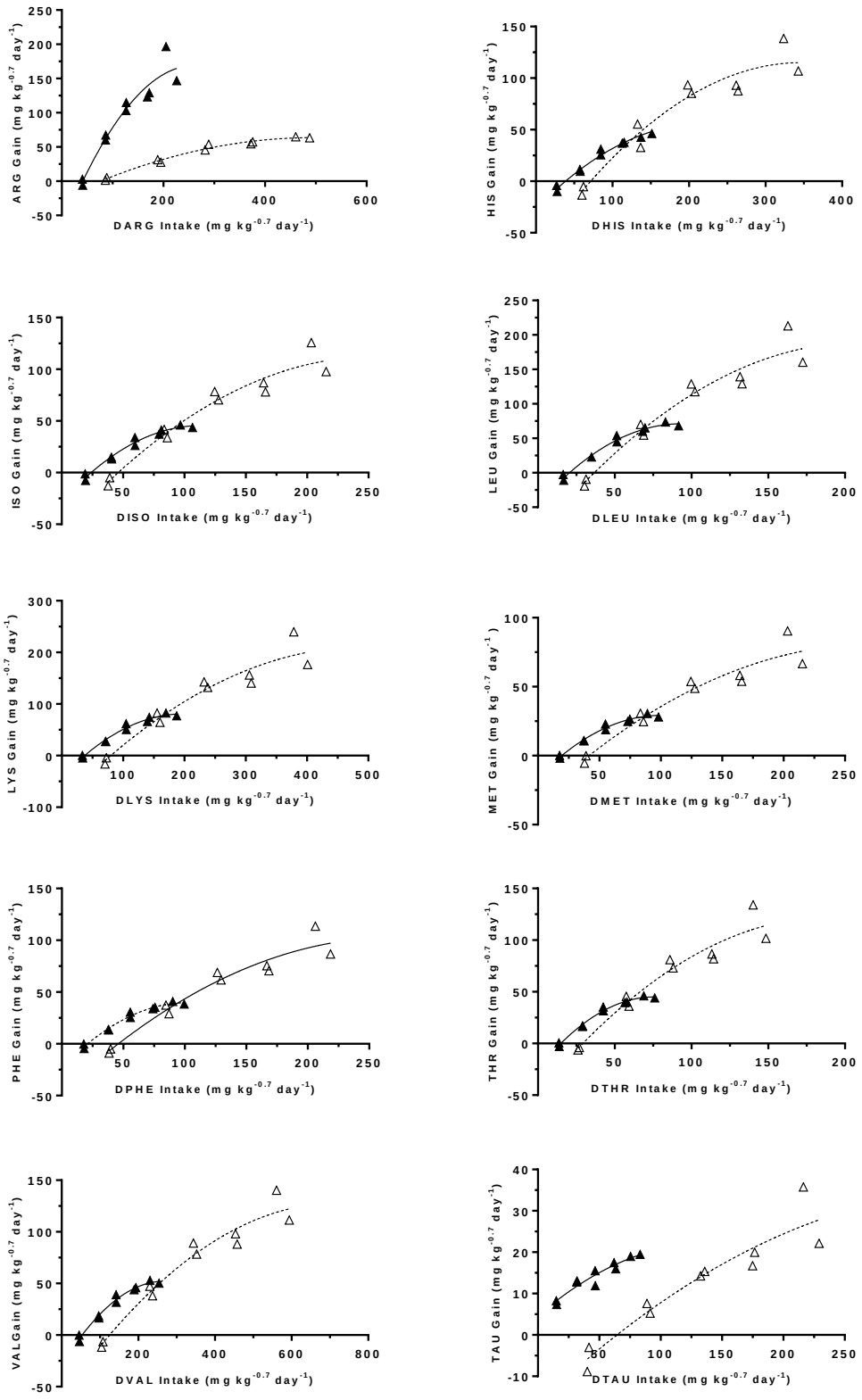


Figure 3.2.2.1.2. Experiment 1; Relationship between whole carcass amino acid gain and digestible amino acid intake including taurine. The rate of amino acid deposition relative to digestible amino acid intake is significantly different between temperature treatments for all essential amino acids except for methionine, lysine and threonine. Solid triangles and solid lines = 15 °C; Open triangles and dashed lines = 24 °C.

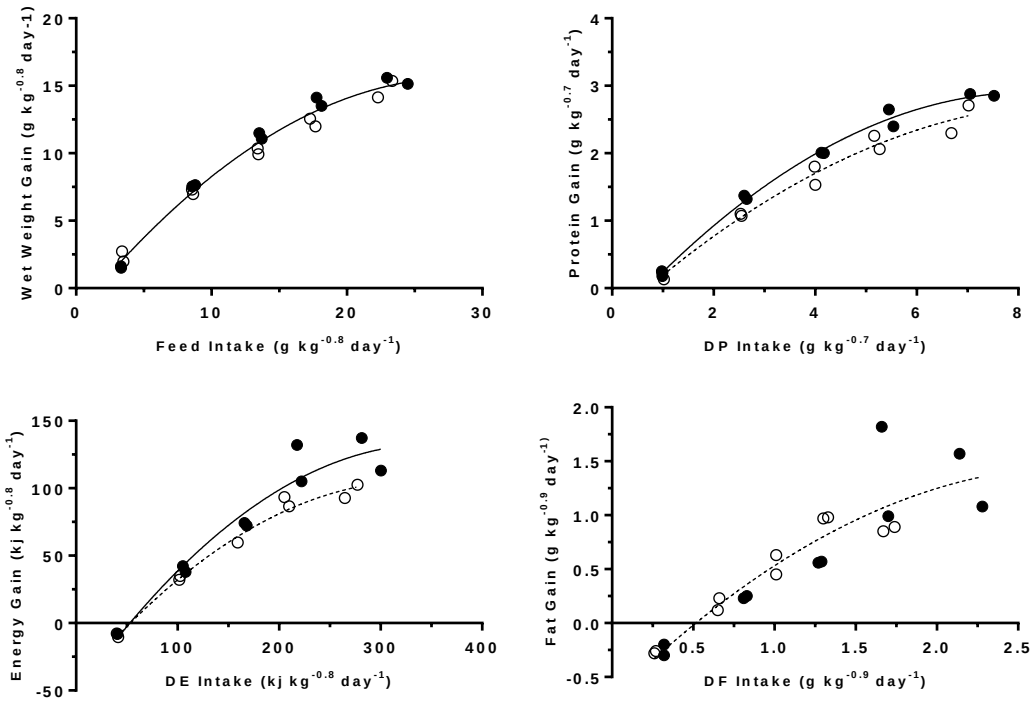


Figure 3.2.2.1.3. Experiment 2; Relationship between whole carcass compositional gain and digestible nutrient intake. The rate of protein and energy deposition relative to digestible protein and energy intake is significantly different between DO treatments. No significant differences in growth responses were observed for feed intake, digestible energy and digestible fat intake when comparing DO treatments. Open circles, dashed lines = 60% saturation; Solid circles, solid line = 100% saturation.

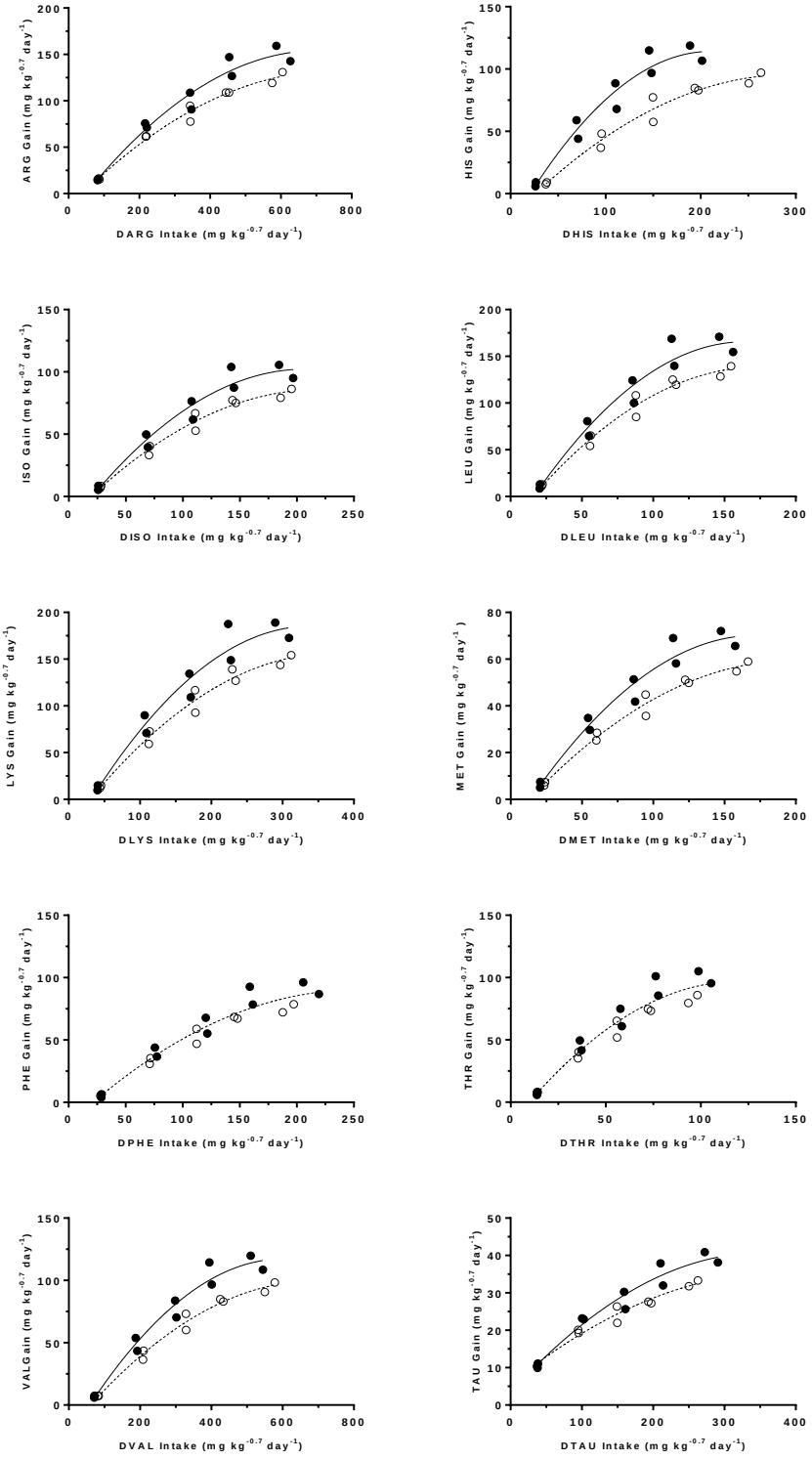


Figure 3.2.2.1.4. Experiment 2; Relationship between whole carcass amino acid gain and digestible amino acid intake including taurine. The rate of amino acid deposition relative to digestible amino acid intake is significantly different between DO treatments for all essential amino acids except for threonine and phenylalanine. Open Circles = 60% saturation; Solid Circles = 100% saturation.

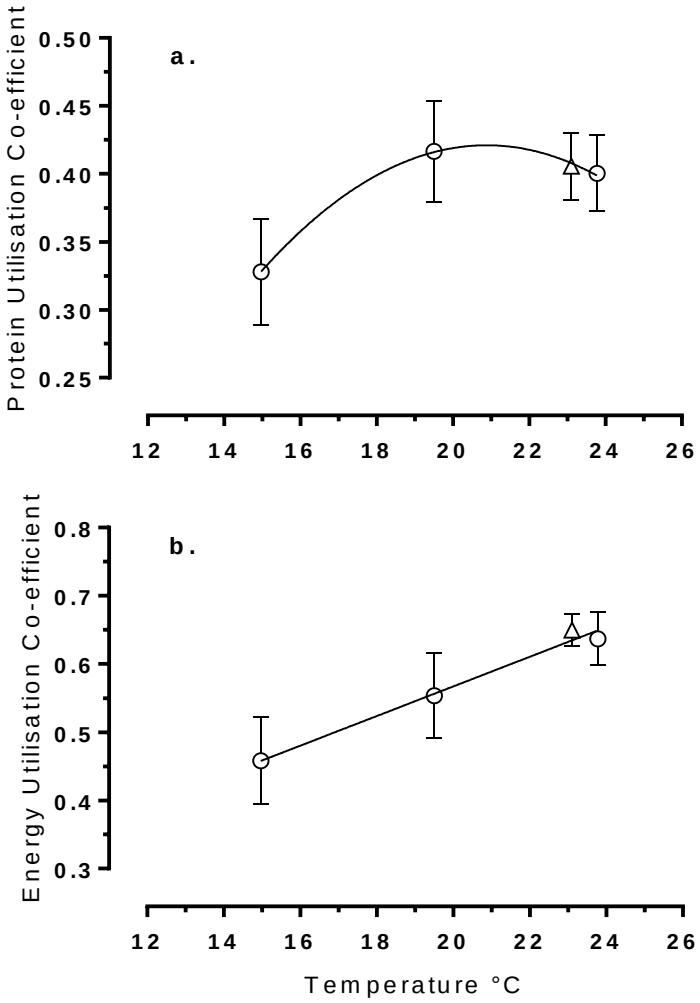


Figure 3.2.2.1.5. Temperature effect on utilisation coefficient values from this study (open circles) from Experiment 1 (15 °C and 24 °C) and Experiment 2 (20 °C; 100% DO treatment) for (a) protein and (b) energy (± SE; *n* = 2). Coefficient values for YTK at 23.1 °C (triangle) derived from Booth et al. (2010). Regression analyses modelled over entire data set (Experiment 1, Experiment 2 this study; Booth et al. (2010) where (a) digestible protein utilisation coefficient = -0.7396 + 0.1112T - 0.00266T^2 (R^2 = 0.998) and; (b) digestible energy utilisation coefficient = 0.1325T + 0.0217 (R^2 = 0.983).

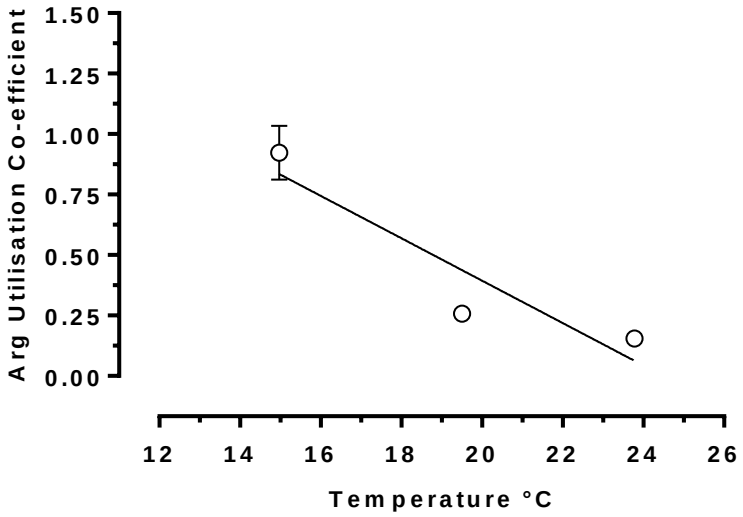


Figure 3.2.2.1.6. Effect of temperature utilisation coefficient values for arginine (± SE; *n* = 2). Data at 15 °C and 24 °C from Experiment 1 (Temperature). Data at 20°C from Experiment 2 (100% DO Saturation Treatment). Regression equation; y = -0.0839T + 2.039 (R^2 = 0.817).

3.2.2.2. Manuscript - Refinement of temperature-dependant growth and bioenergetic model for Yellowtail Kingfish Seriola lalandi.

Igor Pirozzi[a,b] and Mark Booth[a]

[a] New South Wales Department of Primary Industries, Locked Bag 1, Nelson Bay, NSW 2315
[b] College of Science and Engineering and Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville 4801, QLD, Australia.

This manuscript may be referenced as: Pirozzi, I. and Booth, M. (2019). 3.2.2.2. Manuscript - Refinement of temperature-dependant growth and bioenergetic model for Yellowtail Kingfish *Seriola lalandi*. In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.305-329.

Abstract

The aim of this study was to validate an updated version of a bioenergetic model for Yellowtail Kingfish (*Seriola* lalandi; YTK). Bioenergetic models are useful for predicting growth and predicting feed rate at different water temperatures, thereby giving producers a tool by which to benchmark production performance or plan feeding. The existing bioenergetic model for YTK has been updated with new coefficients that account for the effect of water temperature on growth and protein and energy utilisation (Manuscript 3.2.2.1). The model was validated by establishing a series of experimental treatments in which YTK were fed an optimal ration predicted by the model (hereafter prescribed ratio) and compared to the performance of YTK fed to apparent satiation. The validation was done at two temperatures (16 °C and 23 °C) using two formulated feeds; one having 25 g digestible protein (DP) MJ digestible energy^{-1} (DE) and the other 30 g DP MJ DE^{-1}. These diet specifications were selected based on the nutrient requirements of YTK available for the trial, which had a predicted geometric mean body weight (GMBW) of approximately 550 g and 700 g, respectively, when grown at 16 °C and 23 °C for about 7 weeks. Extreme summer ambient air temperatures caused significant temperature fluctuations in the cold water recirculating system (RAS) in the first stages of the trial; however, growth and feed conversion efficiencies of the prescribed ration group were similar to the satiated group, indicating that in cold water, the model is reliable in predicting growth and feed requirement. At 23 °C the model was accurate in predicting growth over the first three weeks of the trial; however, growth rates were overestimated by the end of the trial. It is likely that there was an underlying issue associated with the extreme ambient weather conditions and the handling of fish from this RAS during weight assessments. High mortalities were observed in the warm water RAS towards the later stages of the trial. The growth model was also validated against empirically derived data collected from two long-term pond trials at PSFI (Manuscript 3.2.5.1). In this case there was a strong correlation between the predicted vs the measured response values. Based on growth and feed intake data, the updated bioenergetic model for YTK developed during this project can predict growth and feed demand of YTK within a prescribed range of water temperatures with reasonable accuracy and represent an improvement on previous bioenergetic models for this species.

Introduction

Nutritional models provide a platform to predict nutrient requirements, diet specifications and feed regimes for growing animals in both terrestrial (e.g. van Milgen et al., 2007) and aquaculture settings (e.g. Lupatsch et al., 2001). They also represent a useful management tool by which to assess performance with regard to growth and nutrient conversion efficiencies. Most models are based on empirically derived data sets, therefore, their usefulness and predictive accuracy is often limited to the conditions and environment from which they were collated. For example, there are many models that

have been used to accurately describe the growth of an animal, such as multiphasic or polynomial growth equations (Karkach, 2006). The models often describe growth trajectory with reasonable accuracy until modeling of the data beyond the range of the empirical data set leads to spurious results. This is why there is always a need to further refine and develop models through the collation of response variables under different environmental conditions, ontogeny and nutritional planes.

Booth et al. (2010) developed a bioenergetic model for YTK which estimated the digestible protein (DP) and digestible energy (DE) requirements of YTK growing to 2 kg. That study used a factorial approach, establishing the utilisation efficiencies and maintenance requirements for DP and DE, an assessment of the protein and energy whole body composition as a function of fish size and established the growth potential of YTK under a given set of culture conditions. The utilisation coefficients and growth model both have a significant influence on the accuracy of growth and feed demand predictions in bioenergetic models (Pirozzi et al., 2010). In other words, these models are very sensitive to variations in parameter values. Therefore, it is important to establish these parameters based on robust data and to ensure that the application of the model/s is performed within the limitations of the data sets that they are built from, such as temperature range. One of the limitations of the Booth et al. (2010) study was that it was conducted within a 5 °C temperature range (20-25 °C) and utilisation efficiencies were established across a narrow range of 23.1 ± 1.4 °C. The impact of temperature on model parameters including DP and DE utilisation and maintenance requirements were specifically explored in Manuscript 3.2.2.1.

This Manscript presents the results of an experiment done to refine and validate newer iterations of the existing bioenergetic model for YTK by comparing the growth and feed intake of fish fed to a prescribed ration as dictated by the bioenergetic model to the growth and feed intake of YTK fed to apparent satiation. The trial was undertaken at two temperatures; 16 °C and 23 °C. This Manuscript also integrates the information from Manuscript 3.2.2.1.with that of Booth et al. (2010) to refine the bioenergetic model for YTK with the goal of improving its versatility and predictive accuracy when applied to different a wider range of thermal regimes; regimes that might typically be experienced in YTK farming conditions. Key to this development is the incorporation of a temperature function into the utilisation, maintenance and growth models. Estimations for optimal DP and DE for growing YTK at different temperatures will be derived using the factorial modeling approach. Suggestions for appropriate dietary specifications and feeding regimes for YTK based on the updated models will also be presented in this Manuscript.

Materials and Methods

This study was performed under the NSW DPI Fisheries Animal Care and Ethics (ACEC) Research Authority known as 'Aquaculture Nutrition ACEC 93/5–Port Stephens'. Care, husbandry and termination of fish were carried out according to methods outlined in 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2015).

Factorial bioenergetic modelling - DP and DE requirements

The factorial approach assumes that the requirements for DP and DE can be partitioned into production and maintenance costs. Total nutrient requirement can be described as:

$$a \times BW(kg)^b + c \times Growth \qquad \text{Eq. 1}$$

where a = maintenance requirement; b = weight exponent; c = utilisation coefficient

Maintenance requirements and utilisation coefficients for DP and DE were investigated in Manuscript 3.2.2.1. Weight exponent values for protein (0.7) and energy (0.8) are constants describing the relative metabolic body weight. Parameter values are presented in Table 3.2.2.2.1 and described as a function of temperature.

Growth model

A data set was collated from growth records of YTK used in various tank and pond based feed trials at NSW DPI, Port Stephens Fisheries Institute (PSFI) during the project and also from selected farm data.

Growth data was selected to represent the potential for maximal growth of YTK across a range of temperatures where fish were fed to satiation with nutritionally adequate diets. The growth model incorporates a temperature function in the form of:

$$Weight\ Gain\ (g\ fish^{-1}\ day^{-1} = (a + b \times T + c \times T^2 + d \times T^3) \times BW^e \qquad \text{Eq. 2}$$

where T = temperature, BW = body weight and a and e are constants and b, c and d are coefficients. Model values are presented in Table 3.2.2.2.1 and also Manuscript 3.2.5.1.

The growth model was validated against two longer term field trials with YTK at PSFI (Manuscript 3.2.5.1). Refer to Manuscript 3.2.5.1 for a detailed description of methodology and experiment design related to the field trials. Actual average weight data was compared against the predicted weight data from Eq. 2. Comparisons of predicted vs actual weights were assessed using non-linear regression analyses in Graphpad (Pond Trial 1; Manuscript 3.2.5.1).

Laboratory based validation trial at 16°C and 23°C

A 7 week feeding experiment was done to test and refine the biogenetic model using groups of YTK from a single size class (450 g) reared at 16 °C or 23 °C. Ideally, this trial would have been done in a farm situation; however, at the time that option was not available. Two diets were designed and formulated for the experiment. Both diets contained a similar suite of raw materials and both had the same digestible energy content (12.6 MJ DE kg^{-1} diet), however one diet had a lower digestible protein: digestible energy ratio (DP:DE) than the other (i.e. 25 DP:DE vs 30 DP:DE). The nutrient specifications of the diet were determined based on the requirement of YTK with a geometric mean body weight of 550 g and 700 g grown at 16 °C and 23 °C respectively as predicted by the temperature-dependent growth model. One group of YTK in each temperature regime was fed a ration calculated by the updated bioenergetic model (i.e. a prescribed or optimal ration), while the other group was allowed to consume feed to apparent satiety. Fish on the prescribed ration had their feed intake governed by the digestible energy content of the diets. The experimental design was implemented to determine if the model correctly estimated growth of YTK at two divergent water temperatures based on the nutrient and energy density of the allocated diet. The resultant data was collected to highlight the negative or positive bias within the updated bioenergetic model and which of the model coefficients was responsible for the bias, should any bias occur. This will allow further refinement of the model parameters.

Formulation of experimental feeds - validation trial

To ensure the results had relevance to the Australian YTK industry the two experimental diets were made using intact protein and energy sources (i.e. raw materials being used by the collaborating feed companies). Both diets were based on experimental diets which had previously prepared by Ridley for project trials conducted on larger YTK by SARDI (see Table 3.2.2.2.2 i.e. Mash 1 and Mash 2). The original diet formulations, excluding added fish oil, were batched at Ridley's Narangba plant (QLD, Australia) and shipped to PSFI. Each batch was then combined with other raw materials and fish oil according to formulation constraints to make two diets that differed in DP content but that had a similar amount of DE content (12.6 MJ digestible energy kg^{-1} diet; Table 3.2.2.2.3). The low protein diet had 319 g DP kg^{-1} diet whereas the higher protein diet had 377 g DP kg^{-1} diet. The formula of each experimental diet is presented in Table 3.2.2.2.4.

The DP:DE ratio of the lower protein diet corresponded to the predicted DP:DE ratio required for YTK growing between 450 g to about 1 kg; i.e. the expected change in body weight during this experiment. The diet having the higher DP:DE ratio was included in the experiment to determine if YTK will increase or decrease voluntary consumption based on the amount of available protein, rather than the amount of DE in the diet *per se*. The individual amino acid content of the high DP:DE diet was approximately 18-20% higher, respectively than the low DP:DE diet. The methionine and taurine content of diets was adjusted to reflect the results of project research conducted in Manuscript 3.1.5.3. The nutrient and energy composition of diets is presented in Table 3.2.2.2.5.

Diet manufacture - validation trial

All diets were made at PSFI using laboratory scale equipment. Prior to pellet making all raw materials were finely ground in a high speed hammer mill (Raymond Laboratory Mill, Transfield Technologies, Rydalmere, NSW, Australia; 1.6mm screen). Each mash along with added raw materials was autoclaved for 2 min at 121 °C prior to mixing and pellet making. This was done to promote gelatinisation of starch compounds. The raw materials and supplements were then dry mixed in a Hobart mixer (Hobart Mixer; Troy Pty Ltd, Ohio, USA) before the addition of oil and fresh water to form a moist mash. The mash was then screw pressed into 6 mm pellets (Dolly, La Monferrina, Castell'Alfero, Italy). The moist pellets were then dried at approximately 60 °C to a moisture content of < 10%.

Fish handling and treatment - validation trial

The feed trial was carried in a nutrition laboratory at PSFI using two RAS fitted with 1000 L rectangular polyethylene tanks (IBC's). Both RAS's incorporated 12 tanks. Each RAS was fitted with a similar range of equipment including dedicated bio-filters, particle filtration, foam fractionation and reverse cycle refrigeration units to control water temperature. Effluent water from either RAS was continuously removed and replaced with clean, filtered and disinfected saltwater taken from the Tilligerry Estuary adjacent to PSFI.

The water quality of each RAS was monitored daily with an electronic water quality meter (Horiba). Average water quality (mean ± SD) throughout the experiment was: temperature (22.7 ± 0.5 °C and 16.8 ± 1.4 °C), salinity (32.5 ± 4.9‰), oxygen saturation (99.4 ± 6.2%), pH (8.1 ± 0.2), TAN (0.5 ± 0.2 mg L^{-1}). Note extreme summer ambient conditions influenced water temperature on some days.

Replicate 1000L tanks were each stocked with 10 × 451.0 ± 5.8 g YTK (mean ± SD; average of n = 24 tanks) and there were no significant differences among the average weight of YTK allocated to different treatments at the inception of the trial. Similarly, there were no differences in the starting condition factor of YTK assigned to different treatments (mean ± SD condition factor K = 1.457 ± 0.029; n = 24 tanks). Fish were stocked into systems on the 18th Dec 2017. All fish were progeny of wild-caught YTK broodstock held at PSFI. Prior to stocking, juvenile YTK were fed two to three times daily with commercial aquafeeds and held at water temperatures between 18-22 °C. Representative samples of whole fish were taken at stocking for biochemical analysis.

Tanks of YTK were hand fed once a day (10:00 h) on a prescribed ration calculated by the bioenergetic model or allowed to feed to apparent satiation. The amount of feed given to YTK which had been allocated to the prescribed ration treatments was adjusted on a daily basis according to the models predicted daily increase in body weight of fish reared at 16 ° or 23 °C, respectively. Fish allocated to prescribed rations were offered feed in much the same way as fish fed to apparent satiation. Normally all feed from prescribed rations was consumed, however the entire daily ration was not fed if the fish appeared satiated before the allocated ration was fed in its entirety. In all cases, any uneaten pellets were collected, stored frozen and dried to accurately calculate dry-basis feed intake.

The weight of fish was checked every three weeks to monitor progress against the model predictions. Faecal samples were collected from fish at the end of the experiment to allow the apparent digestibility of test diets to be confirmed.

Major response variables - validation trial

The following performance variables were used to determine the response of YTK to different feeding regimes and different diet specifications;

- Initial weight of fish (g) = individual weight of fish at stocking
- Final weight of fish (g) = individual weight of fish at harvest
- Specific growth rate (% d^{-1}) = [Ln(final weight) – Ln(initial weight)] / days × 100
- Condition factor K = [individual weight of fish (g) / fork length of fish $(mm)^3$] × 10^5
- Relative increase in *K factor* (%) = (Final *K factor* – Initial *K factor*) / Initial *K factor* × 100
- Food conversion ratio (FCR) = feed intake per tank (g) / wet weight gain per tank (g)

Digestibility of test diets - validation trial

A digestibility assay was carried out at the end of the trial using fish remaining from the L5 feed treatment. This part of the trial continued for a further two weeks. Yttrium oxide (Yttrium III oxide, Sigma-Aldrich) was used as an inert marker in the diets. The marker was added at 1 g kg^{-1} of diet and thoroughly mixed. The mixture was then re-pelleted and dried at 40 °C for 8 - 9 h. Prior to stripping, fish were anaesthetised using 5-25 mg L^{-1} Aqui-S ™. Faeces was collected from the posterior intestine of YTK by applying gentle abdominal pressure. Contamination with urine or mucous was minimized and samples were immediately stored at -20° C. This procedure was repeated twice a week until approximately 3 g dry faecal matter was obtained from each tank of fish. Diet and faecal samples were analysed for dry matter, nitrogen, gross energy, fat, ash and amino acids.

Apparent digestibility of the diet was calculated using the formula:

$$ADC(\%) = 100 \times [1 - (\frac{F}{D} \times \frac{D_{marker}}{F_{marker}})]$$

where F = % nutrient in faeces; D = % nutrient in diet; D_{marker} = % marker in diet; F_{marker} = % marker in faeces.

Statistical analyses – validation trial

The interaction between feeding regime and diet specification on performance of YTK was examined using simple and multi-factor ANOVA. Both single and multi-factor ANOVA was done in NCSS 11 Version 11.0.13 (NCSS 11 Statistical Software (2016). NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss). ANOVA was considered significant when $P < 0.05$. When ANOVA was significant the Student Newman-Keuls multiple comparison test was used to compare treatment means at the 95% confidence interval. Regression analysis was done using GraphPad Prism Version 4.01 for Windows (www.graphpad.com).

Results

Factorial bioenergetic modelling - DP and DE requirements

Iterative modelling of DP and DE requirements using the updated parameters presented in Table 3.2.2.2.1 demonstrated a clear temperature dependent effect on requirement, with the ratio of DP to DE increasing with increasing temperature while the DP:DE ratio decreased with increasing body size (Table 3.2.2.2.6; Figure 3.2.2.2.1). This effect was amplified at higher relative water temperatures, above what might be considered thermally optimal for YTK at ~23 °C. The DP:DE requirements in cooler water were not as dissimilar.

Predicted FCR, temperature (12-30°C) and body weight (10 g to 2.0 kg) relationships are presented in Figure 3.2.2.2.2. This relationship is non-linear with FCR tending to increase with decreasing temperature and increasing body weight. There is an optimal thermal zone of ~20-21 °C where FCR is lowest before climbing with increasing temperature. This relationship can be expressed as:

$$FCR = (5.99997 - 0.76316 \times T + 0.03287 \times T^2 + 0.00046 \times T^3) \times BW^{0.25975} \quad \text{Eq.3}$$

Growth model verification

No significant difference ($P > 0.05$) was found between predicted vs actual weight of YTK reared in the field trial at PSFI (i.e. Pond Trial 1; Manuscript 3.2.5.1 (Figure 3.2.5.1.3) when grown from ~80 g to ~750 g over 4 months (refer to Manuscript 3.2.5.1 for ambient temperature profile).

Laboratory based validation trial

This experiment was conducted during summer over 9 weeks at PSFI. Extreme fluctuations in ambient air temperature between December 2017 and January 2018 in NSW made it difficult to precisely control water temperature in this experiment, particularly the 16 °C regime. On several days the air temperature approached or exceeded 40°C (Figure 3.2.2.2.4). The average water temperature recorded in the cool water and warm water RAS over the experiment period was 16.3 °C and 23.2 °C, respectively. The instability in the water temperature of the cool water RAS meant that YTK allocated to the 'prescribed rations' treatment in this RAS had been underfed with respect to model predictions; i.e. the bioenergetic model predicts YTK reared at 17.5 °C would require more feed than required at a nominal temperature of 16 °C. Additional refrigeration units were deployed on the cool water RAS to mitigate for fluctuating summer air temperatures and the incoming temperature of estuarine source water.

Compounding these issues, high numbers of mortalities were observed in the warm water RAS (Table 3.2.2.2.7) following a three week bulk-weight check. This occurrence was likely the result of the extreme ambient weather conditions occurring at the time and potentially exacerbated by the use of anaesthetic. Nonetheless, this data is presented and due consideration of these events is required when interpreting the results of this feeding experiment.

At the conclusion of the trial there were no significant effects of diet type ($P > 0.05$) on growth or feed intake variables. Data was subsequently pooled across diet type and analysed as a two factor ANOVA ($n = 6$) (Figures 3.2.2.2.5, 3.2.2.2.6, 3.2.2.2.8 and 3.2.2.2.11).

The SGR of YTK was significantly affected by water temperature and feeding regime; however there was no effect of diet specification and there were no interactions among the main effects (all $P > 0.05$). Overall the SGR of YTK was significantly higher at 23 °C than 16 °C and higher in fish fed to appetite than fish fed the optimised ration (Figure 3.2.2.2.5).

The intake of YTK varied significantly among temperature and ration treatments. Differences between the prescribed feed intake of YTK and those fed to appetite were relatively greater under the 16 °C regime than for fish reared under the 23 °C regime (Figure 3.2.2.2.6). Intake of YTK was significantly affected by ration type in the cold water treatment but not the warm water treatment (Figure 3.2.2.2.6). There were no interactions among the independent factors (Table 3.2.2.2.7). Water temperature explained the majority of the variance in feed intake. YTK assigned the prescribed ration consumed all feed consistently, particularly in the cold water group; however, towards the last few weeks of the trial YTK the warm water group fed more erratically (Figure 3.2.2.2.7). Feed intake of the prescribed ration group was mostly consistent in the cold water group (Figure 3.2.2.2.7).

FCR ranged from 1.41 to 2.37 depending on temperature and ration. There was a significant interaction ($P < 0.05$) among temperature and ration terms with the magnitude of the effect of ration type on FCR dependent on temperature. This can be seen in Figure 3.2.2.2.8 with FCR significantly higher in the warm water treatment for YTK on the prescribed ration. Apparent FCR was significantly higher under the 23 °C regime than the 16 °C regime (Figure 3.2.2.2.8). This can be partially explained due to the more erratic feeding behaviour of this group (Figure 3.2.2.2.7).

Final condition index (K) was marginally different ($P = 0.04$) between the 16 °C satiated group and the 23 °C prescribed ration group; however, in practical terms they were similar at 1.52 and 1.45 respectively. K tended to vary more in YTK in warm water compared to cold water (Figures 3.2.2.2.9 and 3.2.2.2.10). There were significant differences in the overall change in condition ranging from -1.1% to +4.4% (Figure 3.2.2.2.11).

Survival was affected by temperature but not ration; with the lowest survival occurring in the warm water RAS (Table 3.2.2.2.7). Survival ranged from 90% or greater in the cold water system to 47% for the satiated group reared in warm water. As indicated above, such low survival implies an underlying problem that was unlikely associated with the design of the experiment. Mortalities began occurring during the days immediately following a bulk weight check when ambient air temperatures were extremely high.

Carcass proximate compositions (wet weight basis) are presented in Table 3.2.2.2.8. There was a strong 2nd order interaction ($P < 0.001$) detected for ash composition; however, in absolute terms the ash composition of YTK varied by < 1% on average among all of the experiment treatments (Table 3.2.2.2.8). Fat composition ranged from 6.7-8.3% and ANOVA indicated that there was an interaction ($P < 0.05$) between temperature and ration which was due to the higher fat content response of the

satiated cool water group relative to the warm water group (Table 3.2.2.2.8); however, in absolute terms fat content varied only by 1.6% among all of the experiment treatments. There were no other significant interactions detected when considering other proximate values (Table 3.2.2.2.8).

Figure 3.2.2.2.12 depicts the observed growth of YTK versus the growth of YTK predicted by the bioenergetic growth model. The first three weeks predicted growth in the cold water system was modelled at a temperature of 18 °C; being the average system temperature over that period. This was necessary as high ambient air temperature fluctuations caused temperature instabilities within the RAS. Predicted and actual growth is very similar. As the prescribed ration group were fed to a regime specified for 16 °C their growth over the first three weeks was below that of the satiated group. However, from week three to the final weight measurement at week nine, the growth trajectory closely matches that predicted by the model for growth in the 16 °C system. Conversely, the growth trajectory of YTK held in the warm water RAS closely followed that predicted by the model for the first three weeks, but not from week three to week nine. This is likely influenced by their erratic feeding over this time and symptomatic of the issues described above (Figure 3.2.2.2.7).

Discussion

The objective of this study was to refine an existing bioenergetic model for YTK (Booth et al., 2010) and validate the updated model against actual growth and feed data from experiments and field trials. Ideally the validation would be done at the larger scale, such as on a commercial YTK farm, however this option was not available during the project. The incorporation of temperature functions into the growth, maintenance and nutrient utilisation efficiencies of the model allows for greater flexibility and accuracy in predicting growth and requirements for DP and DE. We found the refined temperature-dependent growth model was generally accurate under fluctuating field conditions such as that experienced in ponds at PSFI, where seasonal water temperature fluctuated between about 12 °C to 26 °C). In this case the actual growth of YTK closely matched that predicted by the updated model (Figure 3.2.2.2.3). While the refined models presented in this study represent a significant improvement for predicting requirement and growth of YTK, it is nonetheless important to understand the context and limitations of the data set that the models are based on.

The ratio of DP:DE increases with increasing temperature however there is a significant amplification of this response at water temperature > 25 °C (Figure 3.2.2.2.1). This implies that temperatures above 25 °C may be considered supra-optimal for YTK. Similar responses to increasing DP:DE demand in supra-optimal temperatures have been demonstrated in Barramundi (Glencross and Bermudes, 2012). Data collated from this current study and that of Manuscript 3.2.2.1 indicate that growth is maximised at 23 °C (Table 3.2.2.2.1 and also see Figure 3.2.5.1.4 Manuscript 3.2.5.1) and FCR's are most efficient at 20 °C (Figure 3.2.2.2.2). Further, Pirozzi and Booth (2009) determined that the routine metabolic rate of YTK is least thermally sensitive at 22.8 °C. Therefore, to optimise feeding efficiencies, growth and performance, farmed YTK should be cultured where possible between 20-23 °C. Such as narrow range of temperature implies YTK production may be based on land-based RAS technology in future; or at least some part of it.

FCR data modelled in Figure 3.2.2.2.2 indicates a deterioration of conversion efficiency at lower temperature relative to YTK held at higher temperatures. While this is generally accepted, the predicted FRCs of approximately 6:1 for 2.5 kg fish held at 12 °C may be overly high. However, Stone et al. (2016) reported FCRs of 4.4:1 for YTK weighing about 1.5 kg held at an average water temperature of 12.8 °C. The data represented in Figure 3.2.2.2.2 also considers a fixed temperature.

The growth model provides a very useful management tool to ascertain if general husbandry and feeding practices are of an adequate standard by comparing actual vs predicted growth rates. Growth rates found to be well below those predicted could indicate problems associated with feed intake such as the quality and/or quantity of the feed offered, underlying health issues or poor water quality.

Incorporation of a temperature function in the model can help farm mangers make decisions about stocking time and time to harvest in sea-cage operations where stock are subjected to year round seasonal fluctuations in water temperature. For example, adapting Eq. 2 to a sea temperature regime off Port Stephens (NSW) can see a difference of in excess of one month for YTK to grow to 2.5 kg if stocked in either summer or winter (Figure 3.2.2.2.13). If animals are grown out for longer than 12 months i.e.

experiencing more than one full seasonal cycle, then days to harvest will realign at some point assuming similar temperature regimes from one year to the next.

The second component of this study was test the bioenergetic growth and feed demand model for YTK by comparing the growth and feed intake of YTK fed to the model specification to that of YTK fed to apparent satiation in a laboratory based feed trial. The trial was conducted at two temperatures; 16 °C and 23 °C. Irrespective of the temperature fluctuations that occurred in the cold water RAS in the first few weeks of the trial, the model adequately described growth and feed demand for YTK held at 16 °C. However, within the context of the current study, growth and feed demand were overestimated for YTK by the conclusion of the trial.

The growth rate and feed demand of YTK held at 23 °C over the first three weeks of the study closely matched that predicted by the model. However by the conclusion of the trial the growth trajectory had decreased significantly. As indicated above, growth models can be useful management tools indicating underlying problems. High mortalities post three week handling combined with highly variable body condition index (Figures 3.2.2.2.9 and 3.2.2.2.10) and extreme ambient conditions may indicate that this was the case.

Two diets were formulated for the validation experiment based on the predicted requirement of YTK growing from 450 g to approximately 700 g in the 16 °C RAS and from 450 g to about a 1 kg in the 23 °C RAS. The DP:DE ratio of YTK declines predictably as the fish grows (Booth et al., 2010), therefore, in theory, many diets could be formulated to match the changing requirement of the growing animal. In practical terms, certainly within the commercial industry, this is not economically viable. As a compromise, the diets in this study were formulated based on the predicted GMBW of the temperature relative size range. The rationale being, that with the option of one diet over the growing period of the study, this would best meet the requirement of YTK within each temperature treatment. However, there were no significant differences found between the diets when considering growth and feed intake variables at the end of the trial indicating that either diet was adequate in terms of supporting the growth of YTK over the culture period. However until further work is done this result should be viewed cautiously. Inherent “noise” in systems, i.e. abiotic or biotic factors, can swamp subtle diet effects (Houlihan et al., 2001).

Feed intake of the prescribed ration was, overall, less than that of the satiated group, irrespective of water temperature. However, the entire prescribed ration oofered to YTK was not always consumed on each day. For example, the intake of the warm water group tended to fluctuate widely after the second weight check and this likely resulted in significantly higher FCRs for this group. On occasion all feeding responses synchronized by dropping across the different treatment groups (Figure 3.2.2.2.7) and this coincided with external influences such as storm events. Further, as growth trajectories for the warm water RAS declined significantly relative to the predicted growth, feed input was, therefore, above the predicted body weight requirement and, hence at this stage, the prescribed ration group in warm water were effectively being fed to “satiation”. In comparison, the cold water group fed very closely to the model throughout the experiment period, which is also reflected in the lowest FCR’s.

The loss of condition (as evidenced by a decrease in *K* factor) in YTK fed the prescribed ration and held in the cold water RAS (Figure 3.2.2.2.11) can be explained by the reduced feed input, relative to temperature, administered during the first three weeks of the trial when extreme ambient weather caused temperature increases within the cold water RAS. Irrespective of statistical significance, overall differences in average *K* values were numerically similar across the different groups. However, individual *K* values varied widely in YTK held in warm water compared to the cold water system, increasing significantly with fork length for the warm water group but independent of fork length for the cold water group. This relationship may be an artefact of an underlying health issue for some individual YTK in the warm water system, which manifested as increased mortalities.

Conclusions and Recommendations

Based on the growth and feed intake data presented in this study, growth and feed demand of YTK can be modelled with some confidence. However it is important to maintain a conservative approach to predicting growth estimations. Bioenergetic models represent useful management tools to benchmark performance of stock and to iteratively design diets based on the DP and DE requirements of rapidly

growing YTK in different thermal regimes. While the models presented in this manuscript represent a significant advancement for our understanding of requirement and growth responses for YTK, there is still some considerable room for improvement. YTK are routinely harvested when they reach a body weight of ~4-6 kg. This size class is underrepresented in our updated model as well as in many similar studies on other species. This limitation with respect to the models presented in this manuscript may be overcome by the provision of reliable farm data. Future studies should consider larger fish sizes and the influence of body size on key model parameters including utilisation efficiencies, growth rates and body composition.

It is likely that the growth and feed intake responses of YTK held in the warm water RAS in this study were influenced by other factors that negatively affected their performance. Nonetheless the cool water group performed in a predictable way. This emphasises the practical application of the models in identifying underperforming fish.

Findings

- Growth and feed demand in YTK can be modelled with reasonable confidence over a range of water temperatures based on knowledge of their digestible protein (DP) and (DE) energy requirements.
- The optimal temperature for YTK is 20-23 °C based on growth and FCR responses
- Supra-optimal water temperatures affect the ratio of DP:DE requirements more so than sub-optimal temperatures.
- Use of models outside the range of data used to populate them can result in incorrect predictions.
- Further model development utilising large YTK >3 kg would greatly improve the application and reliability of the updated bioenergetic model for YTK.
- Growth models present a valuable management tool to benchmark performance of stock.
- Diet specifications can be tailored to match nutrient requirement for growing YTK at different temperatures.
- Stocking time can significantly affect time to harvest particularly if grow-out is < 1 year

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The authors thank Dr Basseer Codabaccus, Ian Russell and Brendan Findlay (NSW DPI) for technical assistance.

References

ACEC, 2015. A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research, 4th Edition, NSW DPI Animal Care and Ethics Committee, pp. 56.

Booth, M.A., Allan, G.L., Pirozzi, I., 2010. Estimation of digestible protein and energy requirements of yellowtail kingfish *Seriola lalandi* using a factorial approach. Aquaculture 307, 247-259.

Glencross, B.D., Bermudes, M., 2012. Adapting bioenergetic factorial modelling to understand the implications of heat stress on barramundi (*Lates calcarifer*) growth, feed utilisation and optimal protein and energy requirements - potential strategies for dealing with climate change? Aquac. Nutr. 18, 411-422.

Houlihan, D., Boujard, T., Jobling, M., 2001. Food intake in fish. Blackwell Science Pty Ltd, Victoria, Australia.

Karkach, A.S., 2006. Trajectories and models of individual growth. Demographic Research 15, 347-400.

Lupatsch, I., Kissil, G.W., Sklan, D., 2001. Optimization of feeding regimes for European sea bass *Dicentrarchus labrax*: a factorial approach. Aquaculture 202, 289-302.

Pirozzi, I., Booth, M.A., 2009. The routine metabolic rate of mulloway (*Argyrosomus japonicus*: Sciaenidae) and yellowtail kingfish (*Seriola lalandi*: Carangidae) acclimated to six different temperatures. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 152, 586-592.

Pirozzi, I., Booth, M.A., Allan, G.L., 2010. A factorial approach to deriving dietary specifications and daily feed intake for mulloway, *Argyrosomus japonicus*, based on the requirements for digestible protein and energy. Aquaculture 302, 235-242.

Stone, D. A., D'Antignana, T., Bansemer, M. S., 2016. *Refining Yellowtail Kingfish feeds and feed management*. Australian Seafood Cooperative Research Centre. 144 p.

van Milgen, J., Noblet, J., Etienne, M., Valancogne, A., Dubois, S., Dourmad, J.Y., 2007. InraPorc: A model and decision support tool for the nutrition of growing pigs and sows. J. Anim. Sci. 85, 440-440.

Table 3.2.2.2.1. Bioenergetic model coefficients for Yellowtail Kingfish; values derived from empirical experiments reported in Manuscript 3.2.2.1.

Term	***Coefficients***				
	a	**b**	**c**	**d**	**e**
Growth model[1]	0.7600	-0.1931	0.0149	-0.0003	0.4840
Energy composition (kJ g^{-1})[2]	2.79	0.16			
Protein composition (g kg^{-1})[3]	188.20				
Metabolic body weight exponent (energy)[4]		0.80			
Metabolic body weight exponent (protein)[5]		0.70			
Utilisation coefficient of protein[6]	-0.7394	0.111	-0.002652		
Utilisation coefficient of energy[7]	0.09437	0.02682	-0.0001683		
Maintenance protein[8]	0.04	-0.31			
Maintenance energy[9]	1.98	16.49			

1. growth = $(a + b\text{xtemp} + c\text{xtemp}^2 + d\text{xtemp}^3) \times \text{body weight}^e$
2. energy gain = $a \times \text{body weight}^b \times$ daily weight gain
3. protein gain = $a / 1000 \times$ daily weight gain
4. metabolic body energy = body weight^b
5. metabolic body weight protein = body weight^b
6. Utilisation coefficient of protein = $a + b\text{xtemp} + c\text{xtemp}^2$
7. Utilisation coefficient of energy = $a + b\text{xtemp} + c\text{xtemp}^2$
8. maintenance demand for protein = $(0.04391 \times \text{temp} - 0.314)$
9. maintenance demand for energy = $(1.977 \times \text{temp} + 16.49)$

Table 3.2.2.2.2. Formulation of SARDI / Ridley mash 1 and 2 as provided to NSW DPI; mash was devoid of fish oil and poultry oil (≈20% of dry matter).

Raw material (%)	**SARDI / Ridley Mash 1**	**SARDI / Ridley Mash 3**
Peruvian fish meal	30.00	30.00
Feather meal	2.86	5.44
Wheat gluten meal	4.92	9.37
Wheat (fine)	20.00	13.57
Fish oil (not included in mash)	4.95	4.90
Vitamin C (Stay C 35%)	0.03	0.03
Choline	0.06	0.06
Vitamin/mineral premix	0.60	0.60
Poultry BP meal	2.04	3.89
Blood meal	6.63	12.64
Lupins (de-hulled)	8.64	0.00
Astaxanthin	0.01	0.01
Poultry oil (not included in mash)	15.60	15.83
Monosodium phosphate	2.06	2.06
Taurine (80%)	1.00	1.00
DL-Met (80%)	0.60	0.60
SUM	**100.00**	**100.00**

Table 3.2.2.2.3. Design and gross nutrient and energy content of diets used in bio-energetic validation experiment.

***Diet specification*[1]**	***Category***	***Apparent digestibility coefficient of diet (ADC)***	***Crude nutrient or gross energy of diet (g kg^{-1} or MJ kg^{-1})***	***Digestible nutrient or energy of diet (g kg^{-1} or MJ kg^{-1})***
High DP:DE formula	Protein	0.65	580.0	377.0
	Energy	0.60	21.0	12.6
		DP:DE	27.6 g protein MJ GE^{-1}	29.9g DP MJ DE^{-1}
Low DP:DE formula	Protein	0.65	490.0	319.0
	Energy	0.60	21.0	12.6
		DP:DE	23.3 g protein MJ GE^{-1}	25.3 g DP MJ DE^{-1}

[1] DP = digestible protein; DE = digestible energy.

Table 3.2.2.2.4. Raw material composition of diets used in bio-energetic validation experiment.

Ingredients and mash	**Low DP:DE formula 25 DP:DE**	**High DP:DE formula 30 DP:DE**
Diatom. earth	3.25	3.36
Fish oil	10.11	8.33
Fishmeal prime	5.85	6.66
Maize starch	0.25	1.28
Methionine	0.44	0.27
Y_2O_3	0.10	0.10
Diet 1 mash (Ridley mash)	80.00	0
Diet 3 mash (Ridley mash)	0.00	80.00
Total (%)	**100**	**100**

Table 3.2.2.2.5. Estimated nutrient and energy composition of diets used in bio-energetic validation experiment.

Nutrients (%) or energy	**Low DP:DE formula 25 DP:DE**	**High DP:DE formula 30 DP:DE**
Ash	13.47	13.92
GE (MJ kg^{-1})	21.0	21.0
Protein	49.0	58.0
Fat	15.0	13.0
Alanine	2.41	3.04
Arginine	2.84	3.34
Aspartic acid	2.93	3.50
Cystine	0.54	0.74
Glutamic acid	6.97	8.24
Glycine	2.44	2.94
Histidine	1.40	1.86
Isoleucine	2.09	2.66
Leucine	2.72	2.93
Lysine	2.56	2.93
Methionine	2.00	2.00
Phenylalanine	1.78	2.10
Proline	2.56	3.40
Serine	2.09	2.60
Taurine	1.19	1.19
Threonine	1.75	2.12
Tyrosine	2.00	2.91
Valine	2.34	2.99

Table 3.2.2.2.6. Protein and energy requirements for Yellowtail Kingfish at different body size and temperature based on bioenergetic factorial modelling of the data presented in Table 3.2.2.2.1.

Temperature °C	**16**				**21**				**26**			
Live weight (g fish^{-1})	**100**	**1000**	**2000**	**3000**	**100**	**1000**	**2000**	**3000**	**100**	**1000**	**2000**	**3000**
Growth (g fish^{-1} day^{-1})	2.38	7.24	10.13	12.33	4.62	14.08	19.69	23.95	5.00	15.25	21.33	25.95
Energy Requirement												
MBW (kg$^{-0.8}$)	0.16	1.00	1.74	2.41	0.16	1.00	1.74	2.41	0.16	1.00	1.74	2.41
DE maintenance (kJ fish^{-1} day^{-1})	7.63	48.12	83.79	115.89	9.19	58.01	101.00	139.69	10.76	67.89	118.21	163.50
Energy gain (kJ fish^{-1} day^{-1})	13.67	59.78	93.21	120.87	26.56	116.17	181.15	234.90	28.77	125.86	196.26	254.50
DE growth (kJ fish^{-1} day^{-1})	28.45	124.43	194.03	251.61	45.53	199.14	310.52	402.66	42.44	185.66	289.50	375.41
DE total (kJ fish^{-1} day^{-1})	36.07	172.56	277.81	367.50	54.72	257.14	411.51	542.36	53.20	253.55	407.70	538.91
%DE for maintenance	21.14	27.89	30.16	31.53	16.80	22.56	24.54	25.76	20.22	26.78	28.99	30.34
Protein Requirement												
MBW (kg$^{-0.7}$)	0.20	1.00	1.62	2.16	0.20	1.00	1.62	2.16	0.20	1.00	1.62	2.16
DP Maintenance g/fish/day	0.08	0.39	0.63	0.84	0.12	0.61	0.99	1.31	0.17	0.83	1.34	1.79
Protein gain (g fish^{-1} day^{-1})	0.45	1.36	1.91	2.32	0.87	2.65	3.70	4.51	0.94	2.87	4.01	4.88
DP growth (g fish^{-1} day^{-1})	1.25	3.81	5.33	6.49	2.06	6.28	8.78	10.68	2.66	8.11	11.34	13.80
DP total (g fish^{-1} day^{-1})	1.33	4.20	5.96	7.32	2.18	6.88	9.77	11.99	2.83	8.94	12.69	15.59
%DP for maintenance	5.84	9.25	10.59	11.45	5.56	8.83	10.12	10.94	5.84	9.26	10.60	11.46
DP:DE	36.82	24.34	21.46	19.93	39.86	26.77	23.73	22.11	53.14	35.26	31.12	28.93

Table 3.2.2.2.7. Average (± SE, *n* = 3) performance data for Yellowtail Kingfish for each temperature (16 or 23 °C), Diet (25 or 30 g DP MJ DE^{-1}) and ration (Prescribed or Satiated). First and second order interaction terms; T = temperature (16 or 23 °C), R = ration (prescribed or satiated), D = diet (25 or 30 g DP MJ^{-1}).

	Diet								Pooled SEM				
Parameter	**16P25**	**16P30**	**16S25**	**16S30**	**23P25**	**23P30**	**23S25**	**23S30**		**TxRxD**	**TxR**	**TxD**	**RxD**
Initial Body Weight (g $fish^{-1}$)	453.6	449.8	456.5	448.2	452.1	449.2	454.2	445.8	2.9	-	-	-	-
Initial K	1.48	1.47	1.45	1.46	1.45	1.44	1.45	1.46	0.02	-	-	-	-
Final Body Weight (g $fish^{-1}$)	712.2	736.2	768.6	761.7	794.6	778.5	887.7	876.9	18.9	ns	ns	ns	ns
Final K	1.46	1.45	1.52	1.52	1.46	1.44	1.44	1.49	0.02	ns	ns	ns	ns
Growth (g $fish^{-1}$ day^{-1})	4.04	4.48	4.88	4.90	5.35	5.14	6.77	6.74	0.30	ns	ns	ns	ns
SGR	8.42	8.57	8.70	8.71	8.84	8.76	9.19	9.19	0.04	ns	ns	ns	ns
Feed Intake (g $fish^{-1}$ day^{-1})	6.40	6.30	7.96	7.60	12.36	11.85	12.95	12.65	0.26	ns	ns	ns	ns
FCR	1.59	1.41	1.64	1.55	2.32	2.37	1.94	1.88	0.09	ns	**	ns	ns
Survival (%)	96.7	100.0	100.0	90.0	73.3	66.7	63.3	46.7	0.0	ns	ns	ns	ns

ns = not significant $P > 005$.

** significant at $P < 0.01$.

Table 3.2.2.2.8. Wet weight carcass composition of Yellowtail Kingfish (mean; *n* = 3) and first and second order interaction terms; T = temperature (16 or 23 °C), R = ration (prescribed or satiated), D = diet (25 or 30 g DP MJ^{-1}).

Diet	Moisture (%)	Ash (%)	Lipid (%)	Protein (%)	Energy (MJ/Kg)
16P25	68.13	3.22	7.05	21.81	7.85
16P30	67.92	3.64	6.71	21.45	7.84
16S25	67.23	3.36	7.64	22.18	8.20
16S30	68.25	3.14	7.33	22.09	8.05
23P25	67.51	3.68	8.28	21.71	8.05
23P30	68.35	3.58	8.31	21.03	7.92
23S25	67.07	4.08	8.07	21.96	7.95
23S30	65.26	4.12	7.83	23.54	8.48
TxRxD	ns	***	ns	ns	ns
TxR	ns	ns	*	ns	ns
TxD	ns	ns	ns	ns	ns
RxD	ns	ns	ns	ns	ns
Pooled SEM	0.68	0.25	0.52	0.70	0.23

ns not significant $P > 0.05$.
* significant at $P < 0.05$.
** significant at $P < 0.01$.
*** significant at $P < 0.001$.

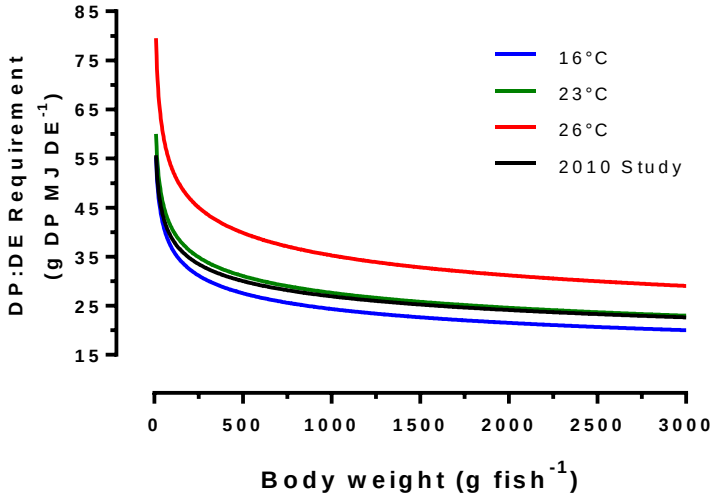


Figure 3.2.2.2.1. Temperature effect on the optimal ratio of digestible protein to digestible energy for growing Yellowtail Kingfish. Response derived from factorial modelling of data presented in Table 3.2.2.2.1. 2010 study refers to that of Booth et al. (2010) which was conducted over a temperature range of 20-25 °C.

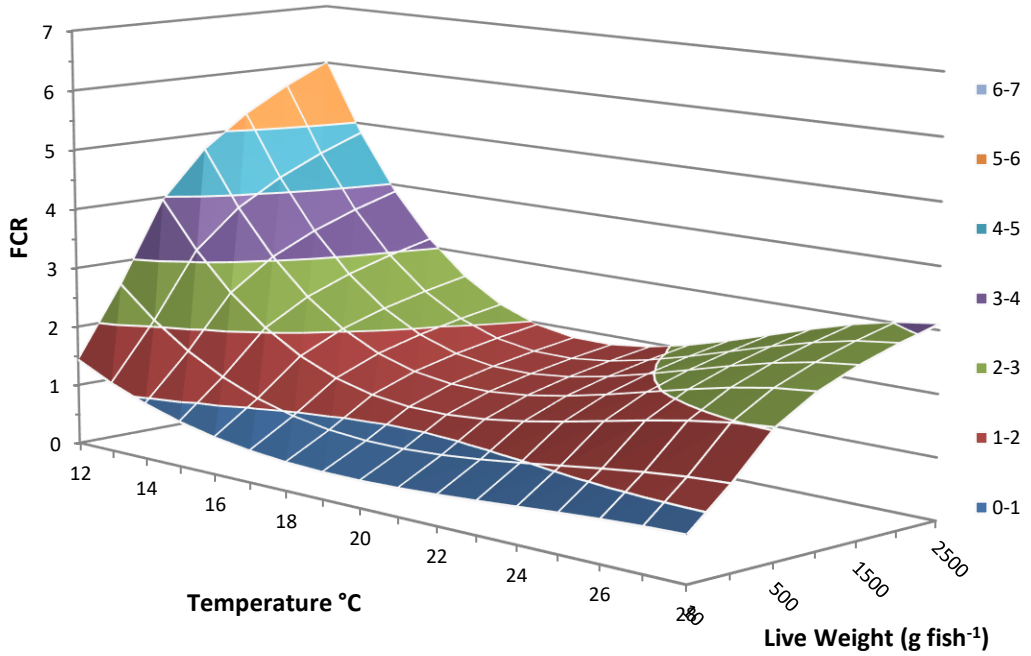


Figure 3.2.2.2.2. Predicted FCR at temperatures ranging from 12 °C – 28 °C for Yellowtail Kingfish growing from 10 g to 2.5 kg.

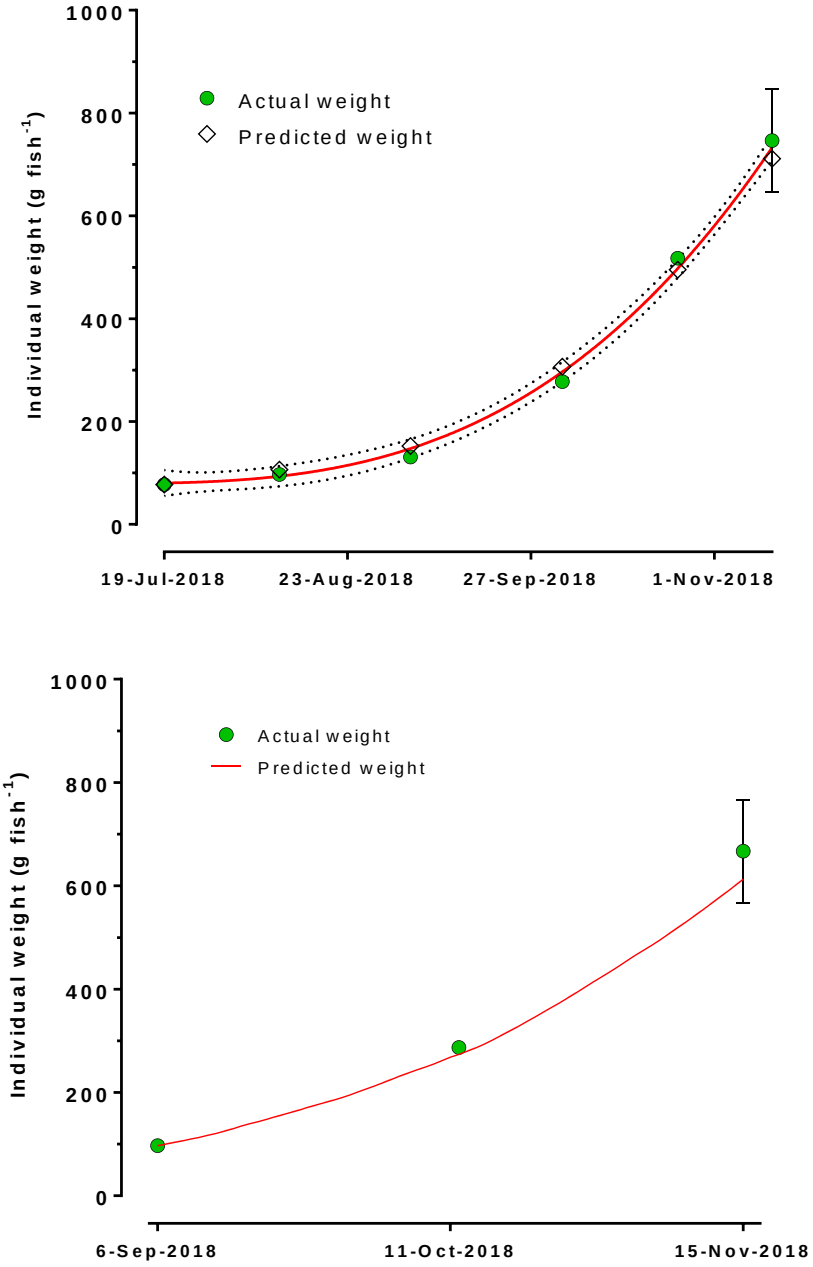


Figure 3.2.2.2.3. Comparative growth of predicted vs actual Yellowtail Kingfish grown in outdoor pond trial at PSFI (Manuscript 3.2.5.1). TOP: Average weight of Yellowtail Kingfish (n = 6 cages) in Pond Experiment 1 Manuscript 3.2.5.1 versus predicted weight. Red regression line represents global shared model ($P > 0.05$), dotted lines indicate 95% confidence intervals. BOTTOM: Pond Trial 2 Manuscript 3.2.5.1 graph presented for illustrative purposes as there were too few data points to meaningfully compare growth trajectory via regression analyses. Harvest data presented as mean ± SD.

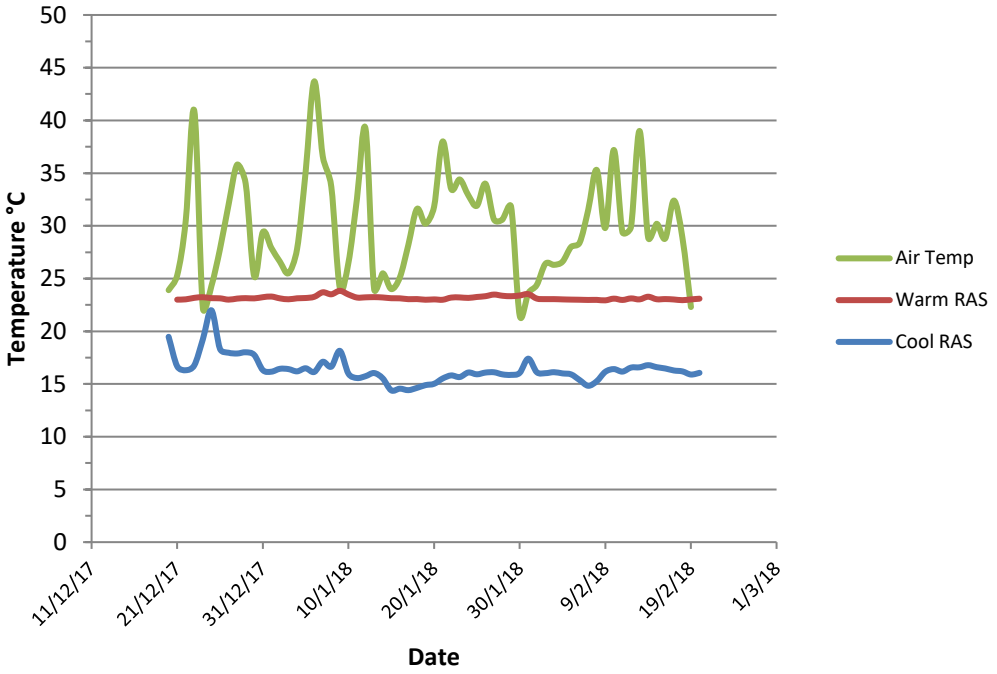


Figure 3.2.2.2.4. Variation in ambient air temperature and water temperature of recirculating aquaculture systems (RAS) used in Experiment 2. Validation trial; cool water RAS fluctuated widely due to extreme fluctuations in ambient air temperature.

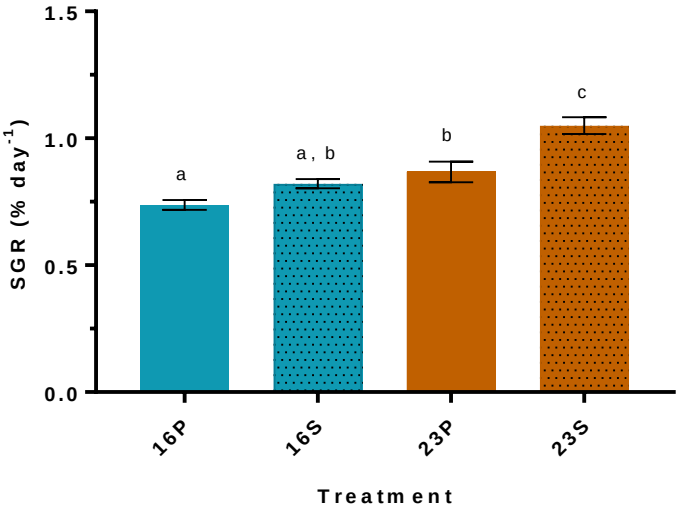


Figure 3.2.2.2.5. Average daily growth of Yellowtail Kingfish (± SE) among temperature (16 or 23 °C) and ration (Prescribed or Satiated) treatments. Data pooled within diet (n = 6). Different letters denote statistical significance ($P < 0.05$).

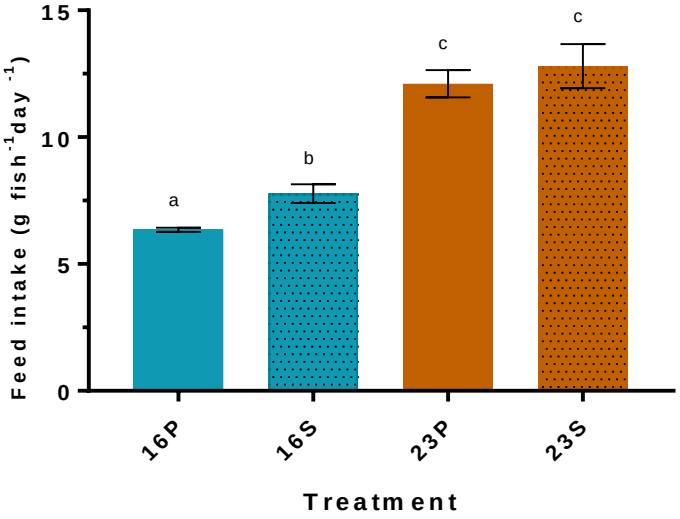


Figure 3.2.2.2.6. Average Yellowtail Kingfish feed intake (± SE) among temperature (16 or 23 °C) and ration (Prescribed or Satiated) treatments. Data pooled within diet (n = 6). Different letters denote statistical significance ($P < 0.05$).

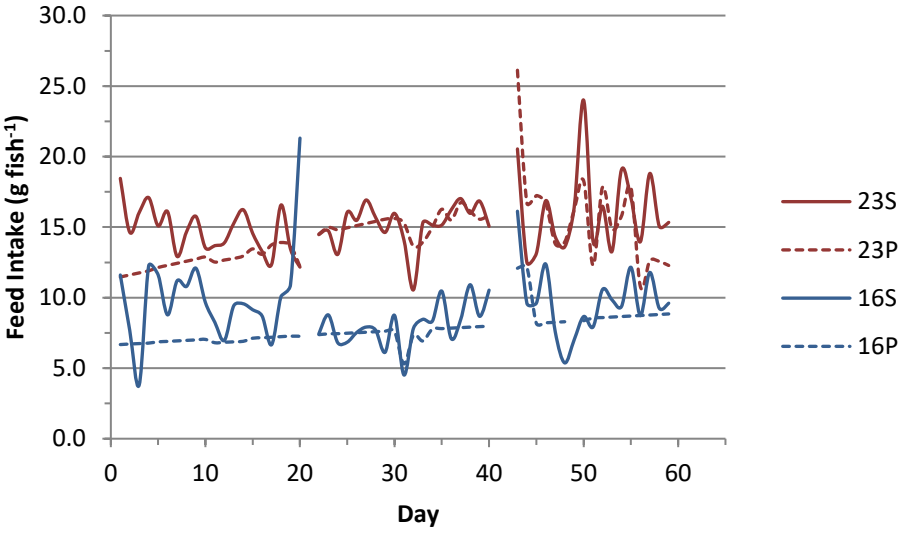


Figure 3.2.2.2.7. Average daily feed intake of Yellowtail Kingfish at different temperature (16 °C, blue line; or 23 °C, brown line) and ration (Prescribed, dashed line; or Satiated, solid line) treatments. Gaps in feed intake data indicate weight check days where no feed was administered. Prescribed ration group were fed a compensatory ration following feed deprivation. Data pooled within diet (n = 6).

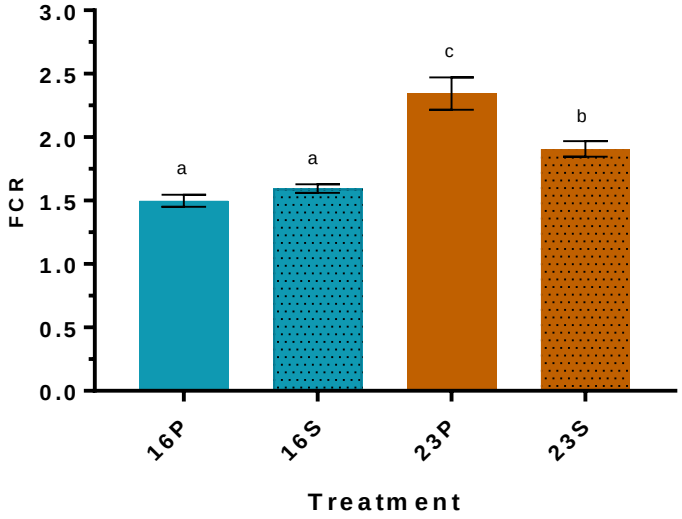


Figure 3.2.2.2.8. Average FCR of Yellowtail Kingfish (± SE) among temperature (16 or 23 °C) and ration (Prescribed or Satiated) treatments. Data pooled within diet (n = 6). Different letters denote statistical significance ($P < 0.05$).

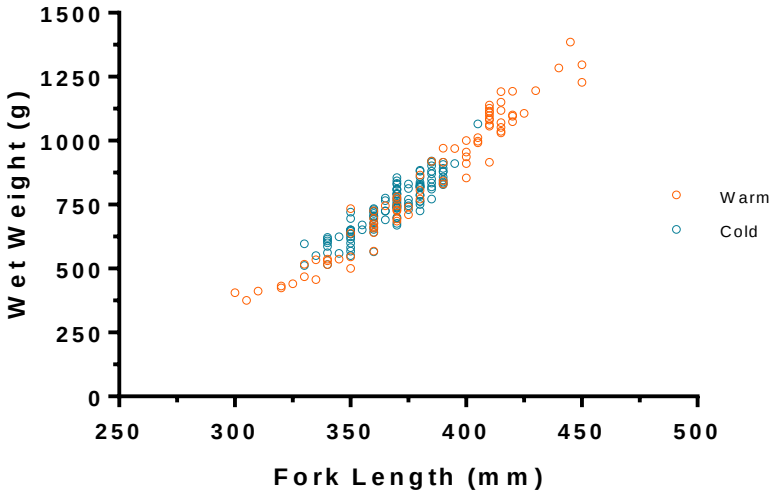


Figure 3.2.2.2.9. Relationship between final body weight and length of individual Yellowtail Kingfish indicating greater size variability of Yellowtail Kingfish reared in warm water at the conclusion of the study.

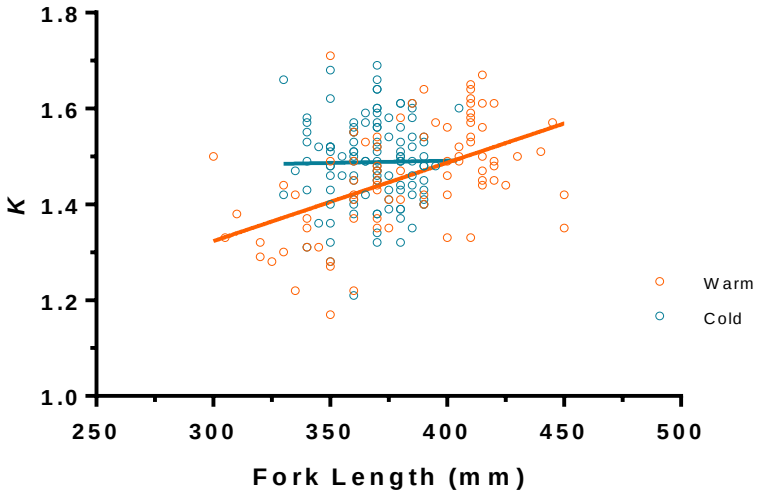


Figure 3.2.2.2.10. Condition (*K*) of individual Yellowtail Kingfish reared in warm and cold water. Regression slopes are significantly different ($P < 0.05$) with the condition of warm water reared Yellowtail Kingfish tending to increase with increasing size compared to cold water fish.

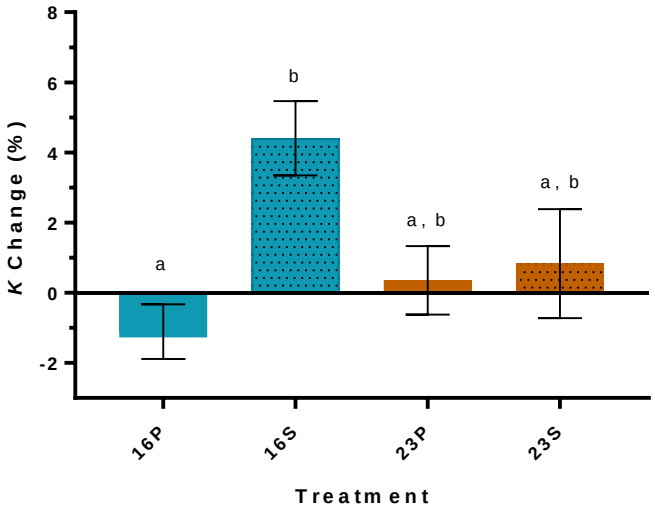


Figure 3.2.2.2.11. Proportional (%) change in condition (*K*) (± SE) of Yellowtail Kingfish after 9 weeks. Data pooled within diet ($n = 6$). Different letters denote statistical significance ($P < 0.05$).

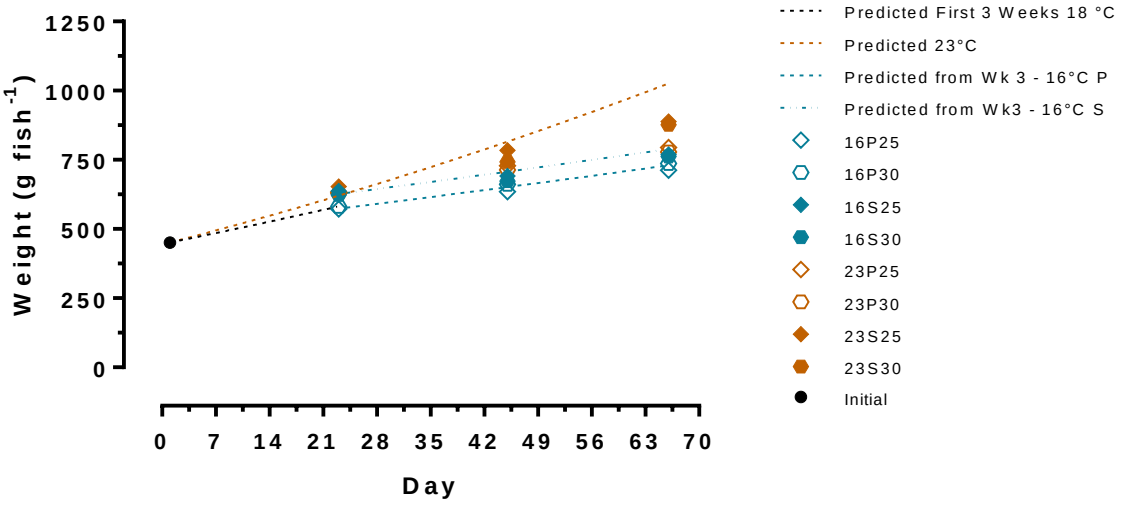


Figure 3.2.2.2.12. Predicted growth (solid and dashed lines) versus measured average body weight of Yellowtail Kingfish over time for diet (25 or 30% CP), ration (S = satiated or P = prescribed) and temperature (16 °C or 23 °C) treatments. Initial cold water growth prediction at 18 °C as the RAS experienced significant temperature fluctuations due to extreme ambient temperatures over the first three weeks.

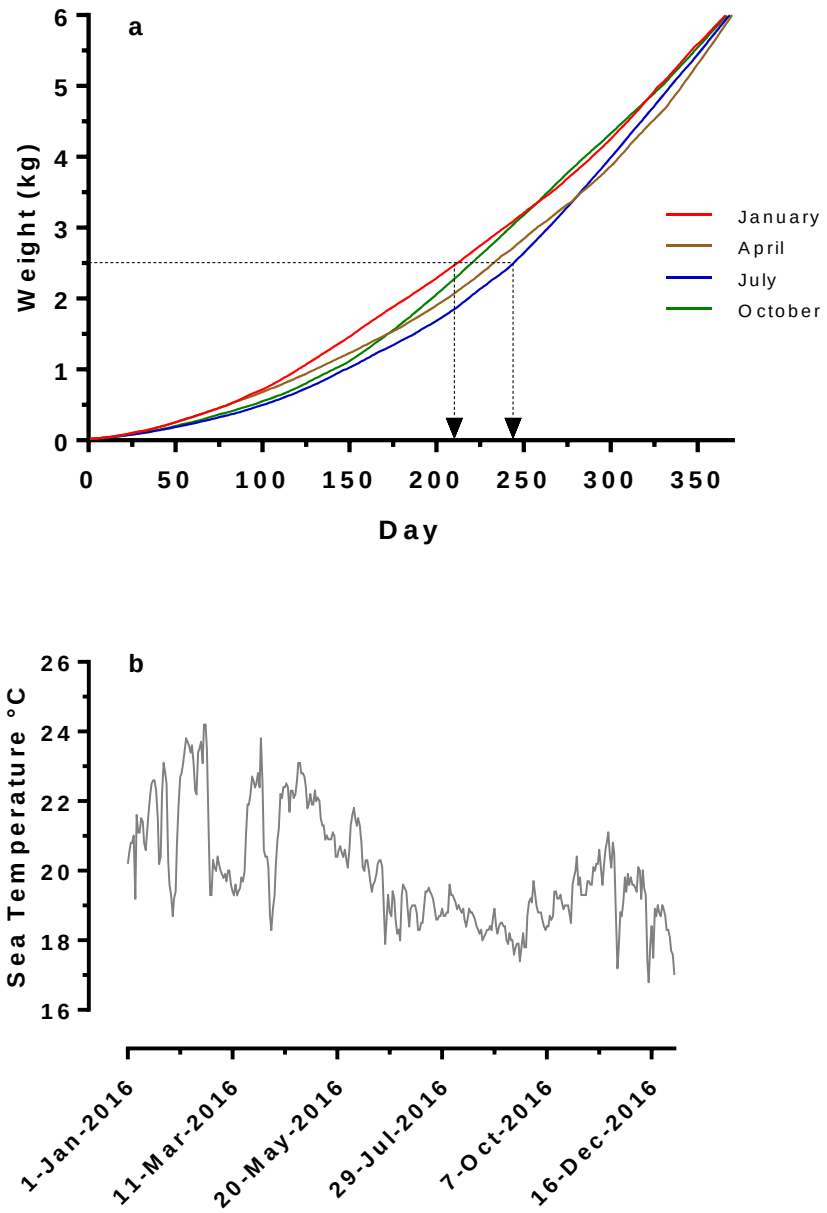


Figure 3.2.2.2.13. (a) Predicted daily growth of Yellowtail Kingfish stocked at different times of the year on the first of the month of; January, April, July or October based on (b) sea temperatures off Broughton Island, Port Stephens 1st January, 2016 to 31st December, 2016. Growth is estimated based on fish with an individual initial weight of 20 g growing to 6 kg. Stocking time has the greatest impact on Yellowtail Kingfish growing to approximately 2.5 kg, i.e. fish experiencing < 12 month seasonal cycle. The modelled data indicates a difference of 33 days for Yellowtail Kingfish to reach 2.5 kg if stocked in summer or winter. Predicted growth of Yellowtail Kingfish assumes optimal conditions (excluding temperature) and uninterrupted access to quality feed throughout grow out.

3.2.2.3. Manuscript - The critical oxygen threshold and hypoxia tolerance of Yellowtail Kingfish (Seriola lalandi).

Caroline L. Candebat[a,c,d], Mark Booth[b], Jane Williamson[c], Igor Pirozzi[a,b]

[a] College of Science and Engineering and Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville 4801, QLD, Australia

[b] Department of Primary Industries, Locked Bag 1, Nelson Bay, NSW 2315

[c] Macquarie University, Balaclava Road, North Ryde, NSW, 2109, Australia

[d] University of Hamburg, Institute for Hydrobiology and Fisheries Science. Hamburg, Germany

This manuscript may be referenced as: Candebat, C.L., Booth, M., Williamson, J. and Pirozzi, I. (2019). 3.2.2.3. Manuscript - The critical oxygen threshold and hypoxia tolerance of Yellowtail Kingfish (*Seriola lalandi*). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027),* Adelaide, June. pp.330-345.

Abstract

This study defines the critical oxygen threshold ($[O_2]_{crit}$) in juvenile Yellowtail Kingfish (*Seriola lalandi*; YTK) with respect to acclimation temperature (15 °C and 20 °C) and dietary lipid source (fish oil and poultry oil). Additionally, observations on the visual and behavioural hypoxia responses in YTK were made. Low saturations of dissolved oxygen are one of the first limiting abiotic factors in land-based aquaculture and mariculture systems, impacting the health and wellbeing of target-species. YTK is a high energy demanding species and the commercial YTK aquaculture is rapidly expanding globally, yet, no information on the hypoxia tolerance for this species is available. YTK aquaculture is commonly carried out in sea pens, in which abiotic factors such as temperature and ambient oxygen can fluctuate substantially. The move away from marine fish oils to more sustainable terrestrial oil sources in aquafeeds implies a change in intake of dietary fatty acid profiles. This shift in dietary fatty acid concentration can impart physiological effects impacting on the stress tolerance of the animal. The critical oxygen threshold is a common method, used to quantify the lower, tolerated threshold of oxygen saturation of an organism. The current study demonstrated that YTK can regulate oxygen consumption until a dissolved oxygen concentration of 1.84 - 2.92 $mg \cdot L^{-1}$, which strongly depends on the acclimation temperature, and to a lesser extent diet. Once these concentrations are surpassed, YTK become oxyconformers, unable to maintain a steady rate of oxygen uptake. Warmer acclimation temperatures led to significantly less hypoxia tolerance compared to YTK held in colder temperatures. Dietary oil source had no significant effect on the critical oxygen threshold; however, YTK fed a poultry-oil based diet showed a strong deviation in routine metabolic rate and $[O_2]_{crit}$. The first behavioural responses exhibited by YTK after passing the $[O_2]_{crit}$ threshold are exaggerated gulp ventilation, mouth breathing on the surface, and operculum movements, followed by a visual change in skin coloration. We strongly recommend rapid oxygenation of the rearing system at the first sign of these behavioural changes, as further onset, such as burst swimming, quickly lead to the final stages of hypoxia and possibly death.

Introduction

The critical oxygen threshold ($[O_2]_{crit}$) is a turning point, separating two phases of oxygen consumption in an organism, the oxyregulating phase and the oxyconforming phase. Throughout the oxyregulating phase, organisms can maintain an optimal oxygen consumption rate independently of the ambient oxygen concentration. Throughout the oxyconforming phase, organisms are directly relying upon the ambient oxygen concentration, and are typically unable to maintain an optimal oxygen consumption rate. Most aquatic organisms maintain a relatively constant oxygen consumption rate with decreasing

ambient oxygen concentration until the $[O_2]_{crit}$ is reached (Perry et al., 2009; Rogers et al., 2016). There are exceptions such as the Inanga (*Galaxias maculatus*); oxygen consumption decreases linearly all the way from an normoxic to a hypoxic environment, indicating that it is a true oxyconformer (Pörtner and Grieshaber, 1993; Urbina and Glover, 2013). Decreasing environmental oxygen past the $[O_2]_{crit}$ provokes behavioural (Kramer, 1987), physiological (Barton and Zwama, 1991; Pörtner and Peck, 2010; Claireaux and Chabot, 2016), molecular (Soitamo et al., 2001; Richards, 2009) and genetic (Wenger, 2000; Nikinmaa and Rees, 2005) changes which can help to improve oxygen delivery, energy expenditure and subsequently, conserve energy to maintain aerobic ATP production.

The sensitivity to environmental hypoxia is species and individual specific (Sylvester et al., 1975), correlating with abiotic factors such as temperature (Beamish, 1964) and salinity (Ern et al., 2014) and biotic factors such as routine metabolic rate, life-stage and biomass (Burton et al., 1980). Abiotic and biotic factors alter the oxygen supply, demand and energy partitioning for and in the animal. For instance, elevated temperatures cause most fish to be less hypoxia tolerant by inducing an increase of the standard metabolic rate (SMR) and reduced oxygen supply through decreased oxygen solubility in the water, causing an imbalance of energy portioning (Rogers et al., 2016).

The impact of dietary oils on the $[O_2]_{crit}$ have not been extensively studied. Dietary lipids for fast-growing, marine, carnivorous fish are mainly provided by fish oil, which is extracted from capture fisheries for the aquafeed industry and is rich in long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA), and arachidonic acid (20:4 n-6, AA). Research on the lipid requirements of marine carnivorous fish have shown the inability to endogenously convert lipids to LC-PUFA and consequently LC-PUFA have to be provided via dietary supplementation to maintain growth, health and physiological functioning (Tocher, 2010). Despite fish oil's essentiality, it has become an unsustainable and costly source of LC-PUFA (FAO 2015). Currently, the aquaculture industry utilises alternative dietary oils, including blends of fish and terrestrial animal and/or plant oils. One of these sources is poultry oil (PO) which is rich in monounsaturated fatty acids (MUFA) and total n-6 PUFA, but devoid of EPA and DHA (Higgs et al., 2006).

Considering the growing importance of sustainable alternatives for marine carnivorous species in aquaculture it is important to know, if dietary oils have a significant impact on physiological stress threshold. Hulbert and Lewis Else (1999) hypothesized that an organism's metabolism is linked to membrane bilayers, emphasizing that DHA is an important building block of the bilayer. Results on larval and juvenile Dover sole demonstrate that various essential fatty acids alter the routine metabolic rate (RMR) and $[O_2]_{crit}$, (McKenzie et al., 2008). However, others have found no influence of dietary fatty acids on metabolism (Valencak and Ruf, 2007; Gonzalez et al., 2015).

Yellowtail Kingfish (*Seriola lalandi*) (hereafter referred to as YTK), is an important emerging species to Australian large-scale mariculture and is grown in New South Wales, South Australia and Western Australia. However, to date, there is no information on the $[O_2]_{crit}$ in YTK. The impact of temperature on the critical oxygen threshold is of particular interest for the aquaculture industry in terms of acute, seasonal and climate change related fluctuations. Further, poultry oil is now routinely being used in commercial diets to partially replace fish oil for this species.

The objective of this study was to quantify the $[O_2]_{crit}$ and hypoxia tolerance of YTK at seasonally relevant temperatures (15 °C and 20 °C). Further, YTK in this study were fed one of two diets containing fish or poultry oil to elucidate the effect of dietary oil source on oxygen metabolism. The results will provide a better understanding of the physiological thresholds of YTK.

Methods

Experiments on YTK in this study were performed under the NSW Department of Primary Industries (Fisheries) Animal Care and Ethics Committee authorization; ACEC REF: 97/8 and 93/5. Care, husbandry and termination of YTK were carried out according to methods outlined in 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2015).

Experimental design

A 2 × 2 factorial design was applied to test the effect of two acclimation temperatures (15 °C or 20 °C) and two dietary oils (fish oil or poultry oil) on the hypoxia tolerance of YTK. The hypoxia tolerances of the four treatments groups were determined over six days. Each treatment was tested in three replicated metabolism chambers in which four individual YTK were stocked. Measurements of the same treatment were repeated twice on different individuals and each run lasted approximately 2.5-4 h. All experimental measurements were conducted between 09:00 and 16:00 h to avoid circadian rhythms in physiology or behaviour.

Experiment diets

Two isonitrogenous (55% crude protein) and isoenergetic (25 MJ kg^{-1}) diets were formulated using practical ingredients (Table 3.2.2.3.1). The dietary nutrient profile was chosen as a commercially relevant specification. With the exception of the dietary oil, the ingredient inclusion was consistent between the diets.

Ingredients were first milled to < 650 µm particles, ensuring homogenous blending. Dry ingredients were then mixed in a batch mixer for 45 min (Ernest Fleming Machinery and Equipment Pty Ltd, batch mixer). Dietary oil and water were then added and thoroughly mixed (Hobart Food Equipment Co., LTD. Mixer). The soft dough was then mechanically extruded with an electric mincing machine to obtain pellets of the desired diameter (6 mm) and strands were then manually broken to < 5 mm pieces. Moist pellets were then dried on perforated trays in convection drier at 38 °C for 8.5 h (moisture content < 10%). Dried pellets were stored in re-sealable containers and kept frozen at -18 °C until used. Feeds were sampled and analysed for proximate (i.e. moisture, total nitrogen, crude lipid and ash content) and fatty acid composition. The proximate composition of the diet is presented in Table 3.2.2.3.1. The fatty acid profiles of the diets are presented in Table 3.2.2.3.2.

Temperature and diet acclimation

Prior to determining $[O_2]_{crit}$ YTK were acclimated to an allocated diet and temperature regime over a period of 51 days. The acclimation phase was as follows. Forty YTK were each stocked in to eight one m^3 net-cages held in two 10 m^3 recirculating aquaculture systems for each respective temperature treatment (15 °C or 20 °C). The initial mean stocking body weight was 250.9 ± 34.5 g (total n = 320). Each diet was randomly allocated to two cages per temperature treatment.

YTK were initially stocked in these systems at an ambient temperature of 18 °C. The temperature of each system was then gradually adjusted at approximately 1 °C per day until temperatures of 15 °C and 20 °C were achieved. Experimental diets were fed after the first day of stocking the pre $[O_2]_{crit}$ trial acclimation. YTK were fed *ad libitum* once per day (12:00 h). The water quality parameters were daily monitored for ammonia (1.1 ppm), dissolved oxygen (DO) saturation (104%), temperature (Table 3.2.2.3.3), pH (8.0) and salinity (32 g L^{-1}). The light regime was based on the natural light regime of the season (11 L:13 D). Due to a technical error, YTK in the warm water system were exposed to a brief hypoxia event resulting in eight mortalities. The remaining YTK recuperated quickly and showed no adverse behaviour and fed normally.

Respirometer

The experimental inventories consisted of one circular-shaped 10 m^{-3} temperature bath holding six floating, 200 L flat-bottomed respirometers (Figure 3.2.2.3.1). Respirometer chambers were utilized to measure the hypoxia tolerance of YTK using a closed static system. The warming/cooling bath was temperature regulated by a reverse cycle heat pump (HWP20-3P, Rheem Pool Heating, Liverpool, NSW, Australia), stabilizing the experiment temperature. Each respirometry chamber was airtight sealed and fitted with a clear Perspex lid. Dissolved oxygen was infused through a ceramic stone, lying beneath the pump in the bath, supplying the respirometer chambers with fresh seawater for the experimental acclimation phase and the post-experiment recuperation phase of the YTK.

The air-water interface in an open-top respirometer has enough boundary layer to reliably measure oxygen consumption in YTK (Pirozzi and Booth, 2009). Nevertheless, additional tests on the hypoxia tolerance of YTK need adjustments of the respirometers, as larger YTK tend to swim to the surface during hypoxic conditions. This "mouth breathing" or gulping behaviour breaks the air water interface. To prevent mixing events and restrict "mouth breathing", respirometers were covered with Plexiglas.

Dissolved oxygen in the respirometer chambers was measured with two FireStingO2 devices (Pyro Science GmbH, Aachen, Germany). Connected to each FireStingO2 device were three optical fibre cables (SPFIB, Pyro Science GmbH, Aachen, Germany) transferring the admitted red light source to lens spot adapters (SPADLNS, Pyro Science GmbH, Aachen, Germany). Lens spot adapters were carefully fixed in place with adhesive glue (Sika Australia Pty. Ltd.) to the outer surface of the Perspex lid. Lens spot adapters have an integrated collimating lens, which allows working with Perspex thicknesses of 2-6 mm. Contactless oxygen sensor spots (COSS, OXSP5, Pyro Science GmbH, Aachen, Germany) were attached with clear silicone glue to the inner side of the Perspex lid. COSS allow measurements within closed containers through a transparent barrier and provide exact measurements. COSS have no intrinsic O_2 consumption and therefore no correction of data was required. Sensors were calibrated individually according to the manufacturer's instructions. Black corflute sheets were installed in-between the respirometers, shielding YTK from external disturbances.

After each experiment the background biological oxygen demand (BOD) in the respirometer was measured. None or low levels of background BOD were found, but where necessary oxygen consumption rates were corrected for background respiration.

Critical oxygen threshold determination

The $[O_2]_{crit}$ level of YTK acclimated to different temperatures and dietary oils was assessed using a total of 88 YTK (483.3 ± 55.1 g) of which four YTK were stocked in each respirometer. There were $n = 3$ replicate respirometers per treatment. Respirometry was repeated twice using different fish.

YTK were fasted prior to respirometry taking into consideration the acclimation temperature (15 °C, 48 h; 20 °C, 36 h). On the same day of hypoxia tolerance determination, fiber-optic sensors (OXSP5, Pyro Science GmbH, Germany) were calibrated [two-point in water at the beginning of the experiment (O_2 at 100% and 0%)]. Additionally, salinity, temperature and pressure were measured by a Hach HQ40D portable meter and these variables were then manually entered in the Pyro Oxygen Logger to refine measurements (FireStingO2, Pyro Science GmbH, Germany).

After the calibration procedure, oxygen saturation was maintained at 100% by controlling the inflow rate of oxygenated water into the respirometers. Respirometers were then systematically stocked with four YTK each over 25 min increments to facilitate a time shifted start for measurements and the chambers were carefully sealed with Perspex lids and spring clamps. YTK were habituated in the normoxic water (O_2 ~100%) until the oxygen consumption stabilised (~45 min). After the habituation phase, respirometer chambers were isolated by stopping the inflow of oxygenated water and outflow of water. Remnant air was removed through an air-outlet on the Perspex lid, which was afterwards sealed. Automatic, computational live measurements recorded oxygen concentration in the water (Pyro Oxygen Logger). The level of oxygen in the static water was decreased by the oxygen consumption of the YTK until the $[O_2]_{crit}$ threshold was passed.

Hypoxia related behavioural observations

Observations of behavioural responses of YTK in the respirometers were made throughout the respirometry trial. These behavioural and visual responses included gulping, change of skin colouration, burst swimming behaviour and the loss of equilibrium. Classifications were based on McKenzie et al. (2008).

Gulping ($[O_2]_{gulp}$) was defined as the oxygen level at which more than two YTK in one respirometer unit showed exaggerated gulping ventilation, mouth breathing on the surface, and operculum movements. Change in skin color ($[O_2]_{color}$) was identified as the oxygen level at which dark spots were developed or a general darker complexion was noticeable in individual YTK. Burst swimming behaviour ($[O_2]_{burst}$)

was the oxygen level at which all YTK displayed confused, disoriented and burst movements. The loss of equilibrium $[O_2]_{LOE}$ was defined as the point at which a YTK started to sink to the bottom of the tank, with no attempt to swim to the water surface.

Immediately after observing an individual YTK displaying $[O_2]_{LOE}$, the respirometer was opened, and water was quickly sampled to test for CO_2 level. Carbon dioxide concentration never exceeded 6 ppm. Fresh O_2 saturated water was then immediately pumped in to the respirometry chamber and saturation levels (100%) quickly restored. After 15 min, YTK were removed from the respirometer to record body weight and length. All YTK fully recovered from hypoxic exposure.

Data analyses

The $[O_2]_{crit}$ and RMR was determined by averaging the outcome of each experimental run and analysed via GraphPad Prism ver. 6 (La Jolla, CA, USA).

The following formula was used to calculate the oxygen consumption (mg $kg^{-1} \cdot h^{-1}$) of each run:

$$Oxygen\ consumption = \frac{([O_2]_t - [O_2]_{t+1}) * v}{k * m}$$

Eq. 1

where; $[O_2]_t$ refers to the oxygen concentration (mg L^{-1}) at time point t, $[O_2]_{t+1}$ is the oxygen concentration at the next time point (the oxygen measurements were calculated according to the O_2 solubility coefficient in water under corresponding temperature, pressure and salinity), v is the volume in litres of the respirometer, k is the time interval between time points t and t+1, and m is the body mass of the YTK in kg. The collected oxygen consumption values were subject to non-linear regression analysis (Koops and Grossman, 1993):

$$Y = A - b * s * \ln(1 + \exp\left(\frac{c-x}{s}\right))$$

Eq. 2

and segmental linear regression ´broken stick` analysis, joining two straight lines at a certain breakpoint:

$$y_i = \begin{cases} intercept_1 + slope_1 x & for\ x \leq c \\ intercept_1 + c(slope_1 - slope_2) + slope_2 x & for\ x > c \end{cases}$$

Eq. 3

Models were then cross-validated for quality and fit via Akaike information criterion (AIC).

Statistical analyses were performed using R software environment for statistical computing (2.13.). The effects and interactions of thermal acclimation and dietary oil on RMR, $[O_2]_{crit}$, body mass, $[O_2]_{LOE}$, $[O_2]_{color}$, $[O_2]_{gulp}$, $[O_2]_{burst}$ were statistically analysed via two-way analysis of variance. Assumptions of homogeneity and normality were tested via Levene's and Shapiro-Wilk test. In case assumptions were not met, data were either log or square-root transformed and then statistically analysed.

Results

Critical oxygen threshold ($[O_2]_{crit}$)

$[O_2]_{crit}$ values are presented in Table 3.2.2.3.3. $[O_2]_{crit}$ was determined by non-linear or segmental regression analysis and then statistically analysed for differences in regression models. Results indicate the chosen analysis method has a significant impact on the $[O_2]_{crit}$ values ($P < 0.05$). All $[O_2]_{crit}$ determined by BSR analysis were significantly higher than $[O_2]_{crit}$ values determined via NLS ($P < 0.05$). Therefore, $[O_2]_{crit}$ values determined by BSR analysis indicate less hypoxia tolerance than $[O_2]_{crit}$ values determined by NLS analysis. After comparing both models by the AIC criterion, results indicated that the NLS model was a better fit of measured oxygen consumption. Hereafter, stated $[O_2]_{crit}$ values are the result of NLS model analysis (Figure 3.2.2.3.2).

No interaction of dietary oil source and temperature on $[O_2]_{crit}$ was detected. Results on the impact of thermal acclimation and lipid source on $[O_2]_{crit}$ in YTK showed varying results.

YTK acclimated to different temperatures showed significant differences in $[O_2]_{crit}$ ($P < 0.05$). YTK were less hypoxia tolerant in warm water than YTK in colder water ($[O_2]_{crit}$; Fish oil diet 2.33 ± 0.11 mg L^{-1} and poultry oil diet: 2.80 ± 0.79 mg L^{-1}), than YTK acclimated to 15°C ($[O_2]_{crit}$; Fish oil diet 1.37 ± 0.14 mg L^{-1} and poultry oil diet: 1.66 ± 0.55 mg L^{-1}).

Even though different dietary oils did not seem to impact $[O_2]_{crit}$, trends were visible ($P < 0.07$). These trends indicated that YTK fed diets containing more fish oil tolerate hypoxia better than YTK fed diets containing poultry oil. Additionally, YTK fed the poultry oil diets demonstrated greater standard deviations than the YTK fed the fish oil diets (Figure 3.2.2.3.2).

The elapsed time, until a critical hypoxic environment was created, differed significantly between the different temperature treatments ($P < 0.01$; Table 3.2.2.3.3).

Routine metabolic rate

RMR data are presented in Table 3.2.2.3.3 and Figure 3.2.2.3.2. No interactions between temperature and dietary oil on the routine metabolic rate of YTK were detected. Nevertheless, the routine metabolic rates differed significantly between temperatures ($P < 0.05$) and almost doubled in YTK acclimated to 20 °C compared to YTK acclimated to 15 °C. YTK acclimated to 20 °C had significantly higher metabolic rates (254.06 ± 4.73 mg O_2 L kg h^{-1} and 263.75 ± 35.26 mg O_2 L kg h^{-1}), than YTK acclimated to 15 °C (167.11 ± 8.94 mg O_2 L kg h^{-1} and 179.29 ± 11.51 mg O_2 L kg h^{-1}). No significant difference in RMR ($P > 0.05$) was detected in YTK acclimated to different dietary oils (Table 3.2.2.3.3).

Behavioural responses

Observations were made on the sequence of behaviours and visual changes responding to hypoxia. Throughout all treatments, YTK, exposed to hypoxia, showed exaggerated gulping, burst swimming behaviour and a change in coloration (dark spots). The sequence of behaviour across all treatments was consistent; firstly YTK started gulping, secondly the YTK's skin coloration changed, thirdly YTK showed burst swimming, and finally YTK lost equilibrium (Table 3.2.2.3.4).

No significant interaction ($P > 0.05$) was determined between dietary oil and temperature when considering behavioural and visual responses. Acclimation temperature and lipid-source had no significant effect ($P > 0.05$) on the gulping behaviour of the YTK. Nevertheless, gulping started generally after transitioning into the oxyconforming phase (Table 3.2.2.3.4).

After gulping, YTK changed coloration by developing dark spots all over the body, starting from the head and/or dorsal fin. The change in coloration was not significantly impacted by lipid-source but showed trends with different acclimation temperatures ($P < 0.06$; Table 3.2.2.3.4).

After or at the same time YTK changed coloration, YTK started to burst swim. Burst swimming was not significantly impacted by the dietary oil, but YTK acclimated to the colder temperature significantly changed the swimming behaviour at a lower environmental oxygen content, than YTK acclimated to warmer temperatures (Table 3.2.2.3.4).

Shortly after the change of swimming behaviour, YTK lost equilibrium and started to sink to the bottom of the tank. The acclimation temperature had a significant impact on the loss of equilibrium ($P < 0.05$). Dietary oil did not have a significant impact ($P > 0.05$). YTK acclimated to colder temperatures lost equilibrium at much lower oxygen content than YTK acclimated to warmer temperatures. YTK displayed a loss of equilibrium from 1.22 mg·L^{-1} up to 1.48 mg·L^{-1} (Table 3.2.2.3.4).

Discussion

Critical oxygen threshold ($[O_2]_{crit}$)

This study provides information on the hypoxia sensitivity of YTK under different temperature and diet regimes, showing that YTK are able to regulate their oxygen consumption to a threshold of 1.84-2.92 mg L^{-1} (equiv. 22.3%-38.8% saturation at 19.4 °C to 14.5 °C, respectively;

https://water.usgs.gov/software/DOTABLES/). These results indicate that YTK hypoxia tolerance is strongly linked to ambient water temperature. This response is species specific. For example, Atlantic Salmon (*Salmo salar*) can regulate their oxygen consumption down to ~35-50% saturation between 14 °C and 22 °C, respectively; below these levels salmon become oxyconformists (Barnes et al., 2011). In almost all studies determining temperature and hypoxia tolerance interactions, warmer temperatures induce less hypoxia tolerance in fish (Barnes et al., 2011; Collins et al. 2013; Rogers et al. 2016). Nevertheless, the overall impact of temperature on hypoxia tolerance is species specific. An increase of temperature by 3°C reduced the hypoxia tolerance in Cardinalfish (*Ostorhinchus doederleini*) dramatically, while an increase of temperature by 20 °C reduced the hypoxia tolerance in Carp only subtly (Nilsson et al., 2010). YTK exhibited a similar reaction to an increase of 5°C as Common Dentex (*Dentex dentex*) (Cerezo et al., 2006), with a relatively strong reduction of hypoxia tolerance.

Neither poultry nor fish oil had significant effects on the hypoxia tolerance of YTK even though results indicate a slight increase of hypoxia tolerance when YTK were fed fish oil at both water temperatures. The non-significant effects of oil sources on $[O_2]_{crit}$ in YTK may be partially explained by the integration of fish meal in both diets. Fish meal contains fish oil which would have contributed to the overall amount of essential fatty acids in the test diets (Table 3.2.2.3.2). In larval and juvenile Dover Sole (*Solea solea)* essential fatty acid enriched diets significantly improved the hypoxia tolerance when acclimated to approximately 18 °C (McKenzie et al., 2008), while deficiencies in polyunsaturated fatty acids reduced hypoxia tolerance and led to significantly higher mortalities when exposed to 10% oxygen saturation (Logue et al., 2000). Even though no significant effect of dietary oil on hypoxia tolerance of YTK was demonstrated in this study, the high variability in hypoxia tolerance of YTK fed the poultry oil-based diet in comparison to the YTK fed the fish oil-based diet was noticeable (Figure 3.2.2.3.2) It is not clear why this occurred and is an area that requires further investigation.

$[O_2]_{crit}$ can be estimated by using segmented linear regression, also called piecewise regression or broken-stick regression (BSR) (Cerezo et al., 2006; Nilsson et al., 2010). This mathematical approach assumes an abrupt change to occur in the animal as it moves from being an oxyregulator to an oxyconformer. In a biological sense, this approach does not seem particularly appropriate for a biochemical and biophysical process. The non-linear regression line (NLS) describes $[O_2]_{crit}$ as the point at which a slope of a function starts to flatten out and at which the slope approaches zero (i.e. the asymptote). Marshall et al. (2013) compared $[O_2]_{crit}$ assessed by BSR and several NLSs', confirming that the respective regression analysis can cause significant differences in the assessment of $[O_2]_{crit}$. It is recommended to choose a regression analysis which results in the best fit by comparing models visually as well as statistically using appropriate criterion such as AIC. Clearly, biphasic slopes, describing the relationship between oxygen consumption and decreasing oxygen availability, show more similar results in $[O_2]_{crit}$ between the two techniques. The smoother a slope becomes, the more it appears that $[O_2]_{crit}$ assessed by NLS and BSR differ (Figure 3.2.2.3.2). NLS incorporates the smoothness of a function with decreasing oxygen availability (Marshall et al., 2013). Results from this study confirm that the selection of regression analysis for the determination of $[O_2]_{crit}$ must be carefully considered. The BSR analysis tended to shift the $[O_2]_{crit}$ further towards the right on the *x*-axis (Figure 3.2.2.3.2), indicating a lower degree of hypoxia tolerance while the $[O_2]_{crit}$ assessed by NLS indicated that YTK had a relatively greater degree of hypoxia tolerance. Due to a better statistical fit, results on the $[O_2]_{crit}$ assessed via non-linear regression analysis were considered more appropriate.

Routine metabolic rate (RMR)

The RMR of YTK is linearly dependent on water temperature, steadily increasing with increasing temperature (Pirozzi and Booth, 2009). RMR of YTK acclimated to 15° C, measured by Pirozzi and Booth (2009), (177.87 ± 34.04 mg O_2 kg^{-1} h^{-1}) were almost half of the RMR of YTK acclimated to 20 °C (253.57 ± 38.46 mg O_2 kg^{-1} h^{-1}) in the same study. The RMR of YTK in that study strongly correlates with the RMR of YTK acclimated to 15 °C and 20 °C measured in this study (Table 3.2.2.3.3). Atlantic Salmon acclimated to 18 °C have a similar RMR as YTK (Barnes et al., 2011). However, the RMR in southern bluefin tuna (*Thunnus maccoyii*), a highly active and pelagic fish species, is four times that of YTK (Gooding et al., 1981; Fitzgibbon et al., 2008; Pirozzi and Booth, 2009). This leads to the conclusion that even though YTK shares morphological characteristics of the tuna family, it does not have the same metabolic expenses. Although YTK do not have the high RMR exhibited by the tuna

family, their RMR is higher than the Dolphinfish (*Coryphaena hippurus*) (Benetti, 1992), confirming YTK are a species with relatively high energy demands

Behavioural responses to hypoxia

YTK exposed to hypoxic conditions showed a sequence of behaviours, starting with exaggerated gulping ventilation, a change in skin coloration and shortly after, burst swimming, followed by the loss of equilibrium (Table 3.2.2.3.4). Characterization of visual responses were based on similar descriptions of Dover Sole exposed to hypoxia (McKenzie et al., 2008), with the exception that this study also observed a change in skin coloration in YTK.

The first observation of unusual behaviour, while gradually decreasing the oxygen content, in YTK was exaggerated gulp ventilation. This response improves the blood oxygen transport during hypoxia (Burggren, 1982). Secondly, YTK showed isolated or complete discoloration during advanced hypoxia. This might be a form of cyanosis, which is a dark discoloration of tissues near the skin surface, due to low oxygen saturations (Lundsgaard and Abbott, 1923). Thirdly, YTK were burst swimming, perhaps in an attempt to avoid the hypoxic zone (McKenzie et al., 2008) or ram ventilate. In contrast to YTK, Dover Sole shows strong burst swimming at 2.64 mg O_2 L^{-1}, while YTK started burst swimming at 1.50-1.97 mg O_2 L^{-1}. The early visual warning sign of burst swimming in Dover sole might give enough time to react to hypoxic conditions, while the late burst swimming, shortly followed by the loss of equilibrium in YTK, makes it even more important to focus on the early visual hypoxia responses such as exaggerated gulp ventilation.

Conclusion and Recommendations

This study has quantified the critical dissolved oxygen threshold of sub-adult YTK. Concomitant with this threshold are a consistent sequence of behavioural responses to hypoxia which are initiated with exaggerated opercula and mouth gulping movements and surface swimming, ending with loss of equilibrium. Standard management practices should ensure aquaculture systems remain saturated (100% dissolved oxygen) at all times; however, if the initiation of these behavioural responses is observed in culture situations, rapid re-oxygenation must be implemented to avoid the onset of lethal hypoxic conditions. YTK are hypoxia sensitive, especially when held at warmer water temperatures; at a temperature of 20 °C a dissolved oxygen concentration of below 2.6 mg O_2 L^{-1} (~38% saturation) will induce hypoxia. YTK have an elevated RMR in warm water compared to YTK held in cool water. The time taken to deplete normoxic saturated (100%) water to $[O_2]_{crit}$ levels at 15 °C is more than double that of YTK at 20 °C; this has significant implications on the reaction time to implement re-oxygenation of a rearing system should a system failure occur. The influence of dietary oil source did not have a significant effect on respiration rates of YTK; however, the consistent trend observed when comparing $[O_2]_{crit}$ of YTK fed poultry oil diets to YTK fed fish oil diets warrants further investigation.

Findings

- YTK can regulate their oxygen consumption to a concentration of ~1.9-2.6 mg O_2 L^{-1} (equiv. ~22-38% saturation) at 20 °C and 15 °C, respectively, after which point they become oxyconformers and transition to a hypoxic state.
- $[O_2]_{crit}$ is strongly dependent on the acclimation water temperature. Warmer acclimation temperatures decrease the hypoxia tolerance of YTK.
- Oil-source (fish oil versus poultry oil) had no significant effect on the critical oxygen threshold of YTK in this study. However, YTK fed a poultry-oil diet showed a relatively large deviation in routine metabolic rate and $[O_2]_{crit}$.
- There is a consistent sequence of behavioural responses in YTK to the onset of hypoxic conditions that is initiated with exaggerated opercula and mouth gulping movements and surface swimming. Rapid oxygenation of the rearing system at first indications of these behavioural changes is strongly recommended.

- Critical DO limits have been defined for sub-adult YTK at water temperatures that bracket the majority of production situations in Australia (15 °C -20 °C).
- Knowledge of these DO limits will provide farm managers with a better understanding of the physiology and behavioural responses of YTK to low DO environments. This knowledge will improve the capacity of farm managers to react to and mitigate low DO challenges.
- The DO limits defined in this research can be used by farm managers to ensure YTK are provided with adequate DO, especially during handling, bathing or transportation procedures.
- The influence of dietary lipid on $[O_2]_{crit}$ may be more pronounced in low fishmeal diets and is an area worth investigating.
- Water temperatures where YTK are farmed in NSW, South Australia and Western Australia often exceed 20 °C, and we recommend conducting further $[O_2]_{crit}$. experiments at higher water temperatures.

Publications

No publications have resulted from this R&D to date.

Acknowledgements

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, New South Wales Department of Primary Industries (NSW DPI) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

We would like to thank Dr. Michael Salini from Ridley for provision of several of the raw materials used in the experimental diets. We also thank Dr. Basseer Codabaccus, Brendan Findlay and Ian Russel who provided valuable technical support and assistance in running experiments and constructing respirometers (NSW DPI) and the team at CSIRO Agriculture and Food (especially Barney Hines) for the chemical analysis of diets.

References

ACEC, 2015. A Guide to Acceptable Procedures and Practices For Aquaculture and Fisheries Research - 4th Edition, 4th ed. NSW Department of Primary Industries (Fisheries) for NSW Fisheries Animal Care and Ethics Committee, Nelson Bay, NSW, Australia.

Barnes, R., King, H., Carter, C.G., 2011. Hypoxia tolerance and oxygen regulation in Atlantic salmon, *Salmo salar* from a Tasmanian population. Aquaculture 318, 397–401.

Barton, B.A., Zwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu. Rev. Fish Dis. 3–26.

Beamish, F.W.H., 1964. Respiration of fishes with special emphasis on standard oxygen consumption: II. Influence of weight and temperature on respiration of several species. Can. J. Zool. 42, 177-188.

Benetti, D.D., 1992. Bioenergetics and growth of dolphin, *Coryphaena hippurus*. University of Miami. https://doi.org/10.16953/deusbed.74839

Burggren, W.W., 1982. "Air Gulping" improves blood oxygen transport during aquatic hypoxia in the goldfish *Carassius Auratus*. Univ. Chicago Press Journals 55, 327–334.

Burton, D.T., Richardson, L.B., Moore, C.J., 1980. Effect of oxygen reduction rate and constant low dissolved oxygen concentrations on two estuarine fish. Trans. Am. Fish. Soc. 109, 552–557.

Cerezo, J., López, F.M., García, B., 2006. Oxygen consumption and ventilatory frequency responses to gradual hypoxia in common dentex (*Dentex dentex*): Basis for suitable oxygen level estimations Aquaculture 256, 542–551.

Claireaux, G., Chabot, D., 2016. Responses by fishes to environmental hypoxia: Integration through Fry's concept of aerobic metabolic scope. J. Fish Biol. 88, 232–251.

Collins, G.M., Clark, T.D., Rummer, J.L., Carton, A.G., 2013. Hypoxia tolerance is conserved across genetically distinct sub-populations of an iconic, tropical Australian teleost (*Lates calcarifer*). Conserv. Physiol. 1, 1–9.

Ern, R., Huong, D., Cong, N., Bayley, M., Wang, T., 2014. Effect of salinity on oxygen consumption in fishes: a review. J. Fish Biol. 84, 1210-1220.

FAO, 2015. Fishmeal and fish oil - Analysis of 3rd quarter 2015 | GLOBEFISH | Food and Agriculture Organization of the United Nations [WWW Document]. URL http://www.fao.org/in-action/globefish/market-reports/resource-detail/en/c/384232/

Fitzgibbon, Q.P., Baudinette, R.V., Musgrove, R.J., Seymour, R.S., 2008. Routine metabolic rate of southern bluefin tuna (*Thunnus maccoyii*). Comp. Biochem. Physiol. 150, 231–238.

Gonzalez, A., Page, B., Weber, J.-M., 2015. Membranes as a possible pacemaker of metabolism in cypriniform fish: does phylogeny matter? J. Exp. Biol. 218, 2563–2572.

Gooding, R.M., Neill, W.H., Dizon, A.E., 1981. Respiration rates and low-oxygen tolerance limits in skipjack tuna, *Katsuwonus pelamis*. Fish. Bull. 79, 31–48.

Higgs, D.A., Balfry, S.K., Oakes, J.D., Rowshandeli, M., Skura, B.J., Deacon, G., 2006. Efficacy of an equal blend of canola oil and poultry fat as an alternate dietary lipid source for Atlantic salmon (*Salmo salar*) in sea water. I: effects on growth performance, and whole body and fillet proximate and lipid composition. Aquac. Res. 37, 180–191.

Koops, W.J., Grossman, M., 1993. Multiphasic allometry. Growth. Dev. Aging 57, 183–92.

Kramer, D.L., 1987. Dissolved oxygen and fish behavior. Environ. Biol. Fishes 18, 81–92.

Logue, J.A., Howell, B.R., Bell, J.G., Cossins, A.R., 2000. Dietary n-3 long-chain polyunsaturated fatty acid deprivation, tissue lipid composition, ex vivo prostaglandin production, and stress tolerance in juvenile Dover sole (*Solea solea* L). Lipids 35, 745–756.

Lundsgaard, C., Abbott, M.E., 1923. Cyanosis. Can. Med. Assoc. J. 2, 1–76.

Marshall, D.J., Bode, M., White, C.R., 2013. Estimating physiological tolerances - a comparison of traditional approaches to nonlinear regression techniques. J. Exp. Biol. 216, 2176–2182.

McKenzie, D.J., Lund, I., Pedersen, P.B., 2008. Essential fatty acids influence metabolic rate and tolerance of hypoxia in Dover sole (*Solea solea*) larvae and juveniles. Mar. Biol. 154, 1041–1051.

Nikinmaa, M., Rees, B.B., 2005. Oxygen-dependent gene expression in fishes. Am J Physiol Regul Integr Comp Physiol 288, 1079–1090.

Nilsson, G.E., Östlund-nilsson, S., Munday, P.L., 2010. Effects of elevated temperature on coral reef fishes: Loss of hypoxia tolerance and inability to acclimate. Comp. Biochem. Physiol. 156, 389–393.

Ott, M.E., Heisler, N., Ultsch, G.R., Physiologic, A., Medizin, M., 1980. A re-evaluation of the relationship between temperature and the critical oxygen tension in freshwater fishes. Comp. Biochem. Physiol. 67, 337–340.

Perry, S.F., Jonz, M.G., Gilmour, K.M., 2009. Chapter 5 - Oxygen sensing and the hypoxic ventilatory response. Hypoxia 27, 193–253.

Pirozzi, I., Booth, M.A., 2009. The routine metabolic rate of mulloway (*Argyrosomus japonicus: Sciaenidae*) and yellowtail kingfish (*Seriola lalandi: Carangidae*) acclimated to six different temperatures. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 152, 586–592.

Pörtner, H.O., Grieshaber, M.K., 1993. Critical PO2 in oxyconforming and oxyregulating animals: Gas exchange, metabolic rate and energy production. Vertebr. gas Transp. cascade Adapt. to Environ. mode life 330–357.

Pörtner, H.O., Peck, M.A., 2010. Climate change effects on fishes and fisheries: towards a cause-and-effect understanding. J. Fish Biol. 77, 1745–79.

Richards, J.G., 2009. Metabolic and Molecular Responses of Fish to Hypoxia. Fish Physiology 27, 443–485.

Rogers, N.J., Urbina, M.A., Reardon, E.E., Mckenzie, D.J., Wilson, R.W., Cooke, S., 2016. A new analysis of hypoxia tolerance in fishes using a database of critical oxygen level (Pcrit). Conserv. Physiol.

Soitamo, A.J., Rabergh, C.M., Gassmann, M., Sistonen, L., Nikinmaa, M., 2001. Characterization of a hypoxia-inducible factor (HIF-1alpha) from rainbow trout. J. Biol. Chem. 276, 19699–705.

Sylvester, J.R., Nash, C.E., Emberson, C.R., 1975. Salinity and oxygen tolerances of eggs and larvae of Hawaiian striped mullet, *Mugil cephalus*. J. Fish Biol. 7, 621–629.

Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquac. Res. 41, 717–732.

Urbina, M.A., Glover, C.N., 2013. Relationship between fish size and metabolic rate in the oxyconforming Inanga *Galaxias maculatus* reveals size-dependent strategies to withstand hypoxia. Physiol Biochem Zool. 86, 740–749.

Valencak, T.G., Ruf, T., 2007. N-3 polyunsaturated fatty acids impair lifespan but have no role for metabolism. Aging Cell 6, 15–25.

Wenger, R.H., 2000. Mammalian oxygen sensing, signalling and gene regulation. J. Exp. Biol. 203, 1253–1263.

Table 3.2.2.3.1. Experiment diet formulations (dry matter basis).

Ingredient (%)	**Fish oil diet**	**Poultry oil diet**
Fishmeal	55.0	55.0
Wheat flour	15.0	15.0
Dehulled lupin	10.0	10.0
Taurine	1.0	1.0
Vit/min premix	0.5	0.5
Monosodium phosphate	0.15	0.15
Choline chloride 70%	0.05	0.05
Rovimix Stay-C 35%	0.05	0.05
Fish oil	18.25	-
Poultry oil	-	18.25

Table 3.2.2.3.2. Measured proximate and fatty acid content of experimental diets (dry matter basis).

Composition (%)	**Fish oil diet**	**Poultry oil diet**
Moisture	6.05	6.23
Crude lipid	22.60	23.89
Crude protein	56.23	53.26
Carbohydrate	13.22	14.85
Ash	7.95	7.99
Total nitrogen	9.00	8.52
Gross energy (MJ kg^{-1})	25.08	24.66
Fatty acid (% of total fatty acids)		
C12:0	0.0	0.1
C14:0	4.0	1.6
C14:1*n*-5	0.0	0.2
C15:0	0.7	0.3
C16:0	19.0	21.3
C16:1*n*-7	4.9	5.0
C17:0	0.5	0.4
C17:1	0.6	0.3
C18:0	4.2	6.0
C18:1*n*-9T	0.2	0.2
C18:1*n*-9C	22.9	38.1
C18:1*n*-7	3.5	2.6
C18:2*n*-6T	0.2	0.1
C18:2*n*-6C	3.2	12.0
C19:0	0.2	0.2
C18:3*n*-6	0.0	0.0
C18:3*n*-3	1.3	2.1
C18:4*n*-3	1.0	0.4
C20:0	0.3	0.2
C20:1*n*-9	7.1	0.8
C20:1*n*-7	0.5	0.1
C20:2*n*-6	0.4	0.2
C20:4*n*-6	1.1	0.6
C20:3*n*-3	0.3	0.1
C20:5*n*-3	6.8	1.8
C22:0	0.3	0.2
C22:1*n*-9	1.1	0.1
C23:0	0.1	0.0
C22:4*n*-6	0.2	0.1
C24:0	1.8	0.4
C22:5*n*-3	0.1	0.1
C22:6*n*-3	12.6	4.4
C22:1*n*-9	1.0	0.1
Total saturated	31.7	30.9
Total monoenes	41.7	47.4
Total PUFA	27.1	21.9
Total *n*-3 PUFA	22.0	8.8
Total *n*-6 PUFA	5.2	13.1
n-3/*n*-6	4.2	0.7

Table 3.2.2.3.3. Mean values (± SD) for routine metabolic (RMR) under normoxic conditions, duration of $[O_2]_{crit}$ trial, critical oxygen level (NLS and BSR) in YTK fed different dietary oils and acclimated to different temperatures. Different superscript letters within columns indicate a significant difference.

Diet	Temp. (°C)	Body wt (g)	RMR (mg O_2 kg^{-1} h^{-1})	Time (h)	$[O_2]_{crit}$ ($mg \cdot L^{-1}$)	
					NLS	BSR
Fish oil	20	538±77[a]	264.0±44.90[a]	2.30[a]	2.24±0.13[a]	2.50±0.07[a]
Poultry oil	20	515±77[a]	255.68±33.71[a]	2.25[a]	2.92±0.73[a]	3.04±0.60[a]
Fish oil	15	445±81[b]	166.95±8.93[b]	5.10[b]	1.84±0.19[b]	2.33±0.67[b]
Poultry oil	15	421±56[b]	179.05±11.47[b]	5.33[b]	2.00±0.33[b]	2.25±0.46[b]
P-value		< 0.01	< 0.01	< 0.01	< 0.05	< 0.05
Dietary oil × temp.		NS	NS	NS	NS	NS

RMR, routine metabolic rate; $[O_2]_{crit}$, critical oxygen level; NLS, Non-linear regression model; BSR, Segmental 'Broken-stick' linear regression model; NS, non-significant.

Table 3.2.2.3.4. Mean values (± SD) for hypoxia induced visual and behavioural changes and the loss of equilibrium in YTK fed different dietary oils and acclimated to different temperatures. Different superscript letters within columns indicate a significant difference.

		Behaviour			
Diet	Temp. (°C)	$[O_2]_{LOE}$	$[O_2]_{color}$	$[O_2]_{gulp}$	$[O_2]_{burst}$
Fish oil	20	1.30±0.09[a]	1.68±0.11[a]	2.14±0.30[a]	1.57±0.15[a]
Poultry oil	20	1.48±0.21[a]	1.92±0.36[a]	2.48±0.44[a]	1.90±0.22[a]
Fish oil	15	1.22±0.08[b]	1.54±0.33[b]	2.15±0.39[a]	1.52±0.03[b]
Poultry oil	15	1.29±0.08[b]	1.59±0.19[b]	2.39±0.15[a]	1.50±0.06[b]
P-value		< 0.05	0.05	NS	< 0.05
Dietary oil × temp.		NS	NS	NS	NS

*$[O_2]_{LOE}$ Loss of equilibrium; $[O_2]_{color}$ oxygen level at which individual YTK showed a change in color; $[O_2]_{gulp}$ oxygen level at which individual YTK started to show stronger movement of operculum and mouth ventilation; $[O_2]_{burst}$ oxygen level at which individual YTK started to transit into a more active swimming phase; NS non-significant.

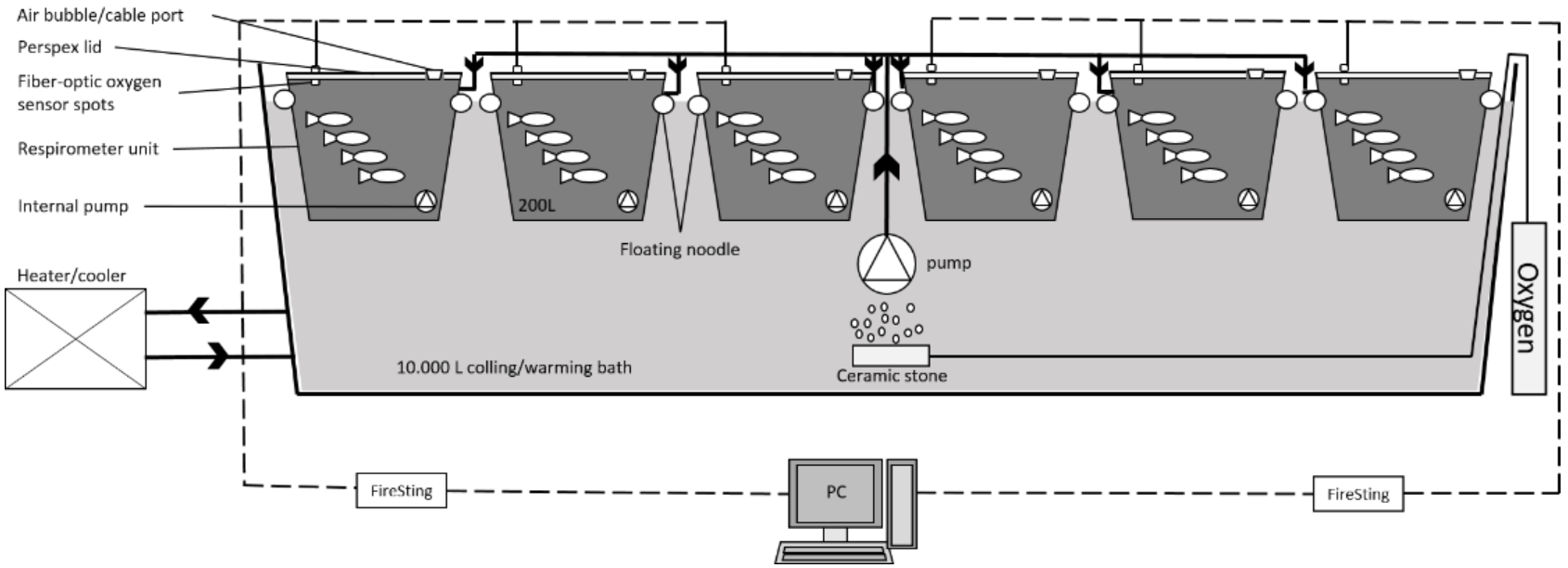


Figure 3.2.2.3.1. Closed static set-up for hypoxia tolerance measurements in Yellowtail Kingfish. One large tank (light grey) housed six respirometer units (dark grey). Respirometer units held four fish and were sealed with clear Perspex lids. Within each respirometer a small submersible pump circulated water. On the inside of each Perspex lid a fibre-optic oxygen sensor-spot was attached and connected to the FireSting unit. Water temperature was maintained via heating/chilling unit. Additionally, water was treated with industrial oxygen ensuring 100% dissolved oxygen in seawater prior to respirometer.

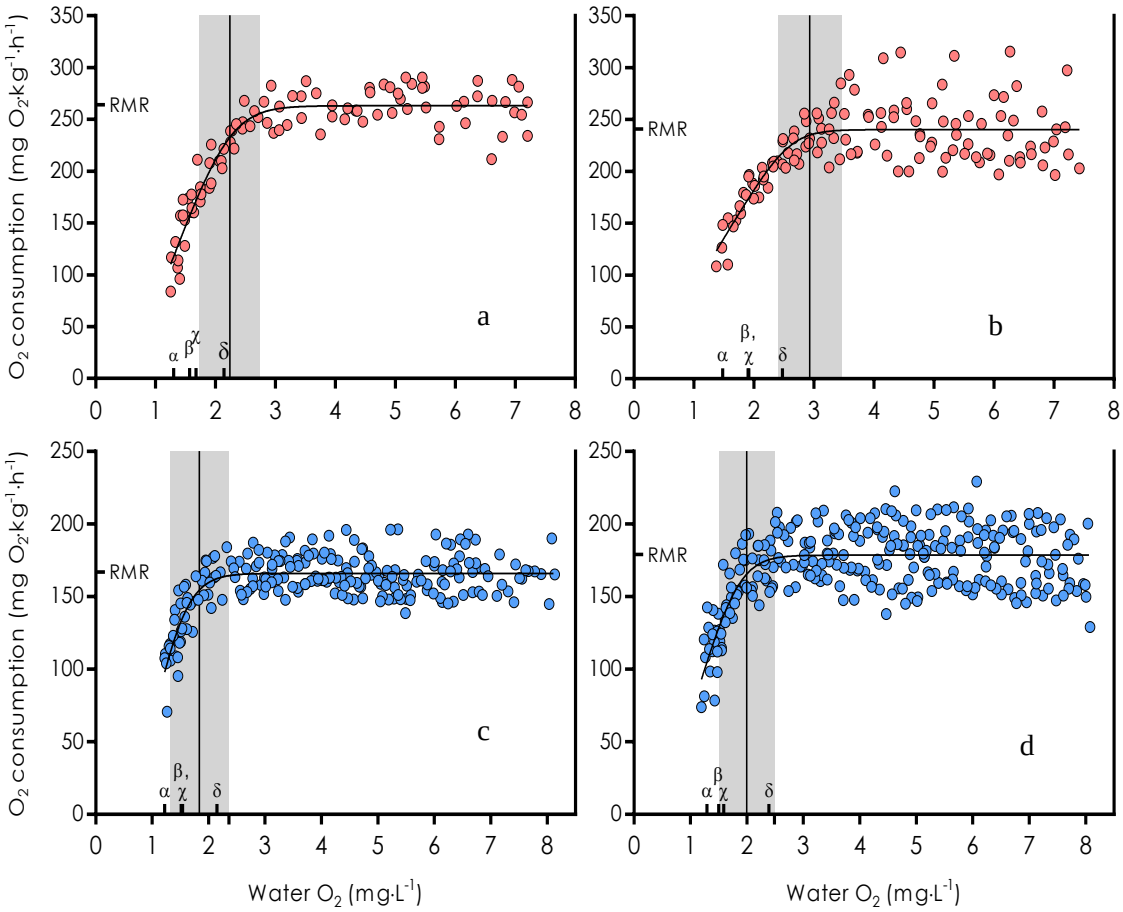


Figure 3.2.2.3.2. Respective $[O2]_{crit}$ plots of Yellowtail Kingfish acclimated to (a) 20 °C and a fish oil based diet, (b) 20°C and a poultry oil based diet, (c) 15 °C and a fish oil based diet, (d)15°C and a poultry oil based diet. Data points indicate the mean mass-specific oxygen consumption rate over a 6 min period. The $[O2]_{crit}$ was assessed via multiphasic linear modelling indicated by the vertical solid line. The grey zone indicates the mean ± 95% confidence interval. Routine metabolic rate (RMR) is indicated by the horizontal regression line. The mean of four different behavioural responses to low dissolved oxygen are indicated as; (α) loss of equilibrium, (β) bursting, (χ) coloration, and (δ) gulping.

3.2.3. Chapter - Optimising feed rations and frequencies for large Yellowtail Kingfish.

3.2.3.1. Manuscript - Optimising feeding strategies for Yellowtail Kingfish (Seriola lalandi) at winter water temperatures.

Matthew S. Bansemer [a], David A.J. Stone [a, b, c ,d], Trent D'Antignana [b,e], Paul Skordas [a], Leigh Kuerschner [b], Krishna-Lee Currie [a, b]

[a] South Australian Research and Development Institute, South Australian Aquatic Sciences Centre, West Beach, SA 5024, Australia

[b] Flinders University, College of Science and Engineering, GPO Box 2100, Adelaide, SA 5001, Australia

[c] University of Adelaide, School of Animal and Veterinary Sciences, Roseworthy, SA 5371, Australia

[d] Marine Innovation Southern Australia

[e] Clean Seas Seafood, PO Box 159, Port Lincoln, SA 5606, Australia

This manuscript may be referenced as: Bansemer, M.S., Stone, A.J., D'Antignana, T.D., Skordas, P., Kuerschner, L. and Currie, K.L. (2019). 3.2.3.1. Manuscript - Optimising feeding strategies for Yellowtail Kingfish (*Seriola lalandi*) at winter water temperatures (FS1; Output 3b). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.346-365.

Abstract

Research on optimising diet formulations and feeding regimes for Yellowtail Kingfish (*Seriola lalandi*; YTK) has primarily focused on warm, optimal water temperatures. Further research is required to determine needs during cool, sub-optimal winter water temperatures. In this 84 day study, the growth performance and feed efficiency of YTK (1.44 kg) fed a commercial formulated diet at seven feed rates, ranging from 0.1% body weight (BW) one day week^{-1} to apparent satiation six days week^{-1}, at cool water temperatures (11.5-16 °C) were investigated. An additional dietary treatment, comprised of feeding thawed and diced Australian Sardines (*Sardinops sagax*; Sardines) to apparent satiation every second day was also tested. YTK fed the commercial formulated diet to apparent satiation six days week^{-1} exhibited significantly higher growth rates and numerically superior FCR than fish fed the same diet at lower feed rates. The maintenance requirement for fish fed the formulated diet was 0.2047% BW d^{-1} (gross energy intake, 39.1 kJ kg fish^{-1}d^{-1} and crude protein intake, 0.92 g kg fish^{-1} d^{-1}). To provide slightly above this rate and ensure growth, fish may be fed to apparent satiation two days week^{-1}. Feeding fish below this maintenance rate during winter, is not recommended, as fish lost weight. Underfeeding may also manifest into nutrient deficiency symptoms and health problems. With regard to YTK fed Sardines, the growth and feed conversion ratio of fish fed Sardines every second day and the formulated diet to apparent satiation six days week^{-1} were similar. However, the fish in-fish out ratio for YTK fed Sardines (7.8) was 50.1% higher than fish fed the formulated diet (5.2), which may impact consumer perception and marketability. We recommend fish are fed the formulated diet to apparent satiation six days week^{-1} during winter. We also recommend further research to validate the current results under commercial conditions.

Introduction

Yellowtail Kingfish (*Seriola lalandi*; YTK) are cultured globally, and are typically produced in sea-cages in Australia, Mexico, Chile and Hawaii, or in indoor facilities in Europe (Premachandra et al., 2017). In Australia, YTK are predominately cultured in South Australia, and are exposed to fluctuating water temperatures that range from 10 °C in winter to 24 °C in summer (Miegel et al., 2010). Water temperature is an important environmental parameter that influences almost every aspect of YTK production (Miegel et al., 2010; Bowyer et al., 2013a; Bowyer et al., 2013b; Bansemer et al., 2015). The optimal water temperature for growth of juvenile YTK (206 g) was reported to be 22.8 °C (Pirozzi and Booth, 2009). Previous studies that aimed to improve diet formulations and feeding regimes, have typically been investigated at water temperatures ≥ 18 °C (Bowyer et al., 2013a; Bowyer et al., 2013b; Bansemer et al., 2015; Stone and Bowyer, 2013; Stone et al., 2016). In contrast, research investigating diet formulations and feeding regimes at sub-optimal winter water temperatures have largely been overlooked.

During winter in South Australia, water temperatures in sea-cages range from 10-14 °C. At these sub-optimal water temperatures, the growth and metabolic rate of YTK is impaired, feed intake reduced, gut transit time prolonged, and the nutritional requirements and digestive physiology differ to fish at summer water temperatures (> 18 °C) (Pirozzi and Booth, 2009; Miegel et al., 2010; Bowyer et al., 2013a; Bowyer et al., 2013b; Bansemer et al., 2015). For example, Bowyer et al. (2013a) reported the feed intake for YTK (22.6 g) at 22 °C was two times greater than at 18 °C, and a 48% higher growth rate was observed in fish at 22 °C than at 18 °C, which are both further impaired as water temperatures reduce (Pirozzi and Booth, 2009; Miegel et al., 2010).

The sustainable and economically viable production of YTK relies on cost effective diets and feeding practices (Stone and Bellgrove, 2013). In the early stages of commercial YTK production in Australia, summer feeding practices involved feeding fish twice daily to apparent satiation (Miegel et al., 2010). Using feeding practices developed for summer water temperatures during winter for YTK may result in overfeeding and inefficient feed utilisation, as the growth, feed intake, gut transit time, nutrient digestibility and numerous other aspects of fish physiology are influenced by water temperature (Jobling, 1994). Recently, there is increased interest to optimise feeding rates and frequencies for aquaculture species, to improve the economical production efficiency (García-Mesa et al., 2014). This has the added benefit of reducing feed wastes and effluent, improved growth rates and feed conversion ratios, and may also reduce fish-size variation at harvest (Güroy et al., 2006). Feed rates and feeding frequencies have been optimised for a closely related *Seriola* sp. the Japanese Yellowtail (*Seriola quinqueradiata*) to maximise economic return (Nakada, 2002). Cultured Japanese Yellowtail are currently fed formulated diets on alternate days, or two to three times a week, at cooler water temperatures (Nakada, 2002). Based on these results, there may also be scope to improve on-farm feeding practices for YTK at winter water temperatures in Australia.

In addition to optimising feed rates and frequencies for YTK using formulated diets, there was also interest to assess YTK growth performance and feed utilisation by feeding Australian Sardines (*Sardinops sagax; Sardines*) during winter. While Sardines may closer resemble the natural diet of YTK, and as a result may provide a superior nutritional profile than commercial formulated diets at times, the nutritional composition of Sardines is dependent on source and season (Nakada, 2008). In contrast, commercial formulated diets are typically formulated and manufactured to optimally and sustainably use marine ingredients (Hardy and Tacon, 2002). Despite these issues, utilising Sardines as feed for YTK may improve growth and feed efficiency at winter water temperatures, which may also improve the sustainable use of marine ingredients (Tacon and Metian, 2008; Tepstra, 2015). Further research in this area was needed.

Aim

The ultimate aim of this research was to better understand winter feed management practices to improve the sustainable production of YTK during winter. More specifically, we aimed to:

(i) Investigate the growth performance and feed efficiency for large YTK (~1.5 kg) fed a commercial production diet at different feed rates and frequencies during low winter water temperatures; and

(ii) Compare the performance of YTK fed the commercial production diet to fish fed a Sardine diet during low winter water temperatures.

Materials and Method

Experimental treatments and feeding techniques

Two diets (biochemical composition Table 3.2.3.1.1 and 3.2.3.1.2) and eight treatment combinations were investigated. A commercial diet formulation (Ridley Clean Seas Pelagica; 30% fish meal; 9% fish oil; referred to as the "formulated diet" herein), was manufactured according to an agreed confidential formulation using a least cost ingredient profile, by Ridley (Narangba, Queensland, Australia) using cooking extrusion technology. Fish fed the formulated diet were fed one of seven feed rates based on % body weight or to apparent satiation, and were adjusted based on monthly weight checks. In addition, freshly frozen Australian Sardines (*S. sagax*) supplied by Sardine Temptations Pty. Ltd. (Port Lincoln, South Australia, Australia) were thawed and diced (~1.5 cm^3) prior to feeding. Fish were fed at 08:30 h. Fish fed to apparent satiation, were fed for four min $tank^{-1}$ to apparent satiation. Fish fed restricted rations were fed until the allocated feed ration was consumed.

Eight treatments were investigated in the current study:

- Treatment 1: Formulated diet fed to apparent satiation six days $week^{-1}$.
- Treatment 2: Formulated diet fed to apparent satiation two days $week^{-1}$ (Mon-Thurs).
- Treatment 3: Formulated diet fed to apparent satiation one day $week^{-1}$ (Mon).
- Treatment 4: Formulated diet fed at 0.1% body weight (BW) one day $week^{-1}$ (Mon).
- Treatment 5: Formulated diet fed at 0.65% BW two days $week^{-1}$ (Mon and Thurs).
- Treatment 6: Formulated diet fed at 0.35% BW two days $week^{-1}$ (Mon and Thurs).
- Treatment 7: Formulated diet fed at 0.12% BW six days $week^{-1}$ (Mon-Sat).
- Treatment 8: Sardines fed to apparent satiation every second day.

Experimental fish and system

Experimental work was conducted in the pool-farm facility at the South Australian Research and Development Institute, South Australian Aquatic Science Centre (SARDI SAASC; West Beach, South Australia, Australia). YTK (mean ± standard deviation; initial weight 1.44 ± 0.13 kg; initial fork length 461.6 ± 15.4 mm; n = 504) were obtained from Clean Seas Seafood (Arno Bay, South Australia, Australia). Upon arrival at SARDI SAASC (25/5/15), YTK were transferred to 5000 L tanks supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated, aerated and oxygenated sea water at ambient temperature and held for ~4 weeks and fed the formulated diet.

Experimental stocking and weight checks

At the commencement of the study (June 2015), YTK were removed from their tank, anaesthetised using AQUI-S® (AQUI-S® New Zealand Ltd., Lower Hutt, New Zealand) at a concentration of 14 mg L^{-1} of seawater. Twenty one fish were measured, weighed and stocked into one of the three replicate 5000 L tanks $treatment\ combination^{-1}$ (n = 24 tanks).

Tanks were supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated sea water at ambient temperature. All tanks were supplied with aeration and oxygenation throughout the study.

At day 28 and day 56, all fish were anaesthetised using AQUI-S® at a concentration of 14 mg L^{-1} of seawater, weighed and measured, and returned back to their respective tanks.

Water quality analyses

Water quality parameters were measured daily at 14:30 h and maintained at appropriate levels for acceptable growth of YTK throughout the study (Table 3.2.3.1.3). Water temperature was measured using a thermometer. Dissolved oxygen (mg L^{-1} and % saturation) was measured using a dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark). The pH was measured daily using a meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, Illinois, United States of America). Salinity (g L^{-1}) was measured weekly using a portable salinity refractometer (model RF20, Extech Instruments, Nashua, New Hampshire, United States of America).

Final harvest sampling

At day 84 (September 2015) fish were anaesthetised using AQUI-S® (14 mg L^{-1} of seawater). All YTK were weighed and measured. Four fish from each tank were collected and stored at -20 °C for subsequent whole body composition analyses. Twelve separate fish from treatment 1, 4, 7 and 8 (n = 3 fish $tank^{-1}$) were sampled for blood haematocrit, and visceral and liver weight in order to calculate visceral index (VSI; %) and hepatosomatic index (HSI; %). These treatments were selected to give a representation of feed rate and frequency restrictions investigated in the current study.

Apparent digestibility coefficients and nutrient digestion

At the conclusion of the growth and feed utilisation experiment, a digestibility experiment was carried out for fish in Treatment 1 and 8 to compare the digestibility of the formulated diet and Sardine diet. After fish were weighed and measured, fish (n = 14 $tank^{-1}$) were returned to their respective tank, and fed their respective diet daily to apparent satiation for six days. After six days, fish were anaesthetised using AQUI-S® at a concentration of 20 mg L^{-1} of seawater, manually stripped and the faecal matter was collected. In brief, manual stripping involved placing the forefinger and thumb on either side of the fish abdomen at the pelvic fin. Moderate pressure was applied by the forefinger and thumb, and at the same time moved towards the anus, this process was repeated three times. Uncontaminated faecal samples (free from blood, urine and mucus) were collected in a 250 mL container and frozen at -20 °C until subsequent analysis. Fish were then revived in their respective tank, and fed their respective diet daily to apparent satiation for a further five days. Fish were manually stripped again after five days to ensure adequate faecal samples were collected.

Biochemical analyses

Diet, and whole initial and final fish (n = 4 fish $tank^{-1}$) were analysed for proximate composition (moisture, protein, lipid, ash, total carbohydrate and energy), taurine and choline, fatty acids and minerals by Asure Quality Laboratories (Auckland, New Zealand). Diets were also analysed for cholesterol and amino acids.

Calculation of performance indices

All data reported for each treatment for animal performance were based on the mean of the three replicate tanks. All calculations using fish weight and diets were based on wet or as fed values:

- Weight gain (g $fish^{-1}$) = final individual weight - initial individual weight
- Biomass gain (g $tank^{-1}$) = final tank weight - initial tank weight
- Specific growth rate (SGR, % d^{-1}) = ([ln final weight - ln initial weight] / days) × 100
- Condition factor = (fish weight [g] / fish length $[cm]^3$) × 100
- Apparent feed conversion ratio (FCR) = feed consumed / fish weight gain
- Apparent protein deposition (%) = ([final soft body protein - initial soft body protein] / protein intake) × 100

- Apparent energy deposition (%) = ([final soft body energy - initial soft body energy] / energy intake) × 100
- Haematocrit count = red blood cell (mm) / total blood (red blood cell and plasma [mm]) × 100
- Visceral index (VSI; %) = wet visceral wt × 100 / final wet fish wt
- Hepatosomatic index (HSI; %) = wet liver wt × 100 / final wet fish wt
- Fish in-fish out ratio (FI-FO) = FCR × 0.75 × 0.5 × [(% fish meal in feed / 22.5) + ((% fish oil in feed - 0.08 × % fish meal in feed) / 5)]

  Where the FI-FO ratio is expressed in reduction fish equivalent and FCR is the feed conversion ratio (kg feed kg-1 fish). The yield of reduction fish is 22.5 % WD fish meal and 5 % fish oil. The factor 0.75 takes into account that about 25% of the WD fishmeal and fish oil is nowadays produced from fish processing by-products, and the factor 0.08 takes into account that WD fish meal contains ~8 % fish oil (Terpstra, 2015).

The apparent digestibility coefficients (ADC) for dietary dry matter, protein and energy were calculated using the following equation and methods described by Maynard and Loosli (1969) and Miegel et al. (2010):

ADC (%) = 100 - (100 × [$\%M_{feed}$ / $\%M_{faeces}$] × [$\%N_{faeces}$ / $\%N_{feed}$])

Where M refers to inert marker (acid insoluble ash [AIA]), and N refers to the nutrient of interest.

Statistical analyses

IBM SPSS, Version 22 for Windows (IBM SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Homogeneity of variances and normality among mean values were assessed using Levene's test and Shapiro-Wilk test, respectively. Difference between treatments for all variables were analysed using one-factor ANOVA. When significant differences were observed, post-hoc tests were used to detect significant differences between treatments (Student-Newman-Keuls). In addition, Pearson's correlation coefficient was used to determine the relationship between feed intake (% BW d^{-1}) and specific growth rate (% d^{-1}) for fish fed the formulated diet. A significance level of $P < 0.05$ was used for all statistical tests. All values are presented as means ± standard error (SE) of the mean unless otherwise stated.

Results

General observations

YTK fed actively during the experiment. Apparent feeding activity increased dramatically as feed rate and frequency decreased. There were no mortalities during the study, and no apparent signs of disease observed.

Growth performance

The initial weight and fork length of YTK were not statistically different between treatments ($P > 0.05$; one-factor ANOVA; Table 3.2.3.1.4). While dietary treatment had no significant effect on the final fork length of fish ($P > 0.05$), other growth performance parameters significantly differed between treatments ($P < 0.05$; one-factor ANOVA; Table 3.2.3.1.4). These growth performance parameters were inclusive of final weight, biomass gain, SGR, length growth rate and final condition factor. In general, fish fed the formulated diet to apparent satiation six days $week^{-1}$ (Treatment 1) and Sardines fed to apparent satiation every second day (Treatment 8) outperformed other dietary treatments. The next best performing treatment was fish fed the formulated diet to apparent satiation two days $week^{-1}$ (Treatment 2). In contrast, fish fed the formulated diet at feed rates below this were not provided with enough feed

to maintain weight (Figure 3.2.3.1.1). Generally, the growth performance of fish fed the formulated diet at 0.1% BW one day week^{-1} (Treatment 4) was inferior to all other treatments investigated (Table 3.2.3.1.4).

In addition, there was a significant positive linear correlation between feed intake (% BW d^{-1}) and SGR (% d^{-1}) for YTK fed the formulated ($P < 0.001$; Pearson's correlation coefficient; $y = 66.051x - 0.1352$, $R^2 = 0.961$, Figure 3.2.3.1.1). The x-intercept (SGR = 0% d^{-1}) was 0.2047% BW d^{-1}. At this point, the gross energy intake was 39.1 kJ kg fish^{-1}d^{-1} and crude protein intake was 0.92 g kg fish^{-1} d^{-1}.

Feed utilisation

Feed consumption rates of YTK were significantly affected by treatment ($P < 0.05$; one-factor ANOVA; Table 3.2.3.1.4). For fish fed the formulated diet, the feed consumption rates of fish were generally related to the treatment feed rates. For example, fish fed to apparent satiation six days week^{-1} had the highest feed consumption rate, while those fed 0.1% BW one day week^{-1} had the lowest. Fish fed Sardines to apparent satiation every second day had significantly higher feed consumption rates compared to fish fed the formulated diet at any feed rate ($P < 0.05$; Table 3.2.3.1.4).

Apparent FCR for YTK was significantly influenced by treatment ($P < 0.05$; one-factor ANOVA; Table 3.2.3.1.4). Fish fed the formulated diet to apparent satiation two or six days week^{-1} (Treatment 2 and 1, respectively) and Sardines to apparent satiation every second day (Treatment 8) exhibited positive FCR. The FCR of fish fed Treatment 1 was significantly superior than those fed to apparent satiation one day week^{-1} (Treatment 3) and 0.65% BW two days week^{-1} (Treatment 5; $P < 0.05$) and numerically superior to other treatments. The FCR for fish in Treatments 3, 4, 5, 6 and 7 were negative, due to the inherent weight loss of fish in these treatments.

Whole fish proximate and energy composition

Lipid and energy content of YTK in Treatment 1, 2 and 8 were significantly higher than Treatment 7 ($P < 0.05$; one-factor ANOVA; Table 3.2.3.1.4). There were no significant differences in lipid and energy content between all other treatments ($P > 0.05$). Furthermore, the tissue taurine level of fish in Treatment 1 and 4 were significantly higher than Treatment 8 ($P < 0.05$; One-factor ANOVA; Table 3.2.3.1.4). Taurine levels were not significantly different between other treatments investigated ($P > 0.05$). Tissue moisture (65.4-69.2%), protein (19.65-20.78% wet), ash (2.3-2.8% wet) and carbohydrate (< 1.5% wet) content of fish were not significantly influenced by treatment ($P > 0.05$; one-factor ANOVA; Table 3.2.3.1.4).

Nutrient utilisation

Apparent protein deposition for YTK was significantly affected by treatment ($P < 0.05$; one-factor ANOVA; Table 3.2.3.1.4). The protein deposition of fish in Treatment 4 was significantly lower than other treatments investigated ($P < 0.05$), which were not significantly different from each other ($P > 0.05$).

Apparent energy deposition was also significantly affected by treatment ($P < 0.05$; one-factor ANOVA; Table 3.2.3.1.4). Apparent energy deposition was significantly higher for fish in Treatment 1, 2 and 8, and significantly lower for fish in Treatment 4 ($P > 0.05$; one-factor ANOVA; Table 3.2.3.1.4). However, fish in Treatment 3 and 6 exhibited statistically similar apparent energy depositions to those in Treatment 1, 2, 5 and 7 ($P > 0.05$).

Whole fish fatty acid composition

There were numerous differences in fatty acid levels of fish between treatments ($P < 0.05$; one-factor ANOVA; Table 3.2.3.1.5). In general, the fatty acid level of YTK in Treatments 1, 2 and 8 were significantly higher than those in Treatment 7 ($P < 0.05$), while the fatty acid level of fish in other

treatments were similar ($P > 0.05$). These levels are consistent with the differences observed in lipid levels between treatments. The long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA; eicosapentaenoic acid [EPA, 20:5n3], docosapentaenoic acid [DPA, 22:5n3], docosahexaenoic acid [DHA, 22:6n3]) were generally significantly higher in YTK fed Treatment 1 and 8 than fish fed Treatment 7.

Whole fish mineral composition

The tissue mineral levels (calcium, copper, iodine, iron, magnesium, manganese, potassium, phosphorus, selenium, zinc) of YTK were not significantly influenced by treatment ($P > 0.05$; one-factor ANOVA, Table 3.2.3.1.6).

Blood haematocrit, gastrointestinal indices and apparent digestibility coefficients

For the four treatments sampled (Treatments 1, 4, 7 and 8), blood haematocrit of YTK were not significantly different between treatments, and ranged from 39.6 to 46.0% ($P > 0.05$; one-factor ANOVA; Table 3.2.3.1.6). For the same four treatments, visceral index (VSI; %) and hepatosomatic index (HSI; %) of YTK were treatment significantly influenced by treatment ($P < 0.05$; one-factor ANOVA; Table 3.2.3.1.7). With regard to VSI, fish fed Treatment 1 and 8 had significantly higher VSI to those fed Treatment 7 ($P < 0.05$), while the VSI of those fed Treatment 4 was statistically similar to Treatment 1, 7 and 8 ($P > 0.05$). In addition, HSI was significantly higher in fish fed Treatment 1 and 8, compared to those fed Treatment 4 and 7 ($P < 0.05$; one-factor ANOVA; Table 3.2.3.1.7).

With regard to the apparent digestibility coefficient (ADC, %), YTK fed the Sardine diet exhibited significantly higher ADC (%) for dietary dry matter, protein and energy, compared to fish fed the formulated diet ($P < 0.05$; one-factor ANOVA; Table 3.2.3.1.6).

Discussion

Our overall aim in the current study was to improve our understanding of feed management practices to improve the sustainable production of YTK at cool water temperatures during winter. To achieve this aim, YTK were fed a formulated diet at different ration levels and feeding frequencies, which ranged from 0.1% BW one day week^{-1} to apparent satiation six days week^{-1}. YTK in the current study exhibited growth rates comparable to, or slightly better than, those observed at Clean Seas Seafood commercial sea-cage facilities during the same period (Dr T. D'Antignana; Clean Seas Seafood, Port Lincoln, South Australia, Australia; *personal communication*). YTK fed to apparent satiation six days week^{-1} (Treatment 1) exhibited significantly higher growth rates and numerically superior FCR, compared to fish fed the formulated diet at all other feed ration levels and feeding frequencies. Fish fed Treatment 1 had greater access to feed, and as a result consumed more feed and nutrients than those fed other feeding regimes. Results from the current study are not surprising as dietary protein and energy are the first growth-limiting factors for fish growth (Webster and Lim, 2002).

The growth rate of YTK is temperature-dependent, and is markedly reduced during winter compared to summer (Pirrozi and Booth, 2009; Bowyer et al., 2013a; Bowyer et al., 2013b). As YTK exhibited depressed growth rates during winter compared to summer, it may be beneficial to feed marginally above the maintenance ration through winter to improve production and avoid potential nutritional deficiency health problems. Based on results from the current study, YTK (1.44 kg) fed the formulated diet required a maintenance feed rate of 0.2047% BW d^{-1} to maintain weight at an average water temperature of 12.8 °C. In order to feed just above the maintenance ration, YTK may be fed the formulated diet to apparent satiation two days week^{-1} or at other feed rates not investigated in the current study.

Based on the maintenance feed rate of 0.2047% BW d^{-1}, the initial weight of YTK (1.44 kg) and the dietary gross energy and crude protein level of the formulated diet (19.1 MJ kg^{-1} and 45.1%, respectively), the gross energy and crude protein maintenance requirements are 39.1 kJ kg fish^{-1}d^{-1} and 0.92 g kg fish^{-1} d^{-1}, respectively at 12.8 °C. Based on the apparent energy (77.0%) and protein (89.3%) digestibility co-efficient for the formulated diet fed to apparent satiation six days week^{-1}, the estimated

digestible energy and protein maintenance requirements are 30.1 kJ kg fish^{-1}d^{-1} and 0.82 g kg fish^{-1} d^{-1}, respectively at 12.8 °C. Pirrozi and Booth (2009) modelled the routine metabolic rate (RMR), based on the oxygen consumption (MO_2), of smaller juvenile YTK (206 g) at 10, 15, 20, 25, 30 or 32.5 °C. Oxygen consumption was reported to be an accurate representation of metabolic rate (Withers, 1992). Pirrozi and Booth (2009) also used mass-specific data, which was scaled using the metabolic body mass exponent of 0.8 reported by Brett and Groves (1979). Based on this research, Pirrozi and Booth (2009) reported a temperature-dependant (T) routine metabolic rate, of these smaller YTK (206 g), as 4.04T-13.14 kJ kg$^{-0.8}$ d^{-1}. Using the average water temperature (12.8 °C) and initial larger fish weight (1.44 kg) in the current study, and the model developed by Pirrozi and Booth (2009), the RMR of YTK was 41.9 kJ d^{-1}. The RMR for YTK reported by Pirrozi and Booth (2009) is 28% higher than results from the current study (30.1 kJ d^{-1}). This result highlights the importance further investigations focused on understanding the specific RMR for YTK under a number of interactive conditions, including weight and water temperature.

There are a number of important factors that need to be considered, before feeding a maintenance ration to YTK during periods of slow growth at sub-optimal water temperatures. Firstly, it is important that the weight and condition of YTK is not compromised at harvest and that fish are supplied with adequate levels of essential nutrients. Previous studies have reported that some fish species, including Channel Catfish (*Ictalurus punctatus*), Red Porgy (*Pagrus pagrus*), Barramundi (*Lates calcarifer*), and Rainbow Trout (*Oncorhynchus mykiss*) fed a restricted feed ration or fasted, exhibited compensatory growth once re-fed to apparent satiation (Kim and Lovell, 1995; Rueda et al., 1998; Nikki et al., 2004; Tian and Qin, 2004). For example, Barramundi at 28 °C exhibited compensatory growth when fed to 50% and 75% satiation for two weeks then re-fed to apparent satiation, compared to the control fed to apparent satiation (Tian and Qin, 2004). Specifically, Barramundi fed to 50% and 75% satiation caught up to the weight of control fish after two and four weeks, respectively. Fish fed to 0% and 25% satiation for two weeks however, did not catch up to control fish weight after five weeks (Tian and Qin, 2004). Although compensatory growth of some fish species during short durations of restricted feed ration is understood, this phenomenon is not reported in the scientific literature for YTK. It may be beneficial in future studies to investigate compensatory growth in YTK, particularly with regard to fish fed a maintenance ration throughout periods of cooler sub-optimal water temperatures, and then switched to apparent satiation feeding daily during of warmer water temperatures during spring and summer.

The second aim of the current study was to compare the performance of YTK fed a formulated diet or a Sardine diet. The growth and FCR of YTK fed the formulated diet to apparent satiation six days week^{-1} (Treatment 1) and fish fed Sardines every second day (Treatment 8) were similar. There were also no significant differences in apparent protein and energy deposition between fish fed Treatment 1 and 8. However, YTK fed Treatment 8 exhibited numerically higher (52%) apparent protein deposition (11.84 and 17.98%, respectively), numerically higher (44%) apparent energy deposition (34.66 and 49.89%, respectively), and superior digestibility than fish fed the formulated diet to apparent satiation six days week^{-1}. It should be noted however, that differences may have become apparent if fish were fed their respective diets at the same feeding regime. Despite numerical improvements in growth and nutrient utilisation, and a feed strategy that may be economical, feeding Sardines to YTK does not represent a sustainably viable option for the production of YTK, and may limit market access. A common measure for the sustainable use of marine ingredients is the fish in-fish out (FI-FO) ratio (Tacon and Metian, 2008; Jackson, 2009; Tepstra, 2015). In the current study, based on the fish meal (30%) and fish oil (9%) content of the formulated diet, and an FCR of 4.43 for fish fed to apparent satiation six days week^{-1}, the FI-FO ratio was 5.2. In contrast, the FI-FO ratio for YTK fed Sardines every second day was 7.8, which was 50.1% higher than those fed the formulated diet. The FI-FO ratio for YTK fed Sardines in this case was based on the reduction fish equivalent component value of the calculation being equal to one, fish were fed Sardines on an as-fed fresh basis and an FCR of 7.8 (Tepstra, 2015). As the aquaculture industry is tending to reduce the use of high fish meal/fish oil diets due to economic and sustainability issues, further consideration to sustainability and customer perception are needed before YTK are fed Sardines under commercial conditions (Hardy and Tacon, 2002).

Conclusions and Recommendations

In conclusion, if commercial producers of YTK aim to capitalise on limited fish growth during periods of cooler sub-optimal water temperatures, it is recommended that fish (~1.5 kg) are fed a formulated diet to apparent satiation six days week^{-1}. In contrast, if the primary aim of farms is to reduce feed and feeding costs, and maintain fish weight, YTK require a maintenance ration of 0.2047% BW d^{-1}·(provided an estimated energy maintenance ration of~ 56.3 kJ fish^{-1} d^{-1}), which was achieved, albeit slightly to excess, by feeding fish the formulated diet to apparent satiation two days week^{-1}. If YTK are fed a maintenance ration, attention to the essential dietary nutrients levels are needed. We do not recommend feeding below this maintenance rate as fish lost weight during the study. With regard to feeding Sardines, the growth performance of YTK fed Sardines every second day was similar to fish fed the formulated diet to apparent satiation six days week^{-1}. However, YTK fed Sardines had a FI-FO ratio that was 50.1% higher than those fed the formulated diet. The higher FI-FO associated with feeding Sardines may have a negative impact on consumer perception, with regard to sustainably, and may present market access problems. The use of Sardines is a decision to be made by YTK producers.

Findings

- The current study provides a much clearer understanding of the optimal feed rates and frequencies for large adult YTK throughout periods of cool winter water temperatures.
- Feeding YTK to apparent satiation two days week^{-1} provided slightly above the maintenance feed rate of 0.2047% BW d^{-1} (provided an estimated energy maintenance ration of~ 56.3 kJ fish^{-1} d^{-1}), which appeared to be the minimum required ration to maintain positive growth. However, fish fed to apparent satiation six days week^{-1} exhibited superior growth and FCRs than fish fed lower feed rates or frequencies.
- An improvement in FCR by careful feed management during winter, based on the information provided within this Manuscript, will assist producers in achieving one of the overarching goals of the K4P project, which was to provide information to assist producers to achieve FCRs of < 2.2 for large YTK between 1.5-3.5 kg.
- As a result of this research, an Australian YTK producer adopted new winter feeding strategies and suggested immediate savings of ~$350,000 each winter (annum) would be achieved with the production of 2,000 tonnes of YTK (Dr C. Foster; former CEO, Clean Seas Seafood; *personal communication*). When this practice is applied to a future targeted production level of 10,000 tonnes of YTK per annum, a saving of $1,750,000 per annum would be achieved.
- With regard to feeding Sardines to large YTK every second day during winter, although fish performed well, they exhibited an inferior fish in fish out ratio (50.1% lower) compared to fish fed the Ridley Pelagica diet to apparent satiation six days per week. Feeding Sardines may not align with the sustainable marketing image Australian YTK producers so proudly wish to promote.
- Further refinements of feeding frequency and feeding rates when fed commercial production diets may further optimise winter feeding strategies.

Publications

To date, one publication has arisen from this Manuscript: Matthew S. Bansemer, David A.J. Stone, Trent D'Antignana, Paul Skordas, Leigh Kuerschner and Krishna-Lee Currie, 2018. Optimising feeding strategies for Yellowtail Jack at winter water temperatures. North American Journal of Aquaculture, 80, 128-140.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The research in this manuscript was also supported by the Australian Seafood Cooperative Research Centre (AS-CRC) and forms part of the Refining YTK feeds and feed management project (AS-CRC, 2013/730). The authors would also like to acknowledge the support of the South Australian Research and Development Institute (SARDI) and Marine Innovation Southern Australia for their financial support of Assoc. Prof. David Stone and the provision of the SARDI South Australian Aquatic Sciences experimental facilities at West Beach, South Australia. We would also like to thank Dr Richard Smullen and Dr Simon Tabrett of Ridley for their input into experimental design, experimental diet formulation and manufacture and Dr Matthew Bransden of Skretting Australia for his input into experimental design. Thanks to Dr Craig Foster and Chester Wilkes of Clean Seas Seafood for their input into experimental design. We also thank Assoc. Prof. James Harris, Wayne Hutchinson, Dr Duong Ngoc Duong, Jessica Buss, Nicole Thompson, Samantha Chown and Aaron Teoh for their technical assistance during the experiment.

References

Bansemer, M.S., Forder, R.E.A., Howarth, G.S., Suitor, G.M., Bowyer, J.N., Stone, D.A.J., 2015. The effect of dietary soybean meal and soy protein concentrate on the intestinal mucus layer and the development of sub-acute enteritis in Yellowtail Kingfish (*Seriola lalandi*) at suboptimal water temperature. Aquaculture Nutrition 21, 300-310.

Bowyer, J.N., Qin, J.G., Smullen, R.P., Adams, L.R., Stone, D.A.J., 2013a. The use of a soy product in Yellowtail Kingfish (*Seriola lalandi*) feeds at different water temperatures: 1. Solvent extracted soybean meal. Aquaculture 384-387, 35-45.

Bowyer, J.N., Qin, J.G., Smullen, R.P., Adams, L.R., Stone, D.A.J., 2013b. The use of a soy product in Yellowtail Kingfish (*Seriola lalandi*) feeds at different water temperatures: 2. Soy protein concentrate. Aquaculture 410-411, 1-10.

Brett, J. R., Groves, T.D.D., 1979. Physiological energetics. Fish Physiology. VIII, 279-344.

García-Mesa, S., Suárez, M.D., Rodríguez-Rúa, A., Cárdenas, S., García-Gallego, M., 2014. Productive and physiological implications of different feeding frequencies in meagre *Argyrosomus regius* (Asso, 1801). Aquaculture Engineering 60, 6-13.

Güroy, D., Deveciler, E., KutGüroy, B., Tekinay, A. A., 2006. Influence of feeding frequency on feed intake, growth performance and nutrient utilization in European sea bass (*Dicentrarchus labrax*) fed pelleted or extruded diets. Turkish Journal of Veterinary and Animal Sciences 30, 171-177.

Hardy, R.W., Tacon, A.G.J., 2002. Fish meal: historical uses, production trends and future outlook for sustainable supplies. In: Stickney RR, McVey JP (eds). Responsible Marine Aquaculture, pp. 311-325. CAB International, Wallingford, UK.

Hulbert, A.J., Else, P.L., 1999. Membranes as possible pacemakers of metabolism. Journal of Theoretical Biology 199, 357-374.

Jackson, A., 2009. Fish in-fish out (FIFO) ratios explained. Aquaculture Europe 34, 5-10.

Jobling, M., 1994. Fish Bioenergetics, Chapman & Hall, London, UK.

Kim, M.K. Lovell, R.T., 1995. Effect of restricted feeding regimens on compensatory weight gain and body tissue changes in channel catfish *Ictalurus punctatus* in ponds. Aquaculture 135, 285-293.

Maynard, L.A., Loosli, J.K., 1969. Animal Nutrition 6th Edn. McGraw-Hill, New York. St. Louis, San Francisco, London, Sydney, Toronto, Mexico, Panama, 613 pp.

Miegel, R.P., Pain, S.J., Van Wettere, W.H.E.J., Howarth, G.S., Stone, D.A.J., 2010. Effect of water temperature on gut transit time, digestive enzyme activity and nutrient digestibility in yellowtail kingfish (*Seriola lalandi*). Aquaculture 308, 145-151.

Nakada, M., 2002. Yellowtail culture development and solutions for the future. Reviews in Fisheries Science 10, 559-575.

Nakada, M., 2008. Capture-based aquaculture of yellowtail. In A. Lovatelli; P.F. Holthus (eds). Capture-based aquaculture. Global overview. FAO Fisheries Technical Paper. No. 508. Rome, FAO. pp. 199-215.

Nikki, J., Pirhonen, J., Jobling, M., Karjalainen, J. 2004. Compensatory growth in juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum), held individually. Aquaculture 235, 285-296.

Pirozzi, I., Booth, M.A., 2009. The routine metabolic rate of mulloway (*Argyrosomus japonicus*: Sciaenidae) and yellowtail kingfish (*Seriola lalandi*: Carangidae) acclimated to six different temperatures. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology 152, 586-592.

Premachandra, H.K.A., Nguyen, N.H., Miller, A., D'Antignana, T., Knibb, W., 2017. Genetic parameter estimates for growth and non-growth traits and comparison of growth performance in sea cages vs land tanks for yellowtail kingfish *Seriola lalandi.* Aquaculture 479, 169-175.

Rueda, F.M., Martinez, F.J., Zamora, S., Kentouri, M., Divanach, P., 1998. Effect of fasting and refeeding on growth and body composition of red porgy, *Pagrus pagrus* L. Aquaculture Research 2, 447-452.

Stone, D. A. J. and Bowyer, J. N. 2013. Final Report. Sustainable Feeds and Feed Management for Yellowtail Kingfish (*Seriola lalandi*). South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2013/000200-1. SARDI Research Report Series No. 751.

Stone, D.A.J. and Bellgrove, E., 2013. A literature review: the current status of knowledge of the nutritional requirements of yellowtail kingfish. In: Stone, D. A. J. and Bowyer, J. N. (Eds). Final Report. Sustainable Feeds and Feed Management for Yellowtail Kingfish (*Seriola lalandi*). South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2013/000200-1. SARDI Research Report Series No. 751. pp. 92-121.

Stone, D.A.J., D'Antignana, T., Bansemer, M.S., 2016. Refining Yellowtail Kingfish feeds and feed management. South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication. CRC Project No. 2013/730.

Tacon, A.G.J., Metian, M., 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. Aquaculture 285, 146-158.

Terpstra, A.H.M., 2015. The use of fish meal and fish oil in aquaculture and calculation of the fish-in-fish-out (FIFO) ratio. PhD. Universitate Vadensi, The Netherlands. pp.19.

Tian, X., Qin, J.G., 2004. Effects of previous ration restriction on compensatory growth in barramundi *Lates calcarifer*. Aquaculture 235, 273-283.

Webster, C.D., Lim, C. 2002. Nutrient Requirements and Feeding of Finfish for Aquaculture. CABI Publishing, USA.

Withers, P.C. 1992. Comparative Animal Physiology. Brooks/Cole Thompson Learning, Pacific Grove, California, USA.

Table 3.2.3.1.1. The proximate, mineral and amino acid composition of the formulated diet and Sardine.

	As fed		Dry basis	
Item[1,]	Formulated diet	Sardines	Formulated diet	Sardines
Analysed proximate composition (g kg^{-1})				
Moisture	68	721	0	0
Crude protein	451	189	484	677
Crude lipid	240	41	258	147
Ash	89	45	95	161
Carbohydrate[2]	152	4	163	14
Gross energy (MJ kg^{-1})	19.10	4.73	20.49	16.95
Crude protein:energy (g MJ^{-1})	23.6	40.0	25.3	40.0
Cholesterol	2.3	1.1	2.5	3.9
Analysed minerals (mg kg^{-1})				
Calcium	24000	6700	25751	24014
Copper	8.7	6.3	9.3	22.6
Iodide (Potassium Iodide) (µg kg^{-1})	1.8	0.69	1.9	2.5
Iron	290	34	311	122
Magnesium	1900	550	2039	1971
Phosphorus	16000	5900	17167	21147
Potassium	4900	3600	5258	12903
Selenium	2.4	1.5	2.6	5.4
Zinc	150	31	161	111
Analysed amino acids (g kg^{-1})				
Alanine	24.40	10.60	26	38
Arginine	26.61	10.68	29	38
Aspartic acid	32.76	14.58	35	52
Glutamic acid	69.08	25.10	74	90
Glycine	26.46	10.78	28	39
Histidine	13.08	9.34	14	33
Isoleucine	16.94	8.27	18	30
Leucine	33.57	14.42	36	52
Lysine	23.87	15.52	26	56
Methionine	10.21	4.31	11	15
Phenylalanine	19.14	7.60	21	27
Proline	28.08	7.34	30	26
Serine	17.65	6.91	19	25
Threonine	17.19	8.16	18	29
Tyrosine	14.15	6.00	15	22
Valine	21.95	9.25	24	33
Total amino acids	395	16.89	424	605

[1] The formulated diet (Ridley Clean Seas Pelagica) was supplied by Ridley (Narangba, Queensland, Australia); Australian Sardines (*Sardinops sagax*; Sardines) were provided by Sardine Temptations Pty. Ltd. (Port Lincoln, South Australia, Australia).
[2] Carbohydrate = 1000 - (moisture + lipid + protein + ash).

Table 3.2.3.1.2. The fatty acid composition of the formulated diet and Sardines.

	As fed		Dry basis	
Item[1,]	Formulated diet	Sardines	Formulated diet	Sardines
Analysed fatty acids (mg 100 g^{-1})				
C4:0 Butyric	<10	<10	<10	<10
C6:0 Caproic	<10	<10	<10	<10
C8:0 Caprylic	<10	<10	<10	<10
C10:0 Capric	<10	<10	<10	<10
C12:0 Lauric	21	<10	23	<10
C13:0 Trisdecanoic	<10	<10	<10	<10
C14:0 Myristic	950	250	1019	896
C15:0 Pentadecanoic	88	56	94	201
C16:0 Palmitic	4980	850	5343	3047
C17:0 Margaric	46	52	49	186
C18:0 Stearic	1310	230	1406	824
C20:0 Arachidic	68	17	73	61
C21:0 Heneicosanoic	<10	<10	<10	<10
C22:0 Docosanoic	31	13	33	47
C24:0 Tetracosanoic	26	<10	28	<10
C10:1 Decenoic	<10	<10	<10	<10
C14:1 Myristoleic	56	11	60	39
C15:1 Pentadecenoic	13	<10	14	<10
C16:1 Palmitoleic	1490	130	1599	466
C17:1 Heptadecenoic	50	<10	54	<10
C18:1n-6 Octadecenoic	20	<10	21	<10
C18:1n-7 Octadecenoic	640	84	687	301
C18:1n-9 Oleic	6430	220	6899	789
C20:1n-9 Eicosenoic	220	12	236	43
C20:1n-11,13 Eicosenoic	30	<10	32	<10
C20:1 (total) Eicosenoic	250	18	268	65
C22:1n-9 Erucic	30	<10	32	<10
C22:1n-11,13 Docosenoic	<10	<10	<10	<10
C24:1 Tetracosenoic	47	32	50	115
C18:2n-6 Linoleic	2220	100	2382	358
C18:2 Conjugated 9c 11t Octadecadienoic	<10	<10	<10	<10
C18:3n-3 Alpha Linolenic	350	80	376	287
C18:4n-3 Steridonic	200	110	215	394
C18:3n-4Octadectrenoic acid	26	<10	28	<10
C18:3n-6 Gamma Linolenic	42	<10	45	<10
C20:2n-6 Eicosadienoic	31	14	33	50
C20:3n-6 Dihomo-gamma-linoleic	34	<10	36	<10
C20:4n-6 Arachidonic	210	47	225	168
C22:4n-6 Docosatetraenoic	54	37	58	133
C22:5n-6 Docosapentaenoic	59	11	63	39
C20:3n-3 Eicosatrienoic	13	<10	14	<10
C20:4n-3 Eicosatetraenoic	180	28	193	100
C20:5n-3 Eicosapentanaeoic	1480	360	1588	1290
C21:5n-3 Heneicosapentaenoic	<10	<10	<10	<10
C22:5n-3 Docosapentaenoic	200	45	215	161
C22:6n-3 Docosahexaenoic	1170	1020	1255	3656
Saturated fat (g 100 g^{-1})	7.6	1.5	8.2	5.4
Mono unsaturated fat (g 100 g^{-1})	9.2	0.6	9.9	2.2
Poly unsaturated fat (g 100 g^{-1})	6.7	1.8	7.2	6.5
Trans fat (g 100 g^{-1})	0.4	0.1	0.4	0.4
Omega 3 total	3590	1660	3852	5950
Omega 6 total	2630	220	2822	789
Omega 9 total	6730	270	7221	968
∑EPA + DPA + DHA	2850	1425	3058	5108
n-3 FA:n-6 FA	1.37	7.55	1.37	7.55

[1] The formulated diet (Ridley Clean Seas Pelagica) was supplied by Ridley (Narangba, Queensland, Australia); Australian Australian Sardines (*Sardinops sagax*; Sardines) were provided by Sardine Temptations Pty. Ltd. (Port Lincoln, South Australia, Australia).

Table 3.2.3.1.3. Summary of water quality parameters.

Item[1]	Temperature (°C)	Dissolved oxygen (mg L^{-1})	Dissolved oxygen (% saturation)	pH	Salinity (mg L^{-1})
Mean	12.8 ± 0.8	8.6 ± 0.3	102.2 ± 3.1	8.12 ± 0.05	36.9 ± 1.3
Range	11.5 - 16.0	7.5 - 10.8	94.0 - 128.0	8.01 - 9.08	34.0 - 38.0

[1] Values means ± standard deviation.

Table 3.2.3.1.4. Growth performance, feed utilisation, proximate composition and nutrient retentions of Yellowtail Kingfish fed the formulated diet or Sardines at varying feed rates and frequencies for 84 days.

Diet	1	1	1	1	1	1	1	2	
Treatment[1,2]	1	2	3	4	5	6	7	8	ANOVA[3]
Growth performance									
Initial weight (kg)	1.44±0.03	1.44±0.02	1.44±0.02	1.44±0.01	1.44±0.02	1.44±0.02	1.44±0.01	1.44±0.02	$P = 1.000$
Final weight (kg)	1.54±0.02^{ab}	1.47±0.02^{bc}	1.42±0.02^{cd}	1.30±0.01^{e}	1.41±0.02^{cd}	1.38±0.03^{cde}	1.36±0.02^{de}	1.56±0.02^{a}	$P < 0.001$
Biomass gain (kg tank^{-1})	1.97±0.18^{a}	0.62±0.10^{b}	-0.50±0.07^{c}	-2.99±0.18^{e}	-0.48±0.13^{c}	-1.32±0.24^{d}	-1.69±0.21^{d}	2.44±0.07^{a}	$P < 0.001$
SGR (% d^{-1})	0.08±0.01^{a}	0.02±0.00^{b}	-0.02±0.00^{c}	-0.12±0.01^{e}	-0.02±0.01^{c}	-0.05±0.01^{d}	-0.07±0.01^{d}	0.09±0.00^{a}	$P < 0.001$
Initial fork length (mm)	463.8±0.5	462.7±2.6	461.3±2.6	460.7±1.3	458.3±2.0	463.4±2.2	463.0±1.3	459.9±1.2	$P = 0.434$
Final fork length (mm)	474.5±0.9	470.9±1.3	469.3±2.7	465.8±0.8	466.2±2.5	470.5±2.6	468.9±1.3	471.1±1.2	$P = 0.074$
Length growth rate (mm d^{-1})	0.13±0.01^{a}	0.10±0.02^{b}	0.10±0.00^{b}	0.06±0.01^{c}	0.09±0.01^{b}	0.08±0.00^{bc}	0.07±0.01^{bc}	0.13±0.00^{a}	$P < 0.001$
Final condition factor	1.44±0.02^{b}	1.40±0.02^{bc}	1.37±0.01^{c}	1.29±0.01^{d}	1.40±0.00^{c}	1.32±0.01^{d}	1.32±0.01^{d}	1.49±0.01^{a}	$P < 0.001$
Feed utilisation (as fed)									
Feed consumption rate (g fish^{-1} d^{-1})	4.89±0.11^{b}	3.34±0.12^{c}	2.48±0.09^{d}	0.41±0.01^{f}	2.52±0.04^{d}	1.45±0.04^{e}	1.56±0.04^{e}	10.83±0.23^{a}	$P < 0.001$
Apparent FCR	4.43±0.35^{ab}	9.92±1.14^{a}	-9.34±1.91^{c}	-0.24±0.01^{b}	-11.54±4.11^{c}	-2.14±0.55^{b}	-1.70±0.28^{b}	7.83±0.24^{a}	$P < 0.001$
Proximate composition (wet basis)[4]									
Moisture (%)	65.4±0.8	66.0±1.6	68.0±1.1	67.9±0.9	67.2±1.5	68.3±1.0	69.2±0.1	65.7±0.9	$P = 0.213$
Protein (%)	19.83±0.34	19.65±0.44	20.66±0.57	19.82±0.21	20.50±0.57	20.69±0.48	20.78±0.87	20.14±0.17	$P = 0.587$
Lipid (%)	13.1±0.3^{a}	12.1±0.8^{a}	9.7±1.5^{ab}	9.8±0.5^{ab}	10.2±1.0^{ab}	9.7±1.2^{ab}	7.8±0.9^{b}	12.1±0.4^{a}	$P = 0.014$
Ash (%)	2.4±0.3	2.4±0.1	2.4±0.3	2.7±0.2	2.8±0.4	2.3±0.1	2.5±0.2	2.4±0.3	$P = 0.872$
Carbohydrate (%)	<1.5	<1.5	<1.5	<1.5	<1.5	<1.5	<1.5	<1.5	-
Energy (MJ kg^{-1})	8.39±0.24^{a}	7.80±0.38^{a}	7.09±0.46^{ab}	6.99±0.15^{ab}	7.25±0.35^{ab}	7.12±0.36^{ab}	6.42±0.20^{b}	7.89±0.13^{a}	$P = 0.008$
Taurine (mg 100 g^{-1})	281±6^{a}	265±7^{ab}	268±6^{ab}	242±8^{ab}	269±21^{ab}	256±9^{ab}	283±22^{a}	222±6^{b}	$P = 0.047$
Nutrient retention[5]									
Apparent PD (%)	11.84±2.19^{a}	4.76±5.09^{a}	11.62±9.38^{a}	-164.59±27.70^{b}	8.36±9.65^{a}	4.28±10.10^{a}	-0.21±19.41^{a}	17.98±1.26^{a}	$P < 0.001$
Apparent ED (%)	34.66±4.23^{a}	24.75±10.11^{a}	-3.91±16.69^{ab}	-164.02±24.99^{c}	3.45±12.86^{b}	-14.52±24.68^{ab}	-56.44±9.18^{b}	49.89±5.15^{a}	$P < 0.001$

[1] Values are mean ± SE; $n = 3$.

[2] Treatment 1: Formulated diet (Ridley Clean Seas Pelagica) fed to apparent satiation six days week^{-1}; Treatment 2: Formulated diet fed to apparent satiation two days week^{-1}; Treatment 3: Formulated diet fed to apparent satiation one day week^{-1}; Treatment 4: Formulated diet fed at 0.1% body weight (BW) one day week^{-1}; Treatment 5: Formulated diet fed at 0.65% BW two days week^{-1}; Treatment 6: Formulated diet fed at 0.35% BW two days week^{-1}; Treatment 7: Formulated diet fed at 0.12% BW six days week^{-1}; Treatment 8: Sardines (thawed and diced) fed to apparent satiation every second day.

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

[4] Initial fish proximate composition (wet basis): Moisture 66.7%, protein 19.62%, lipid 10.0%, ash 2.2%, carbohydrate (by difference) 1.5%, energy 7.04 MJ kg^{-1}, taurine 297 mg 100 g^{-1}.

[5] ED = energy deposition; PD = protein deposition.

Table 3.2.3.1.5. Fatty acid composition (mg 100 g^{-1}) of Yellowtail Kingfish fed the formulated diet or Sardines at varying feed rates and frequencies for 84 days.

Diet		1	1	1	1	1	1	1	2	
Treatment[1,2,3]	Initial	1	2	3	4	5	6	7	8	ANOVA[5]
Saturated Fatty Acids										
C14:0 Myristic	340	423±9^{a}	397±27^{ab}	310±52^{ab}	323±19^{ab}	343±38^{ab}	313±41^{ab}	253±28^{b}	403±13^{a}	P = 0.021
C15:0 Pentadecanoic	36	45±1^{a}	42±3^{ab}	33±6^{ab}	34±2^{ab}	37±5^{ab}	33±4^{ab}	28±3^{b}	49±2^{a}	P = 0.008
C16:0 Palmitic	1620	2063±34^{a}	1897±127^{ab}	1510±228^{ab}	1513±74^{ab}	1620±178^{ab}	1523±179^{ab}	1247±127^{b}	1880±61^{ab}	P = 0.016
C17:0 Margaric	38	49±1^{ab}	46±4^{ab}	36±5^{bc}	36±2^{bc}	39±5^{bc}	35±4b^{c}	29±3^{c}	53±1^{a}	P = 0.003
C18:0 Stearic	530	697±17^{a}	647±57^{a}	513±75^{ab}	527±27^{ab}	553±63^{ab}	513±55^{ab}	410±40^{b}	653±19^{a}	P = 0.013
C20:0 Arachidic	17	23±0^{a}	22±2^{ab}	17±3^{ab}	19±1^{ab}	19±2^{ab}	18±2^{ab}	15±2^{b}	22±1^{ab}	P = 0.024
C22:0 Docosanoic[4]	10	14±1	13±1	4±4	11±0	13±0	12±1	<10	13±2	P = 0.043*
Saturated Fat (g 100 g^{-1})	2.6	3.4±0.1^{a}	3.1±0.2^{a}	2.5±0.4^{ab}	2.5±0.1^{ab}	2.7±0.3^{ab}	2.5±0.3^{ab}	2.0±0.2^{b}	3.1±0.1^{a}	P = 0.013
Mono-unsaturated Fatty Acids										
C14:1 Myristoleic	17	22±1^{a}	20±1^{ab}	16±3^{ab}	16±1^{ab}	17±2^{ab}	16±2^{ab}	13±1^{b}	21±1^{ab}	P = 0.018
C16:1 Palmitoleic	620	787±22^{a}	727±47^{ab}	577±95^{ab}	567±32^{ab}	617±70^{ab}	570±76^{ab}	463±53^{b}	697±27^{ab}	P = 0.024
C18:1n-7 Octadecenoic	320	403±13^{a}	373±28^{a}	293±46^{ab}	293±15^{ab}	317±35^{ab}	290±35^{ab}	233±23^{b}	363±13^{ab}	P = 0.013
C18:1n-9 Oleic	2730	3600±100^{a}	3330±239^{ab}	2700±393^{ab}	2690±150^{ab}	2777±307^{ab}	2673±328^{ab}	2163±214^{b}	3077±112^{ab}	P = 0.027
C20:1 Eicosenoic (total)	250	310±12	310±31	230±40	273±18	263±32	253±39	187±18	300±21	P = 0.077
C20:1n-9Eicosenoic	130	167±3	163±13	126±21	140±6	140±17	133±20	102±9	157±9	P = 0.067
C20:1n-11,13Eicosenoic	110	137±7	147±18	103±21	130±10	127±18	120±20	85±9	143±12	P = 0.129
C22:1n-9 Docosenoic	22	28±0^{a}	28±2^{a}	21±3^{ab}	24±2^{ab}	22±3^{ab}	22±3^{ab}	16±1^{b}	27±1^{ab}	P = 0.035
C22:1n-11, 13 Docosenoic	66	83±2	78±9	47±5	64±3	69±7	63±11	46±7	79±4	P = 0.172
C24:1 Tetracosenoic	31	26±13	39±2	30±2	36±2	33±4	34±2	28±3	43±3	P = 0.351
Mono Unsaturated Fat (g 100 g^{-1})	4.1	5.4±0.1^{a}	5.1±0.4^{a}	4.1±0.6^{ab}	4.1±0.2^{ab}	4.2±0.5^{ab}	4.0±0.5^{ab}	3.2±0.3^{b}	4.8±0.2^{ab}	P = 0.023

[1] Values are mean ± SE; n = 3.

[2] Treatment 1: Formulated diet (Ridley Clean Seas Pelagica) fed to apparent satiation six days week^{-1}; Treatment 2: Formulated diet fed to apparent satiation two days week^{-1}; Treatment 3: Formulated diet fed to apparent satiation one day week^{-1}; Treatment 4: Formulated diet fed at 0.1% body weight (BW) one day week^{-1}; Treatment 5: Formulated diet fed at 0.65% BW two days week^{-1}; Treatment 6: Formulated diet fed at 0.35% BW two days week^{-1}; Treatment 7: Formulated diet fed at 0.12% BW six days week^{-1}; Treatment 8: Sardines (thawed and diced) fed to apparent satiation every second day.

[3] Values for the following fatty acids < 10 mg 100 g^{-1} and were excluded from the table: C4:0 Butyric, C6:0 Caproic, C8:0 Caprylic, C10:0 Capric, C12:0 Lauric, C13:0 Trisdecanoic, C21:0 Heneicosanoic, C24:0 Tetracosanoic, C10:1 Decenoic, C15:1 Pentadecenoic, C17:1 Heptadecenoic, C18:1n-6 Octadecenoic, C18:2 CLA 9c 11t, C18:2 CLA 10t 12c. C21:5n-3 Heneicosapentaenoic acid, C22:2 Docosadienoic, C18:1n-6 Octadecenoic.

[4] Samples below the detectable range were assigned the value of 0; if the values for all three replicates were below detectable range then they are reported here as below the detectable range (e.g. < 10).

[5] A significance level of P < 0.05 was used for all statistical tests, where significant differences were observed post-hoc tests (Student-Newman-Keuls test) were used to detect differences between treatments, values within each row without a common superscript are significantly different (a indicates the highest value; P < 0.05). *One-factor ANOVA detected a significant difference, but Student-Newman-Keuls test was unable to discern significant differences.

Table 3.2.3.1.5. Continued: Fatty acid composition (mg 100 g^{-1}) of Yellowtail Kingfish fed the formulated diet or Sardines at varying feed rates and frequencies for 84 days.

Diet		1	1	1	1	1	1	1	2	
Treatment[1,2,3]	Initial	1	2	3	4	5	6	7	8	ANOVA[5]
Poly-unsaturated Fatty Acids										
C18:2n-6 Linoleic	960	1280±26^{a}	1167±74^{a}	960±136^{ab}	923±48^{ab}	967±94^{ab}	933±114^{ab}	760±81^{b}	1077±37^{ab}	P = 0.015
C18:3n-3 Alpha Linolenic	130	180±6^{a}	157±12^{a}	124±20^{ab}	120±6^{ab}	130±15^{ab}	120±17^{ab}	96±12^{b}	167±7^{a}	P = 0.005
C18:3n-4 Octadecatrienoic	18	22±1^{a}	20±2^{a}	17±3^{ab}	16±1^{ab}	17±2^{ab}	16±2^{ab}	12±1^{b}	22±1^{a}	P = 0.012
C18:3n-6 Gamma Linolenic	17	23±1^{a}	21±1^{a}	17±3^{ab}	17±1^{ab}	17±2^{ab}	17±2^{ab}	13±2^{b}	21±1^{a}	P = 0.008
C18:4n-3 Steridonic	89	113±3^{a}	101±9^{ab}	76±14^{ab}	80±5^{ab}	84±8^{ab}	80±16^{ab}	61±9^{b}	120±6^{a}	P = 0.006
C20:2n-6 Eicosadienoic	20	17±9	25±2	20±3	19±1	21±2	19±3	15±2	27±1	P = 0.396
C20:3n-3 Eicosatrienoic[4]	<10	11±0^{a}	6±4^{b}	<10^{b}	<10^{b}	<10^{b}	3±3^{b}	<10^{b}	11±0^{a}	P < 0.001
C20:3n-6 Dihomo-ɣ-linoleic	15	20±1^{a}	18±1^{a}	15±2^{ab}	15±1^{ab}	15±2^{ab}	15±2^{ab}	11±1^{b}	19±1^{a}	P = 0.016
C20:4n-3 Eicosatetracenoic	100	127±7	130±12	81±20	118±12	108±13	93±24	78±9	112±15	P = 0.169
C20:4n-6 Arachidonic	89	117±3^{a}	110±6^{a}	90±11^{ab}	93±3^{ab}	91±5^{ab}	93±9^{ab}	73±8^{b}	110±6^{a}	P = 0.008
C20:5n-3 Eicosapentaenoic	520	690±21	597±32	480±78	480±21	490±44	473±73	383±59	643±28	P = 0.007
C22:4n-6 Docosatetraenoic	32	44±1^{ab}	41±2^{ab}	33±4^{bc}	36±1^{bc}	34±3^{bc}	35±4^{bc}	27±3^{c}	47±1^{a}	P = 0.001
C22:5n-3 Docosapentaenoic	170	250±10^{a}	227±17^{a}	187±26^{ab}	187±9^{ab}	187±15^{ab}	190±25^{ab}	143±13^{b}	233±9^{a}	P = 0.008
C22:5n-6 Docosapentaenoic	31	36±2^{a}	32±1^{ab}	25±4^{ab}	26±1^{ab}	26±3^{ab}	25±4^{ab}	20±4^{b}	33±1^{a}	P = 0.011
C22:6n-3 Docosahexaenoic	730	1003±44^{ab}	905±55^{bc}	736±93^{bc}	787±20^{bc}	777±56^{bc}	792±105^{bc}	619±78^{c}	1170±40^{a}	P = 0.001
∑EPA + DPA + DHA	1420	1943±69^{ab}	1728±101^{abc}	1403±197^{bc}	1454±46^{abc}	1454±112^{abc}	1456±201^{abc}	1146±150^{c}	2047±76^{a}	P = 0.003
Poly Unsaturated Fat (% m m^{-1})	3.0	4.0±0.1^{a}	3.6±0.2^{ab}	3.0±0.5^{ab}	3.0±0.1^{ab}	3.1±0.3^{ab}	3.0±0.4^{ab}	2.4±0.3^{b}	3.9±0.1^{a}	P = 0.009
Trans Fat content (% m m^{-1})	0.2	0.3±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	P = 0.062
Total Omega 3	1750	2367±81^{a}	2120±132^{ab}	1703±248^{ab}	1777±62^{ab}	1783±136^{ab}	1767±252^{ab}	1397±177^{b}	2470±93^{a}	P = 0.004
Total Omega 6	1150	1530±35^{a}	1393±85^{a}	1143±162^{ab}	1117±57^{ab}	1167±94^{ab}	1123±133^{ab}	907±97^{b}	1317±47^{ab}	P = 0.011
Total Omega 9	2920	3837±102^{a}	3560±253^{ab}	2880±422^{ab}	2890±161^{ab}	2973±326^{ab}	2863±350^{ab}	2310±225^{b}	3307±127^{ab}	P = 0.027

[1] Values are mean ± SE; n = 3.

[2] Treatment 1: Formulated diet (Ridley Clean Seas Pelagica) fed to apparent satiation six days week^{-1}; Treatment 2: Formulated diet fed to apparent satiation two days week^{-1}; Treatment 3: Formulated diet fed to apparent satiation one day week^{-1}; Treatment 4: Formulated diet fed at 0.1% body weight (BW) one day week^{-1}; Treatment 5: Formulated diet fed at 0.65% BW two days week^{-1}; Treatment 6: Formulated diet fed at 0.35% BW two days week^{-1}; Treatment 7: Formulated diet fed at 0.12% BW six days week^{-1}; Treatment 8: Sardines (thawed and diced) fed to apparent satiation every second day.

[3] Values for the following fatty acids < 10 mg 100 g^{-1} and were excluded from the table: C4:0 Butyric, C6:0 Caproic, C8:0 Caprylic, C10:0 Capric, C12:0 Lauric, C13:0 Trisdecanoic, C21:0 Heneicosanoic, C24:0 Tetracosanoic, C10:1 Decenoic, C15:1 Pentadecenoic, C17:1 Heptadecenoic, C18:1n-6 Octadecenoic, C18:2 CLA 9c 11t, C18:2 CLA 10t 12c. C21:5n-3 Heneicosapentaenoic acid, C22:2 Docosadienoic, C18:1n-6 Octadecenoic.

[4] Samples below the detectable range were assigned the value of 0; if the values for all three replicates were below detectable range then they are reported here as below the detectable range (e.g. < 10).

[5] A significance level of P < 0.05 was used for all statistical tests, where significant differences were observed post-hoc tests (Student-Newman-Keuls test) were used to detect differences between treatments, values within each row without a common superscript are significantly different (a indicates the highest value; P < 0.05). *One-factor ANOVA detected a significant difference, but Student-Newman-Keuls test was unable to discern significant differences.

Table 3.2.3.1.6. Mineral composition (mg kg^{-1}) of Yellowtail Kingfish fed the formulated diet or sardines at varying feed rates and frequencies for 84 days.

Diet		1	1	1	1	1	1	1	2	
Treatment[1,2]	Initial	1	2	3	4	5	6	7	8	ANOVA[3]
Calcium	4900	5400±2303	3233±788	4367±845	3867±1471	3900±458	4033±851	3333±977	4600±569	$P = 0.914$
Copper	0.72	0.82±0.06	0.92±0.09	0.78±0.03	0.65±0.04	0.82±0.04	0.75±0.05	0.73±0.05	0.79±0.06	$P = 0.102$
Iodine (mg 100 g^{-1})	0.85	1.09±0.31	0.61±0.03	0.68±0.03	0.79±0.06	0.69±0.04	0.89±0.12	0.80±0.08	0.86±0.17	$P = 0.355$
Iron	19	33±8	22±1	22±1	23±1	21±2	21±1	24±1	25±6	$P = 0.409$
Magnesium	370	360±30	333±13	353±22	330±15	340±6	360±15	333±19	350±10	$P = 0.830$
Manganese	0.67	0.70±0.16	0.43±0.07	0.45±0.05	0.46±0.07	0.45±0.05	0.50±0.06	0.43±0.10	0.53±0.08	$P = 0.435$
Potassium	3700	3533±33	3600±0	3733±145	3633±33	3667±67	3667±33	3733±33	3467±33	$P = 0.086$
Phosphorus	4700	4867±1172	3767±470	4333±433	4000±656	3933±219	4267±467	3633±467	4367±219	$P = 0.853$
Selenium	0.61	0.69±0.02	0.59±0.05	0.68±0.03	0.62±0.02	0.61±0.01	0.63±0.04	0.60±0.04	0.69±0.04	$P = 0.232$
Zinc	14	14±1	11±0	12±1	13±1	12±1	11±0	13±1	12±1	$P = 0.479$

[1] Values are mean ± SE; $n = 3$

[2] Treatment 1: Formulated diet (Ridley Clean Seas Pelagica) fed to apparent satiation six days $week^{-1}$; Treatment 2: Formulated diet fed to apparent satiation two days $week^{-1}$; Treatment 3: Formulated diet fed to apparent satiation one day $week^{-1}$; Treatment 4: Formulated diet fed at 0.1% body weight (BW) one day $week^{-1}$; Treatment 5: Formulated diet fed at 0.65% BW two days $week^{-1}$; Treatment 6: Formulated diet fed at 0.35% BW two days $week^{-1}$; Treatment 7: Formulated diet fed at 0.12% BW six days $week^{-1}$. Treatment 8: Sardines (thawed and diced) fed to apparent satiation every second day.

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests (Student-Newman-Keuls test) were used to detect differences between treatments, values within each row without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

Table 3.2.3.1.7. Blood haematocrit, gastrointestinal indices, and ADC (%) for dietary dry matter, crude protein and gross energy for Yellowtail Kingfish fed the formulated diet or Sardines at varying feed rates and frequencies for 84 days.

Diet	1	1	1	2	
Treatment[1,2]	1	4	7	8	ANOVA[3]
Blood haematocrit and gastrointestinal indices					
Blood haematocrit (%)	46.0±0.3	39.6±1.3	41.4±1.5	43.0±2.6	$P = 0.105$
Visceral index (%)	5.79±0.19[a]	4.92±0.23[ab]	4.53±0.23[b]	5.74±0.26[a]	$P = 0.010$
Hepatosomatic index (%)	1.11±0.02[a]	0.75±0.05[b]	0.78±0.08[b]	1.00±0.06[a]	$P = 0.006$
Dietary digestibility (ADC; %)[4]					
Dry matter	67.6±2.8	NA	NA	79.1±1.5	$P = 0.023$
Crude protein	89.3±0.9	NA	NA	94.7±0.7	$P = 0.010$
Gross energy	77.0±2.0	NA	NA	89.6±1.2	$P = 0.020$

[1] Treatment 1: Formulated diet (Ridley Clean Seas Pelagica) fed to apparent satiation six days week^{-1}; Treatment 4: Formulated diet fed at 0.1% body weight (BW) one day week^{-1}; Treatment 7: Formulated diet fed at 0.12% BW six days week^{-1}; Treatment 8: Sardines (thawed and diced) fed to apparent satiation every second day.
[2] Values are mean ± SE; $n = 3$; NA denotes not analysed.
[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests (Student-Newman-Keuls test) were used to detect differences between treatments, values within each row without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).
[4] ADC = apparent digestibility coefficient.

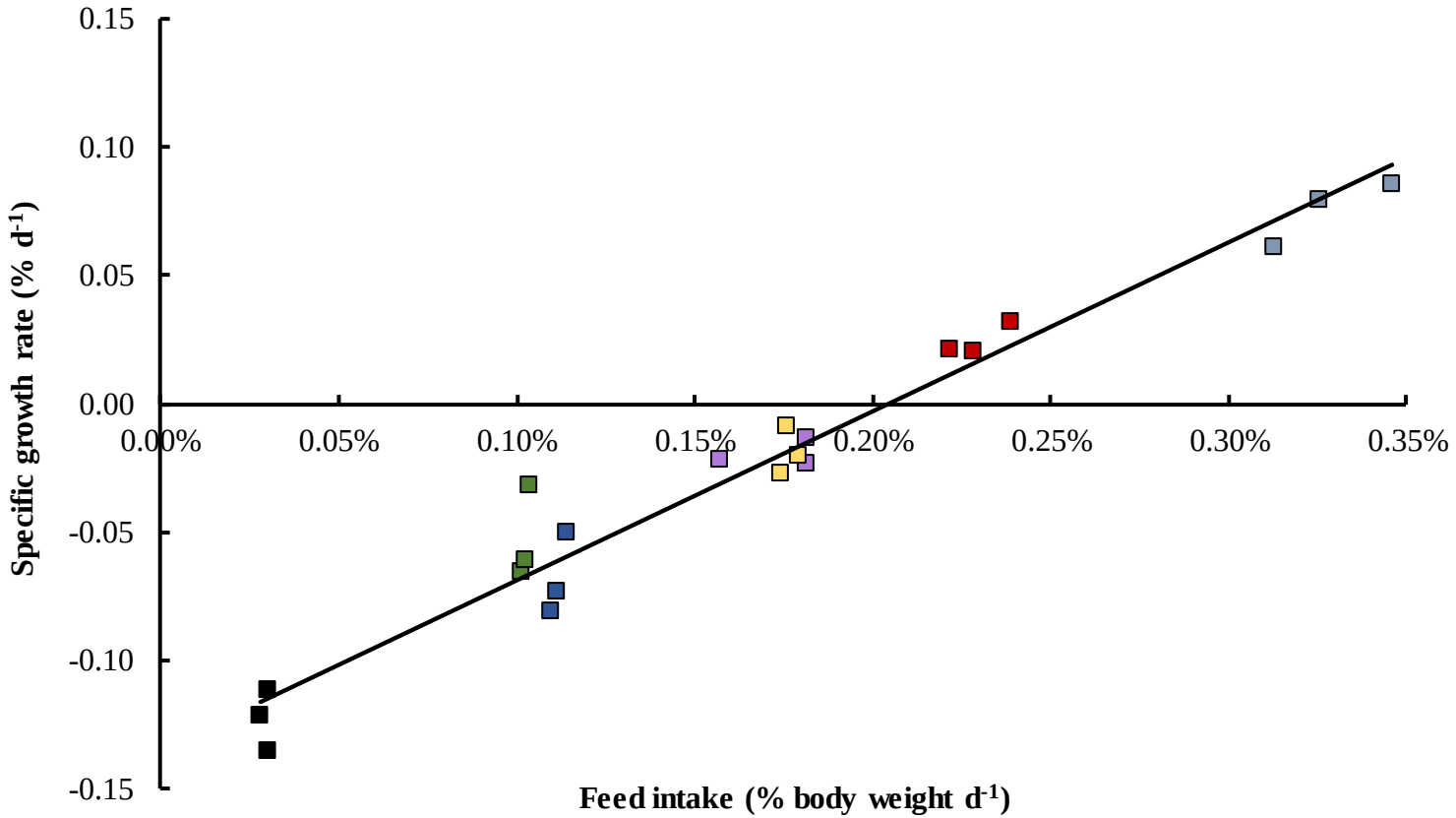


Figure 3.2.3.1.1. The relationship between feed intake (% BW d^{-1}) and specific growth rate (% d^{-1}) for Yellowtail Kingfish fed the formulated diet at varying feed rates and frequencies for 84 days. Linear relationship: $y = 66.051x - 0.1352$, $R^2 = 0.961$, Pearson's correlation coefficient $P < 0.001$.
Feed intake (% BW d^{-1}) was calculated across the entire experiment and includes non-feeding days.

(■) Treatment 1: Formulated diet (Ridley Clean Seas Pelagica) fed to apparent satiation six days $week^{-1}$; (■) Treatment 2: Formulated diet fed to apparent satiation two days $week^{-1}$; (■) Treatment 3: Formulated diet fed to apparent satiation one day $week^{-1}$; (■) Treatment 4: Formulated diet fed at 0.1% body weight (BW) one day $week^{-1}$; (■) Treatment 5: Formulated diet fed at 0.65% BW two days $week^{-1}$; (■) Treatment 6: Formulated diet fed at 0.35% BW two days $week^{-1}$; (■) Treatment 7: Formulated diet fed at 0.12% BW six days $week^{-1}$.

3.2.3.2. Manuscript - Effect of feeding frequency on the growth and feed utilisation for large Yellowtail Kingfish (Seriola lalandi) at warm water temperatures.

David A.J. Stone [a, b, c, d], Matthew S. Bansemer [a], Paul Skordas [a], Krishna-Lee Currie [a], Leigh Kuerschner [a]

[a] South Australian Research and Development Institute Aquatic Sciences Centre, West Beach, SA 5024, Australia
[b] Flinders University, College of Sciences and Engineering, GPO Box 2100, Adelaide, SA 5001, Australia
[c] University of Adelaide, School of Animal and Veterinary Sciences, Roseworthy, SA 5371, Australia
[d] Marine Innovation Southern Australia

This manuscript may be referenced as: Stone, D.A.J., Bansemer, M.S., Skordas, P., Currie, K.L. and Kuerschner, L. (2019). 3.2.3.2. Manuscript - Effect of feeding frequency on the growth and feed utilisation for large Yellowtail Kingfish (*Seriola lalandi*) at warm water temperatures. (N4; Output 3b). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.366-383.

Abstract

In this 84 day study, the growth performance and feed utilisation for large (3.11 kg) Yellowtail Kingfish (*Seriola lalandi*; YTK) fed a formulated diet at four different feed frequencies/ration sizes were investigated. In the first three treatments, fish were fed to apparent satiation once, twice or three times daily 7 days week^{-1}. In the fourth treatment, fish were fed the equivalent ration size to those fed once daily to apparent satiation, but split over two feeds (66.67% at 8:00 h and 33.33% at 16:00 h). Over the entire experiment, the specific growth rate (SGR) of YTK was not significantly different between treatments. However, between stocking to the first weight check (28 days), fish fed twice and three times daily to apparent satiation exhibited significantly higher SGR compared to those fed to apparent satiation once daily and those fed the equivalent split ration. Compared to single ration feeding, split ration feeding once a day did not lead to any significant improvements in SGR or FCR. In contrast, between the first weight check to the final harvest (56 days), there were no significant differences in SGR between treatments. These response differences may be related to decreasing water temperature throughout the experiment. From stocking to the first weight check (28 days), the average water temperature was 21.8 °C (range 23-20 °C), while from the first weight check to final harvest; (56 days) the average water temperature was 18.3 °C (range 22-16 °C). Over the course of the experiment, the feed intake rate (% BW day^{-1}) significantly increased as feeding frequency increased from once to twice to three times a day to apparent satiation. With regard to the feed conversion ratio (FCR) over the entire experiment, fish fed to apparent satiation three times daily exhibited a significantly higher FCR than other treatments investigated. Based on these results, it is recommended that fish of this size are fed to apparent satiation twice daily at water temperatures > 20 °C, and fed to apparent satiation once daily as water temperatures drop from 20 to 16 °C. Further research in pilot scale commercial trials are needed to validate results from the current study before implementing these altered feeding strategies under commercial conditions.

Introduction

A high priority for the Australian Yellowtail Kingfish (*Seriola lalandi*; YTK) industry is to improve the feeding practices, ultimately improving the sustainable and economical production of YTK (Stone and Bellgrove, 2013; Stone et al., 2016). Under commercial conditions, it is important that feeding practices are optimised to ensure the species is provided with sufficient feed to promote optimal feed utilisation, growth and survival (Huang et al., 2015; Stone et al., 2016).

In the early stages of commercial YTK production in Australia, summer water temperature feeding practices involved feeding large fish twice daily to apparent satiation (Miegel et al., 2010). Currently, under Australian commercial conditions, large (> 2 kg) YTK (*Seriola lalandi*) are predominantly fed once daily (Stone et al., 2016). Research conducted with the closely related Japanese Yellowtail (50-80 g; *Seriola quinqueradiata*), indicated this species exhibit two main peaks in feeding behaviour during the day, predominantly associated with change in light intensity (dawn and dusk) (Kohbara et al., 2003). Under experimental conditions, YTK (2.12 kg) at 20.8 °C fed a 67% fish meal based diet readily accepted two feeds per day (08:00 h and 16:00 h), whereas they would only feed consistently every second day (08:00 h) at winter water temperatures (< 15 °C) (Miegel et al., 2010). A recent study by Teoh (2016), linked with Manuscript 3.2.2.1., in experimental facilities at SARDI SAASC reported that YTK (3.7-3.9 kg at ~18 °C) readily accepted feed when fed to apparent satiation twice daily (09:00 h and 16:00 h).

Numerous studies have investigated the effect of feeding frequency and ration size on the performance of aquaculture species (Jarboe and Grant, 1996; Azzaydi et al., 1999; García-Mesa et al., 2014; Arnold et al., 2015; Stone et al., 2016; Bansemer et al., 2018). Recent research on the effect of restricting feed rates for large YTK has successfully demonstrated that fish fed to targeted 80% satiation (achieved: ~90% satiation) exhibited similar growth performance, but superior feed utilisation to those fed 100% satiation (Stone et al., 2016). In the second experiment in the same project, Stone et al. (2016) reported that when fed at the 80% satiation level, large fish exhibited inferior growth rates compared to fish fed to 100% apparent satiation. Stone et al. (2016) concluded that there appears to be a fine line between feeding fish to sub-satiation to improve feed utilisation, and to apparent satiation, without compromising growth. This conclusion is supported by results from Imsland et al. (2011) who reported decreased growth performance for Atlantic Salmon (*Salmo salar*) fed every second day (50%), fed every fourth day (25%), and starved, compared to fish fed every day (100%). In contrast, increasing feeding frequency for juvenile shrimp (~3 g; *Penaeus monodon*) from twice a day to six times a day, and fed a restricted ration (60 or 80% satiation), resulted in significantly improved growth and FCR (Arnold et al., 2015). However, when the feed ration was increased to 100% apparent satiation there was no difference in growth of juvenile shrimp between the two feeding frequencies (two or six times a day).

In addition to the feeding frequency, the ration size per feed is also an important factor that influences growth performance (Li et al., 2014). Blunt Snout Bream (*Megalobrama amblycephala*) fed the same ration size (6-8% BW d^{-1}) exhibited superior growth when fed the ration split over 3-5 feeding events, compared to fish fed the same ration over 1, 2 or 6 feeding events (Li et al., 2014).

Based on previous research, there is a fine line between optimal feeding practices, in terms of frequency and feed rate, and overfeeding or underfeeding. Overfeeding leads to poor feed utilisation and increased feed costs, while underfeeding impacts growth and survival (Huang et al., 2015). If feeding frequencies and ration size can be optimised for cultured YTK it may be possible to improve the growth and feed utilisation. However, due to the large differences in fish species and size, environmental parameters and experimental design in previous feeding frequency and ration studies, it is difficult to apply results for other species to practical feeding practices (frequency and ration) for large YTK (> 2 kg) under commercial conditions. Based on previous research that has reported that large YTK (> 2 kg) readily accept two meals per day, there is a physiological potential to increase the feeding frequency to twice a day to improve productivity. Furthermore, due to logistical constraints, feeding three times a day is the maximum a feed barge can realistically achieve in Australia (Dr Trent D'antignana, Nutrisea; *personal communication*).

Aims

The aim of this study was to investigate the effects of feeding frequency and ration size on growth performance, feed utilisation and health of large YTK (~3 kg) at warm water temperatures. The effects of a split ration feeding regime on nutrient utilisation and growth performance was also assessed.

Methods

Experimental design and diets

In the current study, four feeding frequency treatments were investigated:

- Apparent satiation once daily 7 days week^{-1} (08:00 h)
- Apparent satiation twice daily 7 days week^{-1} (08:00 h and 16:00 h)
- Apparent satiation three times daily 7 days week^{-1} (08:00 h, 12:00 h and 16:00 h)
- Fed twice daily to the ration provided in Treatment 1. Ration was split by 66.67 and 33.33% and was fed at 08:00 h and 16:00 h, respectively).

Fish fed to apparent satiation, involved feeding fish for four min tank^{-1} or until a feed refusal response was observed. Tanks were cleaned every second day. This study ran for a total of 84 days. Fish fed Treatment 4 were fed the same ration provided to Treatment 1, which was calculated daily (but split and delivered two meals). Fish were fed a 9 mm pellet diameter experimental extruded diet manufactured by Skretting Australia (Cambridge, Tasmania, Australia). The experimental diet was formulated to contain highly palatable and digestible ingredients at commercially applicable inclusion levels. The diet contained 30% fish meal, 45% crude protein [39% digestible protein] and 25% crude lipid [24% digestible lipid]). Diet composition is displayed in Table 3.2.3.2.1 and Table 3.2.3.2.2.

Experimental fish

Experimental work was conducted in the pool-farm facility at the South Australian Research and Development Institute, South Australian Aquatic Science Centre (SARDI SAASC; West Beach, South Australia, Australia). YTK (n = 264; 3.11 ± 0.23 kg; 575 ± 17 mm (fork length; mean ± standard deviation) were obtained from Clean Seas Seafood (Port Lincoln, South Australia, Australia). Upon arrival at the SARDI SAASC facility, YTK were transferred to 5000 L tanks supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated, aerated sea water at ambient temperature and held for ~8 months and fed a standard Ridley Pelagica diet (Narangba, Queensland, Australia; crude protein 46%; crude lipid 24%; gross energy 19.30 MJ kg^{-1}).

Skin and gill fluke treatment

Upon arrival at SARDI SAASC, YTK were inspected, and were observed to have a low burden of skin flukes (*Benedenia seriola*) and gill flukes (*Zeuxapta seriola*). Treatment was necessary, and was prescribed by Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd, Ballina, New South Wales, Australia).

Experimental stocking and intermediate weight checks

At the commencement of the study (March 2017), YTK were anaesthetised in 5000 L tanks (total water volume 2500 L) using AQUI-S® (AQUI-S® New Zealand Ltd., Lower Hutt, New Zealand) at a concentration of 14 mg L^{-1} of seawater. Twenty two fish were removed from their tank, measured, weighed and stocked into one of the three replicate 5000 L tanks treatment combination^{-1} (n = 4 treatments; n = 12 tanks).

Tanks were supplied with partial flow-through/recirculating (100% system water exchange d^{-1}), sand filtered, UV treated sea water at ambient temperature. All tanks were supplied with aeration and oxygenation throughout the study.

At weight checks at four weeks and eight weeks post-stocking, all fish were anaesthetised using AQUI-S® at a concentration of 14 mg L^{-1} of seawater. YTK were measured and weighed, and returned back to their respective tanks.

Water quality analyses

Water quality parameters were measured daily at 10:30 h, and maintained at appropriate levels for acceptable growth of YTK throughout the study (Table 3.2.3.2.3). Water temperature was measured using a thermometer and the temperature profile for the study is displayed in Figure 3.2.3.2.1. Dissolved oxygen (mg L^{-1} and % saturation) was measured using a dissolved oxygen meter (OxyGuard International A/S, Birkerød, Denmark). The pH was measured daily using a meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, IL, USA). Salinity (g L^{-1}) was measured weekly using a portable salinity refractometer (model RF20, Extech Instruments, Nashua, NH, USA).

Final harvest sampling

At day 84, all fish were anaesthetised using AQUI-S® at a concentration of 14 mg L^{-1} of seawater and weighed and measured. Three fish from each tank (n = 3 fish $tank^{-1}$; n = 12 tanks; n = 36 fish) were collected whole and stored frozen at -20 °C for biochemical analysis. Blood samples from three separate fish per tank (n = 3 fish $tank^{-1}$; n = 12 tanks; n = 36 fish) were collected using a 19 G needle with a 5 mL syringe at the conclusion of the experiment. Blood samples were transferred to three separate Vacuette® or BD vacutainer® tubes (Z serum clot activator, EDTA or SST™ II advance tubes). A sub-sample of blood collected in EDTA Vacuette® tubes was analysed for blood haematocrit at SARDI SAASC. Serum was analysed for blood biochemistry and whole blood was analysed for blood haematology conducted by IDEXX (Unley, South Australia, Australia). Serum was also analysed for osmolality and pH conducted by Gribbles (Wayville, South Australia, Australia). The blood sampled fish were then dissected and the visceral organs, liver and visceral fat were weighed in order to calculate visceral index (VSI; %), hepatosomatic index (HSI; %) and intraperitoneal fat (%), respectively. The stomach from these fish were opened longitudinally, and were subjectively scored for gastric dilation (Chown, 2015). In addition, $1cm^2$ longitudinally opened hindgut sections were collected from blood sampled fish for histology. In brief, hindgut samples were fixed in 10% seawater formalin for > 48 h, processed and embedded in paraffin wax. Tissue sections were cut using a microtome and floated onto Starfrost® glass slides and dried for > 24 h at room temperature before being stained with hematoxylin and eosin (H and E) and periodic acid-schiff alcian blue (PAS/AB pH 2.5). Gastrointestinal morphological parameters in the hindgut including muscle and serosa thickness, villi length, lamina propria thickness, total goblet cell number, eosinophilic droplets in epithelial cells and melanomacrophage centres were measured.

Apparent digestibility coefficients and nutrient digestion

At the conclusion of the 84 day growth experiment, a digestibility experiment was undertaken. After fish (n = 16 $tank^{-1}$) were weighed and measured they were returned to their tank, and fed their respective treatment for four days. After four days, fish were anaesthetised using AQUI-S® at a concentration of 20 mg L^{-1} of seawater (to enable handling and faecal matter collection), manually stripped and the faecal matter was collected. In brief, manual stripping involved placing the forefinger and thumb on either side of the fish abdomen at the pelvic fin. Moderate pressure was applied by the forefinger and thumb, and at the same time moved towards the anus, this process was repeated six times. Uncontaminated faecal samples (free from blood, urine and mucus) were collected in a 250 mL container and stored frozen at -20 °C for biochemical analysis. Fish were then revived in their respective tank, and fed their respective treatments for a further four days. Fish were manually stripped again to ensure adequate samples were collected. Faecal material from all fish from a tank from both stripping events were pooled for analysis.

Biochemical and histological analyses

The proximate composition analyses of diets and whole body tissue were conducted according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardization (DIN) (2000). A one kg sample of the experimental diet was collected, ground and analysed for proximate composition (moisture, protein, fat, ash, carbohydrate and energy), amino acid profile, taurine level, fatty acid profile and rancidity (p-anisidine and peroxide value). In addition, a total of twelve fish (n = 12 fish) at the start of the experiment, and three fish from each tank (n = 3 fish tank^{-1}; n = 12 tanks; n = 36 fish) at the conclusions of the experiment were collected and stored frozen at -20 °C. Whole fish samples were partially thawed, homogenised and analysed for proximate composition (moisture, protein, fat, ash, carbohydrate [by difference] and energy) and fatty acid profile.

Performance indices

All data reported for each treatment for animal performance were based on the mean of the replicate tanks. All calculations using fish weight and diets were based on wet or as fed values, respectively:

- Biomass gain (kg tank^{-1}) = (final weight + ∑mortality weight) - (initial weight + ∑replacement weight)
- Specific growth rate (SGR, % d^{-1}) = ([ln final weight - ln initial weight] / d) × 100
- Length growth rate (mm d^{-1}) = (final fish length - initial fish length) / d
- Condition factor = (fish weight [g] / fish length [cm]^3) × 100
- Apparent feed conversion ratio (FCR) = feed consumed / fish weight gain
- Apparent protein deposition = ([final whole protein - initial whole protein] / protein intake) × 100
- Apparent energy deposition = ([final whole energy - initial whole energy] / energy intake) × 100
- Haematocrit count = red blood cell (mm) / total blood (red blood cell and plasma [mm]) 100 × 100
- Intraperitoneal fat (%) = wet intraperitoneal fat wt × 100 / final wet fish wt
- Visceral index (VSI; %) = wet visceral wt × 100 / final wet fish wt
- Hepatosomatic index (HSI; %) = wet liver wt × 100 / final wet fish wt

Statistical analyses

IBM SPSS (version 24 for Windows; IBM SPSS Inc., USA) was used for all statistical analyses. Homogeneity of variances and normality among mean values were assessed using Levene's test for equality of variance errors and Shapiro-Wilk test, respectively. Data were compared across all treatments using a one-factor ANOVA. When significant effects were observed, the Student-Newman-Keuls post-hoc test was used to detect significant differences between all treatments. A significance level of $P < 0.05$ was used for all statistical tests. All values are presented as means ± standard error (SE) of the mean unless otherwise stated.

Results

General observations

There were no significant differences in the initial weight and length of YTK between treatments in the current study ($P > 0.05$; one-factor ANOVA; Table 3.2.3.2.4). The average initial weight and length were 3.11 ± 0.23 kg and 575 ± 17 mm (fork length; mean ± standard deviation; n = 264), respectively.

Fish fed actively, and there were no mortalities or apparent signs of disease throughout the duration of the experiment.

Growth performance

From stocking to final harvest, the specific growth rate (SGR), final weight, biomass gain, final fork length and length growth rate of YTK were not significantly different between feeding treatments ($P > 0.05$; one-factor ANOVA; Table 3.2.3.2.4). YTK fed to apparent satiation three times daily (Treatment 3) and twice daily (Treatment 2) tended to grow faster than fish fed to apparent satiation once a day (Treatment 1) and those fed the equivalent 66.67% and 33.33% split ration (Treatment 4). Final Condition factor was significantly influenced by feeding treatment ($P = 0.002$; Treatment 2 = 3 > 1 = 4; Table 3.2.3.2.4).

Between stocking and the first weight check (28 days), when the average water temperature was 21.8 °C (range 23-20 °C), the SGR of YTK was significantly affected by treatment ($P < 0.001$; Treatment 3 > 2 > 1 = 4; Figure 3.2.3.2.2 and 3.2.3.2.3). In contrast, between the first weight check and final harvest (56 days), when the average water temperature was 18.3 °C (range 22-16 °C; only five days when water temperatures exceeded 20 °C during this period), the SGR of YTK was not influenced by feeding treatment ($P > 0.05$; Figure 3.2.3.2.2 and 3.2.3.2.4).

Feed utilisation

From stocking to final harvest, apparent feed consumption and feed intake significantly increased as feeding frequency increased from once (Treatment 1), to twice (Treatment 2) to three times a day (Treatment 3) when fed to apparent satiation daily ($P < 0.001$; Table 3.2.3.2.4). There was no significant difference for feed intake between fish fed Treatment 1 or the split ration of Treatment 4 ($P > 0.05$; one-factor ANOVA; Table 3.2.3.2.4).

Apparent FCR of YTK fed to apparent satiation three times daily (Treatment 3) was significantly higher than fish fed other feeding treatments ($P < 0.05$; one-factor ANOVA; Table 3.2.3.2.4). There were no significant difference in FCR between fish fed Treatment 1, 2 and 4 ($P > 0.05$; Table 3.2.3.2.4).

Whole fish proximate and energy composition

Feeding treatment did not significantly influence moisture (61.7-62.4%), protein (19.5-20.4% wet), lipid (15.9-16.9% wet), ash (2.2-3.1% wet), carbohydrate (< 1% wet), or energy content of whole fish (9.33-9.60 MJ kg^{-1}) ($P > 0.05$; one-factor ANOVA; Table 3.2.3.2.4).

Nutrient utilisation

Apparent protein deposition of YTK was significantly influenced by treatment ($P = 0.003$; one-factor ANOVA; Table 3.2.3.2.4). The apparent protein deposition of fish was significantly higher when fed the split ration treatment (Treatment 4), compared to all other treatments. The apparent protein deposition of fish fed to apparent satiation tended to decrease as feeding frequency increased, and was significantly lower in fish fed three times daily to apparent satiation (Treatment 3) than those fed once daily (Treatment 1). Apparent protein deposition of fish fed twice daily (Treatment 2) was not significantly different than those fed three times daily (Treatment 3) or than those fed once daily to apparent satiation (Treatment 1; $P > 0.05$). The apparent energy deposition was not significantly influenced by feeding treatment ($P = 0.135$; one-factor ANOVA; Table 3.2.3.2.4), but tended to be lower in fish fed three times daily to apparent satiation. There was no significant difference for energy deposition between fish fed Treatment 1 or the split ration of Treatment 4 ($P > 0.05$; one-factor ANOVA; Table 3.2.3.2.4).

Whole fish fatty acid composition

Whole fish fatty acid levels were not significantly influenced by feeding treatment ($P > 0.05$; one-factor ANOVA; Table 3.2.3.2.5).

Blood haematology and biochemistry

All blood haematology and biochemistry parameters, osmolality (mosm kg^{-1}) and pH were not significantly influenced by feeding treatment ($P > 0.05$; one-factor ANOVA; Table 3.2.3.2.6).

Gastrointestinal morphology

Intraperitoneal fat (1.50-2.17%) and visceral index (5.76-6.60%) of YTK was not significantly influenced by feeding treatment ($P > 0.05$; one-factor ANOVA; Table 3.2.3.2.7). Hepatosomatic index (HSI; %) for fish in Treatment 1 was significantly lower than those fed Treatment 3 ($P = 0.031$), while there were no significant differences in HSI between other treatments ($P > 0.05$; one-factor ANOVA; Table 3.2.3.2.7). All fish in the current study were assessed for gastric dilation, and were determined to be in Stage 0 (healthy/no gastric dilation), which is defined as having pronounced/well defined folds throughout the phylorus, anterior and distal stomach.

Thickness of muscle and serosa, villus length, lamina propria thickness, total goblet cell number, eosinophilic droplets in epithelial cells and melanomacrophage centres in the hindgut were not significantly influenced by feeding treatment ($P > 0.05$; one-factor ANVOA; Table 3.2.3.2.7).

Apparent digestibility coefficients

Apparent digestibility coefficients for dry matter (59.9-70.4%), protein (81.3-88.2%) and energy (76.2-84.5%) were not significantly influenced by feeding treatment ($P > 0.05$; one-factor ANOVA; Table 3.2.3.2.7).

Discussion

The aim of this research was to improve the sustainable production of YTK by improving our knowledge of feeding frequencies and ration size at warm water temperatures. To achieve this, four feeding frequencies were investigated. YTK were fed to apparent satiation once, twice or three times daily, 7 days per week, or fed a split ration twice daily that was based on the ration of the fish fed to apparent satiation once daily (ration was split 66.67% and 33.33% and fed at 08:00 h and 16:00 h).

In the current study, the health of fish, in terms of blood haematology and biochemistry, and gastrointestinal morphology was not compromised by differences in feeding strategies. With regard to growth performance, the SGR of fish was not significantly different between treatments over the whole experiment. However, weight checks were conducted at four and eight weeks post-stocking. Growth differences between treatments of fish were observed between stocking and the first weight check at 28 days. During this 28 day period, fish fed to apparent satiation three times daily had superior SGR, but inferior FCR, than the other feeding strategies. Over the same period, fish fed to apparent satiation twice daily had superior SGR than fish fed once daily to apparent satiation, and fish fed the equivalent ration split over two feeds (Treatment 4). Importantly, fish fed to apparent satiation once and twice daily had similar FCR. Between the first weight check to the final harvest (56 days), when water temperatures decreased below 20 °C, there appeared to be no difference in growth by feeding large YTK to apparent satiation more than once daily. However, feeding fish to apparent satiation three times daily led to inferior FCR, compared to other treatments.

One of the most important factors that influenced these results was the change in water temperature throughout the experiment. Water temperature influences numerous metabolic and physiological parameters, feed consumption, growth and survival of YTK (Pirozzi and Booth, 2009; Bansemer et al.,

2015; Bowyer et al., 2013a; Bowyer et al., 2013b; Stone and Bowyer, 2013; Stone et al., 2016). Throughout the experiment, water temperatures decreased from 23 °C to 16 °C. However, during the 28 day period between stocking and the first weight check, the average water temperature was 21.8 °C (range 23-20 °C). During this period of warmer water temperature, YTK exhibited a higher growth potential, which may have been limited by feed intake (Bowyer et al., 2013; Stone et al., 2016). During the 56 day period between the first weight check and final harvest, the water temperature only exceeded 20 °C on five days, and the average water temperature was 18.3 °C (range 22-16 °C). In agreement with collaborating industry partners, Stone and Bowyer (2013) defined sub-optimal water temperatures for juvenile YTK as 18 °C. Bowyer et al. (2013a) reported the feed intake for juvenile YTK (22.6 g) at 22 °C was two times greater than at 18 °C, while the growth rate was 48% higher. In the current study, between the first weight check and final harvest when water temperatures were considered predominantly sub-optimal, growth potential may have been limited by factors other than feed intake. At sub-optimal water temperatures, the growth and metabolic rate of YTK is impaired, feed intake reduced, gut transit time prolonged and digestive enzyme activity is decreased compared to YTK at optimal warm water temperatures (Pirozzi and Booth, 2009; Miegel et al., 2010; Booth et al., 2010; Bowyer et al., 2013a; b; Bansemer et al., 2015; Stone and Bowyer, 2013; Stone et al., 2016).

With regard to digestive efficiency, no significant effects were observed in apparent diet digestibility due to feeding treatments (Table 3.2.3.2.6). Doherty (2018; Honours project related to Manuscript 3.2.3.2), also reported no significant effect of feeding treatment on lipase or trypsin activity in the digestive tract. However, Doherty (2018) reported both digestive enzymes were significantly higher in the pyloric caecae than the posterior region of the digestive tract. Trypsin activity was also reported to be significantly correlated with feed intake rate ($P = 0.018$; $r = 0.757$) and FCR ($P = 0.043$; r = 0.682), corroborating findings from other studies involving YTK (Bowyer et al., 2012; Bowyer et al., 2014; Doherty, 2018).

In the current study, Doherty (2018) also measured the activity and distribution of the digestive enzyme dipeptidyl peptidase-4 (DPP4) in response to increasing feed frequency and split rationing. The role of DPP4 is to regulate bioactive peptides during digestion and was measured in adult YTK for the first time during this study (Doherty, 2018). Membrane-bound DPP4 activity was shown to significantly increase as feed intake increased with feeding treatment. Activities of DPP4 were highest in the membrane-bound component of gastrointestinal tissues and lowest in the blood serum of YTK (Doherty, 2018). Consistent with the distribution of lipase and trypsin activities, DPP4 activity was highest in the pyloric caecae (6.12 nm U mg protein^{-1}) and lowest in the mid-section of the gastrointestinal tract (0.44 nm U mg protein^{-1}). Cytoplasmic DPP4 activity was not significantly different between any gastrointestinal sections within any feeding treatment, nor was blood serum DPP4 activity. However, cytoplasmic DPP4 activity was negatively correlated with specific growth rate ($P = 0.006$, $r = -0.824$). Doherty (2018) suggested these finding warrants further investigation into role of DPP4 in regulating bioactive peptides in relation to feed intake and digestion in YTK.

When results for growth performance from this Manuscript are compared to those for slightly smaller YTK (~1.5 kg fish^{-1}; Bansemer et al., Manuscript 3.2.3.1) fed at a range of frequencies at cooler water temperatures (11.5-16 °C), it appears that daily feeding to apparent satiation, or at least for 6 days per week, may also be advantageous to ensure health and optimum growth at temperatures below 16 °C. However, due to differences in metabolic rate between fish sizes between Manuscripts, and the associated impacts on feed utilisation and growth performance (Jobling, 1994; Pirozzi and Booth, 2009), further research is warranted in this area.

With regard to the split ration feeding regime, under the experimental temperature regime, which mirrored commercial conditions on-farm in South Australia, results clearly demonstrate that there was no benefit in adopting this method. Fish from the split ration treatment were fed at a rate of 0.75% BW d^{-1}. This intake rate was based on the voluntary apparent satiation feed intake rate of fish fed once daily, and was split by 66.67% and 33.33% and fed daily at 08:00 h and 16:00 h, respectively. On a physiological basis, even though there was a significant improvement in protein deposition when fish were fed the split ration, there were no advantages gained in terms of improved energy deposition, nutrient digestibility, FCR or ultimately growth for large YTK (Table 3.2.3.2.3). Split ration feeding may be more beneficial for smaller juvenile YTK, which possess a higher metabolic rate (Jobling, 1994; Pirozzi and Booth, 2009). Further research in this area is warranted.

Conclusions and Recommendations

In conclusion, there is scope to improve commercial productivity for large YTK production by altering the frequency of feeding practices in response to seasonal fluctuations in water temperatures. With regard to SGR and FCR, it is recommend that large YTK are fed to apparent satiation twice daily at water temperatures > 20 °C, and fed to apparent satiation once daily as water temperatures drop from 20 to 16 °C and possibly lower. However, further research in pilot scale commercial trials are needed to validate results from the current study before implementing these altered feeding strategies under commercial conditions. There did not appear to be any benefit in adopting a split ration feeding strategy for large YTK.

Findings

- There is considerable scope to improve the productivity of large (≥1.5 kg fish^{-1}) YTK production by altering apparent satiation feeding frequency in response to fluctuations in seasonal water temperatures.
- In order to improve growth rate and FCR, large fish may be fed to apparent satiation at least twice daily at water temperatures > 20 °C, and fed to apparent satiation once daily as water temperatures drop from 20 to 16 °C, and potentially lower. The logistics and economics associated with the adoption of multi-meal feeding strategies will need to be assessed on a case by case basis by YTK producers.
- An improvement in FCR based on the information provided within this Manuscript, will assist producers developing feeding strategies that achieve one of the overarching goals of the K4P project, which was to provide information to assist producers to achieve FCRs of < 2.2 for large YTK between 1.5-3.5 kg.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The authors would also like to acknowledge the support of the South Australian Research and Development Institute (SARDI) and Marine Innovation Southern Australia for their financial support of Assoc. Prof. David Stone and the provision of the SARDI SAASC experimental facilities at West Beach, South Australia. Thanks to Dr Leo Nankervis and Dr Nicole Ruff of Skretting Australia for input into experimental design, diet formulation and diet manufacture. We would also thank Dr Richard Smullen and Dr Michael Salini of Ridley and Dr Trent D'Antignana Clean Seas Seafood for input into experimental design and diet formulation. Thanks to Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd.) for the diagnosis and prescription of skin and gill fluke treatments. We also thank Ben Crowe, Jackson Doherty, Filipa Duarte, Mark Purvis, and Jessica Buss for their technical assistance during the experiment.

References

Arnold, S., Smullen, R., Briggs, M., West, M., Glencross, B., 2015. The combined effect of feed frequency and ration size of diets with and without microbial biomass on the growth and feed conversion of juvenile *Penaeus monodon.* Aquaculture Nutrition, 1340-1347.

Azzaydi, M., Martínez, F.J., Zamora, S., Sánchez-Vázquez, F.J., Madrid, J.A., 1999. Effect of meal size modulation on growth performance and feeding rhythms in European sea bass (*Dicentrarchus labrax*, L.). Aquaculture 170, 253-266.

Bansemer, M.S., Forder, R.E.A., Howarth, G.S., Suitor, G.M., Bowyer, J.N., Stone, D.A.J., 2015. The effect of dietary soybean meal and soy protein concentrate on the intestinal mucus layer and the development of sub-acute enteritis in Yellowtail Kingfish (*Seriola lalandi*) at suboptimal water temperature. Aquaculture Nutrition 21, 300-310.

Bansemer, M.S., Stone, D.A.J., D'Antignana, T., Skordas, P., Kuerschner, L., Currie, K-L., 2018. Optimising feeding strategies for Yellowtail Jack (*Seriola lalandi*) at winter water temperatures. North American Journal of Aquaculture 80, 128-140.

Booth, M.A., Pirozzi, I., Allan, G.L., Fielder, S. 2010. Aquafin CRC project 1B5: Feed technology for temperate fish species. Vol. 2, diet development. Aquafin CRC. FRDC Project No. 2004/220. July 2010, pp. 376.

Bowyer, J.N., Qin, J.G., Adams, L.R., Thomson, M.J., Stone, D.A.J., 2012. The response of digestive enzyme activities and gut histology in yellowtail kingfish (*Seriola lalandi*) to dietary fish oil substitution at different temperatures. Aquaculture 368, 19-28.

Bowyer, J.N., Qin, J.G., Smullen, R.P., Adams, L.R., Stone, D.A.J., 2013a. The use of a soy product in Yellowtail Kingfish (*Seriola lalandi*) feeds at different water temperatures: 1. Solvent extracted soybean meal. Aquaculture 384-387, 35-45.

Bowyer, J.N., Qin J.G., Smullen, R.P., Adams, L.R., Stone, D.A.J., 2013b. The use of a soy product in Yellowtail Kingfish (*Seriola lalandi*) feeds at different water temperatures: 2. Soy protein concentrate. Aquaculture. 410-411, 1-10.

Bowyer, J.N., Booth, M.A., Qin, J.G., D'Antignana, T., Thomason, M.J.S., Stone, D.A.J., 2014. Temperature and dissolved oxygen influence growth and digestive enzyme activities of yellowtail kingfish *Seriola lalandi* (Valenciennes, 1833). Aquaculture Research 45, 2010-2020.

Chown, S., 2015. Impact of diet retention on gastric evacuation time, growth and the development of gastric dilation in Yellowtail Kingfish (*Seriola lalandi*). Honours thesis, Flinders University, South Australia, Australia. 85 pp.

Doherty, J.G., 2018. Digestive enzyme activity of Yellowtail Kingfish (*Seriola lalandi*) in response to different feeding strategies. Honours thesis, Flinders University, South Australia, Australia. 74 pp.

García-Mesa, S., Suárez, M.D., Rodríguez-Rúa, A., Cárdenas, S., García-Gallego, M. 2014. Productive and physiological implications of different feeding frequencies in meagre *Argyrosomus regius* (Asso, 1801). Aquacultural Engineering 60, 6-13.

Huang, Q., Huang, K., Ma, Y., Qin, X., 2015. Feeding Frequency and Rate Effects on Growth and Physiology of Juvenile Genetically Improved Farmed Nile Tilapia. North American Journal of Aquaculture 77, 503-512.

Imsland, A.K., Våge, K.A., Handeland, S.O., Stefansson, S.O., 2011. Growth and osmoregulation in Atlantic salmon (*Salmo salar*) smolts in response to different feeding frequencies and salinities. Aquaculture Research 42, 469-479.

Jarboe, H.H., Grant, W.J., 1996. Effects of feeding time and frequency on the growth of Channel Catfish *Ictalurus punctatus* in closed recirculating raceway systems. Journal of the World Aquaculture Society 27, 235-239.

Jobling, M. 1994. Fish Bioenergetics, Chapman & Hall, London, UK.

Kohbara, J., Hidaka, I., Matsuoka, F., Osada, T., Furukawa, K., Yamashita, M., Tabata, M., 2003 Self-feeding behavior of yellowtail, *Seriola quinqueradiata*, in net cages: diel and seasonal patterns and influences of environmental factors. Aquaculture 220, 581-594.

Li, X-F., Tian, H-Y., Zhang D-D., Jiang, G-Z., Liu, W-B., 2014. Feeding frequency affects stress, innate immunity and disease resistance of juvenile blunt snout bream *Megalobrama amblycephala.* Fish and Shellfish Immunology 38, 80-87.

Miegel, R.P., Pain, S.J., Van Wettere, W.H.E.J., Howarth, G.S., Stone, D.A.J., 2010. Effect of water temperature on gut transit time, digestive enzyme activity and nutrient digestibility in yellowtail kingfish (*Seriola lalandi*). Aquaculture 308, 145-151.

Pirozzi, I., Booth, M.A., 2009. The routine metabolic rate of mulloway (*Argyrosomus japonicus*: Sciaenidae) and yellowtail kingfish (*Seriola lalandi*: Carangidae) acclimated to six different temperatures. Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology152, 586-592.

Stone, D.A.J., Bellgrove, E., 2013. A literature review: the current status of knowledge of the nutritional requirements of yellowtail kingfish. In: Stone, D.A.J. and Bowyer, J.N. (Eds). Final Report. Sustainable Feeds and Feed Management for Yellowtail Kingfish (*Seriola lalandi*). South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2013/000200-1. SARDI Research Report Series No. 751. pp. 92-121.

Stone, D.A.J., Bowyer, J.N., 2013. Final Report. Sustainable Feeds and Feed Management for Yellowtail Kingfish (*Seriola lalandi*). South Australian Research and Development Institute (Aquatic Sciences), Adelaide. SARDI Publication No. F2013/000200-1. SARDI Research Report Series No. 751. 220 pp.

Stone, D.A.J., D'Antignana, T., Bansemer, M.S., 2016. Final Report. Refining Yellowtail Kingfish feeds and feed management. Prepared by the South Australian Research and Development Institute (Aquatic Sciences), Adelaide. AS-CRC Project No. 2013/730. 144 pp.

Teoh, A., 2016. Effects of feed induced intermittent hypoxia on digestive physiology and stress response in yellowtail kingfish, *Seriola lalandi.* Honours thesis, Flinders University, Adelaide, Australia. 86 pp.

Table 3.2.3.2.1. Analysed proximate composition, rancidity test and amino acid composition of the formulated diet used in the current study.

Item (as fed)	Control diet
Analysed proximate composition (g 100 g^{-1})	
Moisture	8.7
Crude protein	45.4
Crude lipid	24.1
Ash	8.9
Carbohydrate[1]	13.0
Gross energy (MJ kg^{-1})	18.80
Rancidity test	
p-Anisidine Value	5.3
Peroxide Value (mEqO2 kg^{-1})	6.3
Analysed amino acids (g 100 g^{-1})	
Alanine	1.93
Arginine	2.26
Aspartic Acid	2.92
Glutamic Acid	6.63
Glycine	2.01
Histidine	1.28
Hydroxyproline	0.34
Isoleucine	1.41
Leucine	3.06
Lysine	2.41
Methionine	1.08
Phenylalanine	1.86
Proline	2.27
Serine	1.61
Threonine	1.47
Tyrosine	1.13
Total Amino Acids	35.60
Taurine	1.02

[1] Carbohydrate = 100 - (moisture + lipid + protein + ash).

Table 3.2.3.2.2. Analysed fatty acid composition of the formulated diet used in the current study.

Item (as fed)	Control diet
Analysed fatty acids (mg 100 g^{-1})	
Saturated Fatty Acids	
C4:0 Butyric	<10
C6:0 Caproic	<10
C8:0 Caprylic	<10
C10:0 Capric	<10
C12:0 Lauric	<10
C14:0 Myristic	795
C15:0 Pentadecanoic	72
C16:0 Palmitic	4989
C17:0 Margaric	96
C18:0 Stearic	1542
C20:0 Arachidic	48
C22:0 Behenic	48
C24:0 Lignoceric	24
Mono-unsaturated Fatty Acids	
C14:1 Myristoleic	24
C16:1 Palmitoleic	1326
C17:1 Heptadecenoic	<10
C18:1 Oleic	6965
C20:1 Eicosenic	193
C22:1 Docosenoic	24
C24:1 Nervonic	72
Poly-unsaturated Fatty Acids	
C18:2n6 Linoleic	2531
C18:3n6 gamma-Linolenic	24
C18:3n3 alpha-Linolenic	458
C20:2n6 Eicosadienoic	24
C20:3n6 Eicosatrienoic	24
C20:3n3 Eicosatrienoic	<10
C20:4n6 Arachidonic	193
C20:5n3 Eicosapentaenoic	1542
C22:2n6 Docosadienoic	<10
C22:4n6 Docosatetraenoic	<10
C22:5n3 Docosapentaenoic	241
C22:6n3 Docosahexaenoic	1615
∑LC n-3 PUFA	3398
Total Saturated	7664
Total Mono-unsaturated	8628
Total Poly-unsaturated	6724
Omega 6 Fatty Acids	2844
Omega 3 Fatty Acids	3880
Total Mono Trans Fatty Acids	217
Total Poly Trans Fatty Acids	241
P:M:S Ratio	0.9:1.1:1

Table 3.2.3.2.3. Summary of water quality parameters.

Item[1]	Temperature (°C)	Dissolved oxygen (mg L^{-1})	Dissolved oxygen (% saturation)	pH	Salinity (mg L^{-1})	Ammonia (ppm)	CO_2 (mg L^{-1})
Mean	19.5 ± 2.1	7.9 ± 0.9	103.3 ± 8.5	7.74 ± 0.12	38 ± 0	0.22 ± 0.13	1 ± 0
Range	16.0 - 23.0	5.5 - 11.6	80.0 - 139.0	7.41 - 7.97	36 - 38	0.00 - 1.00	0 - 3

[1] Values means ± standard deviation.

Table 3.2.3.2.4. Growth performance, feed utilisation, proximate composition and nutrient retention of Yellowtail Kingfish fed different feed rates for 84 days at warm water temperatures.

Treatment[1,2]	1	2	3	4	ANOVA[3]
Growth performance					
Initial weight (kg)	3.12±0.01	3.11±0.02	3.11±0.01	3.12±0.04	$P = 0.990$
Final weight (kg)	4.17±0.06	4.23±0.02	4.27±0.01	4.18±0.08	$P = 0.503$
Biomass gain (kg tank^{-1})	23.17±1.06	24.61±0.46	25.58±0.50	23.38±0.73	$P = 0.142$
SGR (% d^{-1})	0.35±0.01	0.37±0.01	0.38±0.01	0.35±0.01	$P = 0.089$
Initial fork length (mm)	576±2	574±1	576±1	576±2	$P = 0.892$
Final fork length (mm)	622±4	620±2	623±1	623±3	$P = 0.847$
Length growth rate (mm d^{-1})	0.55±0.02	0.55±0.01	0.57±0.02	0.57±0.02	$P = 0.709$
Final Condition factor	1.73±0.01^{a}	1.77±0.01^{b}	1.77±0.00^{b}	1.72±0.00^{a}	$P = 0.002$
Feed utilisation (as fed)					
Apparent feed consumption (kg tank^{-1})	46.70±0.22^{c}	54.95±0.57^{b}	67.84±0.94^{a}	46.70±0.00^{c}	$P < 0.001$
Apparent feed intake (% BW d^{-1})	0.75±0.01^{c}	0.87±0.01^{b}	1.07±0.02^{a}	0.75±0.01^{c}	$P < 0.001$
Apparent FCR	2.02±0.09^{b}	2.23±0.03^{b}	2.65±0.04^{a}	2.00±0.06^{b}	$P < 0.001$
Whole proximate composition (wet basis)[4]					
Moisture (%)	62.0±0.4	62.2±0.3	61.7±0.3	62.4±0.9	$P = 0.827$
Protein (%)	19.5±0.5	19.7±0.0	19.6±0.3	20.4±0.2	$P = 0.338$
Lipid (%)	16.5±0.3	16.9±0.6	16.8±0.5	15.9±0.8	$P = 0.605$
Ash (%)	3.1±0.5	2.2±0.1	2.7±0.1	2.4±0.3	$P = 0.224$
Carbohydrate (%)	<1	<1	<1	<1	-
Energy (MJ kg^{-1})	9.43±0.20	9.60±0.20	9.53±0.23	9.33±0.24	$P = 0.837$
Nutrient retention[5]					
Apparent PD	23.98±1.56^{b}	22.06±0.33^{bc}	18.40±0.91^{c}	27.86±1.47^{a}	$P = 0.003$
Apparent ED	27.45±1.25	26.18±2.05	21.44±1.40	26.60±2.02	$P = 0.135$

[1] Values are mean ± SE; $n = 3$.

[2] Feed rate treatments: Treatment 1, apparent satiation once daily 7 days week^{-1} (08:00 h); Treatment 2, apparent satiation twice daily 7 days week^{-1} (08:00 h and 16:00 h); Treatment 3, apparent satiation three times daily 7 days week^{-1} (08:00 h, 12:00 h and 16:00 h); Treatment 4, fed twice daily to the ration provided in Treatment 1. Ration was split by 66.67 and 33.33%) and fed at 08:00 h and 16:00 h.

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests (Student-Newman-Keuls test) were used to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

[4] Initial fish proximate composition (wet basis): Moisture 64.0%, protein 18.7%, lipid 15.9%, ash 2.7%, carbohydrate <1%, energy 9.10 MJ kg^{-1}.

[5] ED = energy deposition; PD = protein deposition.

Table 3.2.3.2.5. Fatty acid composition (mg 100 g^{-1}) of whole Yellowtail Kingfish fed different feed rates for 84 days at warm water temperatures.

Treatment[1,2]	1	2	3	4	ANOVA[3]
Saturated Fatty Acids					
C4:0 Butyric	<0.1	<0.1	<0.1	<0.1	-
C6:0 Caproic	<0.1	<0.1	<0.1	<0.1	-
C8:0 Caprylic	<0.1	<0.1	<0.1	<0.1	-
C10:0 Capric	<0.1	<0.1	<0.1	<0.1	-
C12:0 Lauric	<0.1	<0.1	<0.1	<0.1	-
C14:0 Myristic	429.9±8.3	434.2±9.2	441.8±11.7	418.7±23.9	$P = 0.735$
C15:0 Pentadecanoic	49.6±1.0	50.8±1.8	50.4±1.6	47.7±2.3	$P = 0.605$
C16:0 Palmitic	2904.9±71.6	2956.0±81.9	3008.6±65.4	2812.6±137.6	$P = 0.530$
C17:0 Margaric	66.1±1.3	67.7±2.3	67.2±2.1	63.6±3.1	$P = 0.605$
C18:0 Stearic	986.7±23.7	1020.8±24.8	1045.7±8.5	947.7±37.7	$P = 0.115$
C20:0 Arachidic	33.1±0.6	39.3±5.0	33.6±1.0	31.8±1.5	$P = 0.280$
C22:0 Behenic	33.1±0.6	27.8±4.9	28.0±5.7	26.7±5.8	$P = 0.785$
C24:0 Lignoceric	<0.1	<0.1	<0.1	<0.1	-
Total Saturated	4530.8±106.4	4630.7±112.9	4720.1±96.0	4413.4±222.5	$P = 0.509$
Mono-unsaturated Fatty Acids					
C14:1 Myristoleic	<0.1	<0.1	<0.1	<0.1	-
C16:1 Palmitoleic	1008.5±19.4	1049.3±28.2	1046.3±26.6	1001.5±55.5	$P = 0.692$
C17:1 Heptadecenoic	<0.1	<0.1	<0.1	<0.1	-
C18:1 Oleic	5980.3±133.8	6135.7±220.4	6101.9±171.5	5754.6±280.1	$P = 0.587$
C20:1 Eicosenic	176.2±3.0	169.3±5.8	168.0±5.2	159.0±7.6	$P = 0.276$
C22:1 Docosenoic	16.5±0.3	16.9±0.6	16.8±0.5	15.9±0.8	$P = 0.605$
C24:1 Nervonic	37.1±5.3	37.1±5.3	37.1±5.3	31.8±0.0	$P = 0.802$
Total Mono-unsaturated	6953.6±19.1	6974.8±41.4	6996.0±96.7	6985.4±135.3	$P = 0.987$
Poly-unsaturated Fatty Acids					
C18:2n6 Linoleic	1727.8±21.2	1701.3±9.2	1680.1±23.1	1727.8±29.5	$P = 0.403$
C18:3n6 gamma-Linolenic	15.9±0.0	21.2±5.3	15.9±0.0	15.9±0.0	$P = 0.441$
C18:3n3 alpha-Linolenic	249.1±5.3	249.1±5.3	243.8±10.6	254.4±15.9	$P = 0.908$
C20:2n6 Eicosadienoic	31.8±0.0	31.8±0.0	31.8±0.0	31.8±0.0	-
C20:3n6 Eicosatrienoic	15.9±0.0	26.5±5.3	21.2±5.3	21.2±5.3	$P = 0.487$
C20:3n3 Eicosatrienoic	<0.1	<0.1	<0.1	<0.1	-
C20:4n6 Arachidonic	111.3±0.0	106.0±5.3	106.0±5.3	100.7±10.6	$P = 0.728$
C20:5n3 Eicosapentaenoic	551.2±10.6	551.2±21.2	514.1±52.2	535.3±53.0	$P = 0.892$
C22:2n6 Docosadienoic	<0.1	<0.1	<0.1	<0.1	-
Omega 3 Fatty Acids	1876.2±33.1	1881.5±74.8	1770.2±190.4	1807.3±216.9	$P = 0.939$
Omega 6 Fatty Acids	1950.4±21.2	1923.9±15.9	1892.1±33.1	1934.5±45.3	$P = 0.616$
C22:4n6 Docosatetraenoic	15.9±0.0	21.2±5.3	15.9±0.0	21.2±5.3	$P = 0.596$
C22:5n3 Docosapentaenoic	233.2±5.3	227.9±5.3	222.6±24.3	217.3±23.1	$P = 0.921$
C22:6n3 Docosahexaenoic	832.1±23.1	826.8±40.0	773.8±110.7	789.7±128.6	$P = 0.955$
Total Poly-unsaturated	3826.6±43.4	3805.4±90.6	3662.3±223.4	3741.8±260.3	$P = 0.913$
Trans Fatty Acids					
Total Mono Trans Fatty Acids	95.4±0.0	90.1±5.3	95.4±0.0	95.4±0.0	$P = 0.441$
Total Poly Trans Fatty Acids	137.8±5.3	143.1±0.0	132.5±5.3	132.5±5.3	$P = 0.363$

[1] Values are mean ± SE; $n = 3$.

[2] Feed rate treatments: Treatment 1, apparent satiation once daily 7 days $week^{-1}$ (08:00 h); Treatment 2, apparent satiation twice daily 7 days $week^{-1}$ (08:00 h and 16:00 h); Treatment 3, apparent satiation three times daily 7 days $week^{-1}$ (08:00 h, 12:00 h and 16:00 h); Treatment 4, fed twice daily to the ration provided in Treatment 1. Ration was split by 66.67 and 33.33%) and fed at 08:00 h and 16:00 h.

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests (Student-Newman-Keuls test) were used to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

Table 3.2.3.2.6. Blood haematology and biochemistry of Yellowtail Kingfish fed different feed rates for 84 days at warm water temperatures.

Treatment[1,2]	1	2	3	4	ANOVA[3]
Biochemistry[4]					
Sodium (mmol L^{-1})	200±1	201±2	199±2	197±2	$P = 0.456$
Potassium (mmol L^{-1})	3.3±0.3	4.4±0.5	4.5±0.5	5.1±1.7	$P = 0.610$
Urea (mmol L^{-1})	1.2±0.0	1.4±0.1	1.3±0.1	1.2±0.1	$P = 0.158$
Creatinine (mmol L^{-1})	0.018±0.001	0.020±0.001	0.019±0.000	0.019±0.001	$P = 0.364$
Calcium (mmol L^{-1})	3.25±0.02	3.17±0.02	3.14±0.03	3.16±0.06	$P = 0.250$
Protein (g L^{-1})	41±1	42±1	41±1	42±1	$P = 0.781$
Albumin (g L^{-1})	14±0	14±0	14±1	13±0	$P = 0.627$
Globulin (g L^{-1})	27±1	28±1	27±1	29±1	$P = 0.296$
Total Bilirubin (mmol L^{-1})	1±0	2±0	2±0	4±3	$P = 0.653$
ALT (IU L^{-1})	8±1	8±1	9±2	10±3	$P = 0.868$
ALP (IU L^{-1})	13±1	13±1	14±0	14±1	$P = 0.606$
Magnesium (mmol L^{-1})	1.48±0.05	1.51±0.17	1.51±0.07	1.43±0.03	$P = 0.922$
Cholesterol (mmol L^{-1})	6.4±0.3	6.4±0.2	6.4±0.3	6.9±0.5	$P = 0.603$
Triglyceride (mmol L^{-1})	1.19±0.08	1.43±0.12	1.21±0.10	5.87±4.59	$P = 0.438$
Bile Acids (mmol L^{-1})	2.6±0.3	3.6±1.0	3.3±0.9	2.5±0.0	$P = 0.629$
Osmolality (mosm kg^{-1})	408±1	414±3	406±3	407±3	$P = 0.193$
pH	7.73±0.03	7.70±0.03	7.72±0.05	7.63±0.01	$P = 0.205$
Haematology[5]					
RBC ($\times 10^{12}$)	2.89±0.15	2.98±0.13	2.69±0.31	3.10±0.11	$P = 0.515$
HGB (g L^{-1})	124±4	132±3	123±2	131±2	$P = 0.101$
PCV (L L^{-1})	0.525±0.028	0.534±0.028	0.488±0.052	0.561±0.020	$P = 0.541$
MCV (fl)	181.9±0.7	179.3±4.1	181.6±1.7	181.3±1.1	$P = 0.851$
MCH (pg)	43.4±1.2	45.1±2.9	47.9±5.9	42.5±1.2	$P = 0.698$
MCHC (g L^{-1})	239±7	252±21	265±34	234±7	$P = 0.712$
WBC ($\times 10^{9}$)	7.5±1.2	9.4±2.5	13.2±2.2	11.3±2.5	$P = 0.346$
Granulocytes (%)	3±3	3±3	12±7	9±6	$P = 0.511$
Lymph (%)	83±4	81±3	74±9	75±8	$P = 0.708$
Mono (%)	14±3	16±3	14±3	16±3	$P = 0.931$
Eosin (%)	0±0	0±0	0±0	0±0	-
Baso (%)	0±0	0±0	0±0	0±0	-
Platelets ($\times 10^{9}$)	95±27	96±20	59±29	48±15	$P = 0.398$

[1] Values are mean ± SE; $n = 3$.

[2] Feed rate treatments: Treatment 1, apparent satiation once daily 7 days $week^{-1}$ (08:00 h); Treatment 2, apparent satiation twice daily 7 days $week^{-1}$ (08:00 h and 16:00 h); Treatment 3, apparent satiation three times daily 7 days $week^{-1}$ (08:00 h, 12:00 h and 16:00 h); Treatment 4, fed twice daily to the ration provided in Treatment 1. Ration was split by 66.67 and 33.33%) and fed at 08:00 h and 16:00 h.

[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests (Student-Newman-Keuls test) were used to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).

[4] ALT = alanine aminotransferase; ALP = alkaline phosphatase

[5] Smear content: red and white cell normal; Baso = basophil; Eosin = eosinophil; HGB = haemoglobin; Lymph = lymphocytes; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; MCV = mean corpuscular volume; Mono = monocytes; PCV = packed cell volume; RBC = red blood cell count; WBC = white blood cell count.

Table 3.2.3.2.7. Visceral somatic parameters, gastrointestinal morphology and apparent digestibility coefficient of Yellowtail Kingfish fed different feed rates for 84 days.

Treatment[1,2]	1	2	3	4	ANOVA[3]
Visceral somatic parameters					
Intraperitoneal fat (%)	2.03±0.09	1.50±0.25	2.17±0.15	1.94±0.14	$P = 0.095$
Viscerosomatic index (VSI; %)	5.76±0.10	6.35±0.21	6.60±0.14	5.99±0.27	$P = 0.056$
Hepatosomatic index (HSI; %)	0.94±0.03[b]	1.06±0.05[ab]	1.13±0.02[a]	1.03±0.03[ab]	$P = 0.031$
Stomach morphology					
Gastric dilation score[4]	0±0	0±0	0±0	0±0	-
Hindgut morphology					
Thickness of muscle and serosa (µm)	846±49	766±47	785±94	806±15	$P = 0.683$
Villi length (µm)	1561±146	1966±73	1940±13	1757±58	$P = 0.064$
Lamina propria thickness (µm)	31±3	29±2	35±2	29±3	$P = 0.544$
Mucus cells per 100 µm	2.88±0.22	3.60±0.40	4.12±0.60	4.64±1.00	$P = 0.303$
Eosinophilic droplets in epithelial cells	1±0	2±0	2±0	2±0	$P = 0.083$
Melanomacrophage centres	3±0	3±0	4±0	4±0	$P = 0.094$
Apparent digestibility coefficient (ADC; %)					
Dry matter	68.9±6.0	70.4±0.9	65.6±4.3	59.9±7.7	$P = 0.546$
Protein	87.6±1.2	88.2±1.1	84.9±2.2	81.3±3.7	$P = 0.207$
Energy	80.6±4.4	84.5±1.2	80.2±2.8	76.2±4.5	$P = 0.468$

[1] Values are mean ± SE; $n = 3$.
[2] Feed rate treatments: Treatment 1, apparent satiation once daily 7 days week^{-1} (08:00 h); Treatment 2, apparent satiation twice daily 7 days week^{-1} (08:00 h and 16:00 h); Treatment 3, apparent satiation three times daily 7 days week^{-1} (08:00 h, 12:00 h and 16:00 h); Treatment 4, fed twice daily to the ration provided in Treatment 1. Ration was split by 66.67 and 33.33%) and fed at 08:00 h and 16:00 h.
[3] A significance level of $P < 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments, values without a common superscript are significantly different (a indicates the highest value; $P < 0.05$).
[4] Gastric dilation score based on Chown (2015).

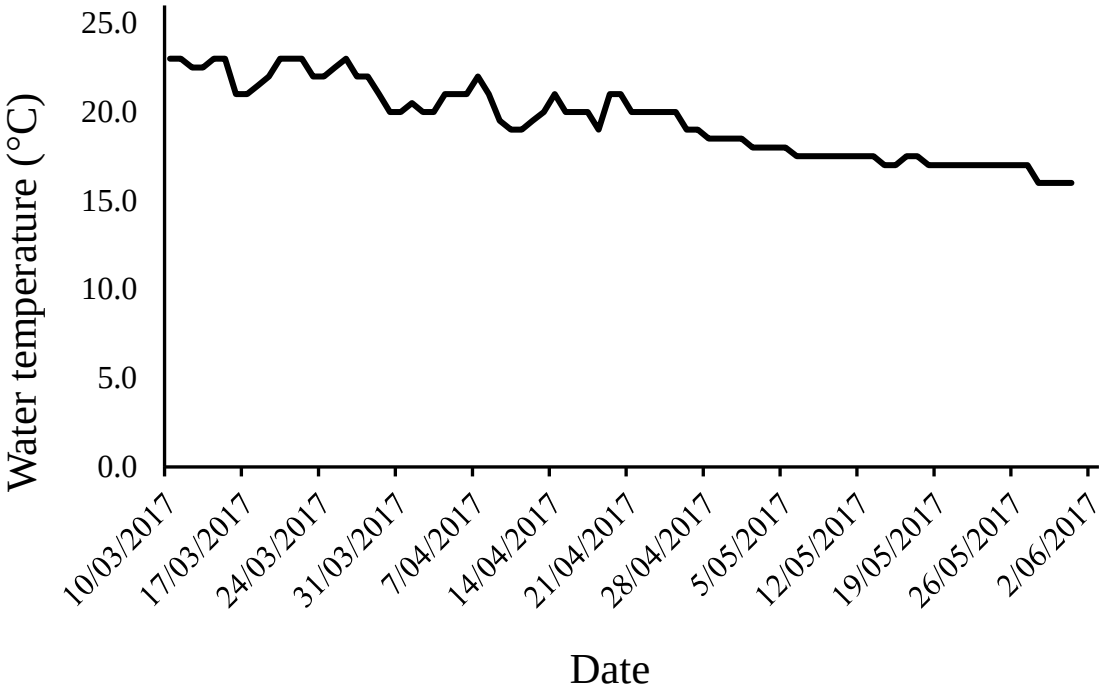


Figure 3.2.3.2.1. Water temperature profile in the experimental system between stocking and the final weight check (84 days) (average 19.5 °C [range 23-16 °C]).

3.2.4. Chapter - Feeding frequencies for sub-adult Yellowtail Kingfish at different water temperatures.

3.2.4.1. Manuscript - Evaluating the impact of feeding strategy and pellet diameter on the growth and feed utilisation of sub-adult Yellowtail Kingfish (Seriola lalandi) reared at 16 °C and 24 °C.

Mark Booth [a], Basseer Codabaccus [a, b], Igor Pirozzi [a, c]

[a] New South Wales Department of Primary Industries, Port Stephens Fisheries Research Institute, Taylors Beach 2316, NSW, Australia.

[b] IMAS Fisheries and Aquaculture Centre, University of Tasmania, Taroona 7053, TAS, Australia.

[c] College of Science and Engineering and Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville 4801, QLD, Australia.

This manuscript may be referenced as: Booth, M., Codabaccus, B., and Pirozzi, I. (2019). 3.2.4.1. Manuscript - Evaluating the impact of feeding strategy and pellet diameter on the growth and feed utilisation of sub-adult Yellowtail Kingfish (*Seriola lalandi*) reared at 16 °C and 24 °C (Output 3c). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.384-405.

Abstract

In this paper we present the results of two independent, but closely related feeding experiments with juvenile Yellowtail Kingfish (*Seriola lalandi*; YTK). The experiments were designed to evaluate the effect of different feeding regimes and different pellet diameters on the performance and feed utilisation of YTK. Each experiment used the same commercial feeds and each experiment was done in a similar recirculating aquaculture system; one at a water temperature of 16 °C and the other at a water temperature of 24 °C. Adjunct trials were done at the conclusion of each feeding experiment to determine the digestibility of the commercial feed and to examine gastric evacuation rate (GER). The experiments have demonstrated that growth rate (SGR) and feed conversion ratio (FCR) of YTK are better, respectively, in fish reared at 24 °C as opposed to 16 °C. The results also provide strong evidence that feeding sub-adult YTK a single meal to apparent satiety once per day supports acceptable growth rate and feed utilisation, irrespective of water temperature. There was no evidence that dividing meals into equal sized portions during the day benefited SGR or FCR. The apparent proximate digestibility of the commercial diet determined using stripping methods was relatively unaffected by water temperature. However, lipid apparent digestibility was slightly depressed at 16 °C. The GER of sub-adult YTK is slower at 16 °C than at 24 °C. In addition, regardless of the temperatures investigated, fish weighing between 150-500 g were unable to consume more than about 3% of their body weight in a single meal. Refed fish appear to consume only as much food as has been evacuated. This wide ranging study provides an extensive data set that will assist YTK farm managers improve their on-farm feeding practices. Importantly, this study demonstrates the biological plasticity of YTK with respect to daily feeding regime and pellet size; abiotic factors that can be manipulated to improve the economic and environmental outcomes of farm raised fish. The YTK industry should continue to feed at least twice daily in farm situations to ensure the all fish have an opportunity to consume enough feed to support their growth potential. Ideally, more research should be done to understand the impact of water temperature on the digestibility of commercial diets and raw materials. Until better methods are developed, such studies should be done using stripping rather than dissection techniques.

Introduction

Choice of feed and feeding strategy represent major economic risks in intensive marine fish farming. Feeding preferences in fish are known to be affected by ontogeny, especially in hatchlings and very small juveniles (Bolliet et al., 2001; Fielder et al., 2002). Feeding frequency and duration also affect the performance and feed efficiency of larger farmed fish (Biswas et al., 2010). Therefore, it is critical to match an appropriate feeding strategy to each stanza of growth. It is also important to select an appropriate diet or feeding strategy to mitigate for prevailing or changing environmental conditions experienced by farmed fish (Kestemont and Baras, 2001; Watanabe et al., 2001a; de la Gandara et al., 2002; Yamamoto et al., 2007; Salama, 2008), otherwise fish performance and feed utilisation can be negatively affected. Typical abiotic factors influencing feeding behaviour that are often beyond the control of farmers operating in open waters include water temperature, dissolved oxygen, pH, salinity and photoperiod. Many biotic factors can also interact positively or negatively on the feeding behaviour of fish. These include stocking density, social or hierarchical structures within rearing pens, presence of predators and human disturbance (Kestemont and Baras, 2001).

It has been shown that feeding frequency in fish is strongly correlated with gastric clearance time (Lee et al., 2000; Riche et al., 2004) and that return of appetite is linked to gastric emptying rate (Riche et al., 2004). Thus matching the timing or frequency of feeding with peak appetite may improve the efficiency of production by enhancing growth and reducing feed conversion ratio (Bolliet et al., 2001; Dwyer et al., 2002; Booth et al., 2008). Optimising feeding regimes may also minimise feed wastage, leading to improvements in water quality and or reductions in size heterogeneity among cohorts of fish (Tucker et al., 2006; Booth et al., 2008), whereas sporadic feeding may lead to increased hunger, intra-specific aggression and increased rates of cannibalism (Folkvord and Ottera, 1993).

Little is known about the optimal feeding strategies for Australian farmed YTK. Common practice within the industry is to hand-feed or mechanically feed (e.g. air blowers) fish at least once a day, preferably in the morning and then offer fish a top-up feed in the afternoon. This twice-daily routine is mostly used on larger fish > 0.5 kg. Juvenile fish under this weight are often offered feed up to three times daily to ensure they are satiated and that all fish have an equal opportunity to feed. Different farming groups often use different feeding strategies to feed their YTK. The reasons for doing so are varied, but they are generally based on anecdotal rather than scientific evidence.

One of the most important abiotic factors influencing the feeding behaviour of farmed Yellowtail Kingfish (*Seriola lalandi*; YTK) is water temperature (Miegel et al., 2010; Bowyer et al., 2014;). Seasonal water temperature can range from 10 °C to as high as 28 °C in the locations that YTK are farmed (i.e New South Wales, South Australia, and Western Australia). Such environmental variability makes it inherently difficult to determine an appropriate feeding strategy for this species. For example, low water temperature often leads to prolonged decreases in feed intake resulting in reduced weight gain and elevated FCR. Better or more targeted feeding strategies may overcome or mitigate these responses. A biotic factor that may interact with feeding strategy is pellet size; i.e. when is it appropriate to increase pellet size? Small diameter pellets may be more digestible than larger pellets *per se* due to their high surface to volume ratio and their susceptibility to attack by gastric juices and digestive enzymes.

Two independent, but closely linked experiments were done to evaluate the effect of different feeding regimes and different pellet diameters (6 mm versus 9 mm) on growth, feed intake and feed utilisation in sub-adult YTK. One experiment was done at a water temperature of 16 °C and the other at a water temperature of 24 °C. The experiments were done in different but similar research laboratories. In addition to the primary aims, the effect of water temperature and feeding regime on the apparently digestibility and gut transit time of diets was examined, specifically to elucidate if feeding regime (once versus twice daily) or faecal collection method (i.e. stripping versus dissection technique) affected the results. Some of the data from each experiment was combined *post priori* to examine the wider implications of the selected feeding regimes on performance of YTK.

Methods

This study was performed under the NSW DPI Fisheries Animal Care and Ethics (ACEC) Research Authority known as ‘Aquaculture Nutrition ACEC 93/5–Port Stephens’. Care, husbandry and

termination of fish was carried out according to methods outlined in 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2015). Experiments were performed at NSW DPI's Port Stephens Fisheries Institute (PSFI).

Commercial aquafeeds selected for experiments

Two commercial aquafeeds, each with a different pellet size (6 mm versus 9 mm diameter) were chosen for experiments. These feeds were being used by our collaborating partner Huon Aquaculture Pty Ltd to feed YTK on the NSW DPI - Huon Aquaculture Marine Aquaculture Research Lease (MARL). The feeds were manufactured for Huon Aquaculture by Skretting Australia (Cambridge, Tasmania). The 6 mm and 9 mm diameter aquafeeds were composed of the same raw materials; however they had minor formulation differences (David Whyte; Huon Aquaculture; *personal communication*). The 9 mm feed had an approximately 3% increase in oil content which was offset by slight reductions in fishmeal, dehulled lupin and SPC content compared to the 6 mm feed.

Feeding regimes

Four feeding regimes were applied in each experiment and they were replicated across pellet diameter (see Table 3.2.4.1.1). Groups of fish allocated to regime 'A' were fed to apparent satiation once every day at 08:00 h. Groups of fish allocated to regime 'B' were fed to apparent satiation twice every day, once at 08:00 h and once at 16:00 h. The average amount of feed consumed by regime 'B' on any day was then used as the basis for feeding fish allocated to regime 'C' and regime 'D'. This was done by calculating the average individual feed intake of fish from the three replicate tanks assigned to regime 'B', then multiplying that value by the number of fish in each replicate tank assigned to regime 'C' and regime 'D'. This was done for each pellet size. This method accounted for any mortality. The quantum of feed allocated to regime 'C' and regime 'D' on any day was fed differentially; being split 50:50 in tanks allocated to regime 'C' and split 33:33:33 in tanks allocated to regime 'D'. If for any reason tanks of fish allocated to regime 'C' or regime 'D' did not consume their entire daily ration, feeding was ceased and uneaten feed weighed to allow determination of actual feed intake.

Experimental rearing systems

Both experiments were done in similar recirculating aquaculture systems (RAS), however the systems were located in separate laboratories. The warm water trial (24 °C) was done in a greenhouse enclosure while the cold water trial (16 °C) was done in an enclosed laboratory. Both RAS incorporated 24 × 1000 L experiment tanks (square polyethylene food grade international bulk containers (IBC)). Each tank in the 16 °C RAS was fitted with an air-stone diffuser and a mesh screen to prevent fish escaping. Tanks in the 24 °C RAS had an additional air-stone diffuser added to inject industrial grade oxygen into the water column to ensure dissolved oxygen levels were > 5 mg L^{-1} at all times. Each RAS incorporated a similar range of equipment including fluidized-bed bio-filters, particle filtration, foam fractionation and reverse cycle refrigeration units to control water temperature. Effluent water from either RAS was continuously removed and replaced with clean, filtered and disinfected top-up saltwater pumped from the estuary adjacent to PSFI

Photoperiod in each experiment followed ambient day length; however for safety reasons the laboratories were lit during working hours using overhead fluorescent lights. Water quality was monitored daily using an electronic water quality meter (either Horiba U10 or U50) whereas total ammonia nitrogen (TAN) was measured every other day using an off the shelf test kit. Mean ± SD water temperature during the cool water trial was 16.1 ± 0.5 °C. Salinity ranged from 27.8‰ to 33.3‰. Mean ± SD dissolved oxygen concentration was 8.2 ± 1.0 mg L^{-1} and mean ± SD oxygen saturation was 108.8 ± 14.6%. pH ranged from 7.24 to 7.83. Total ammonia nitrogen concentration (TAN) ranged from 0.1-2.0 mg L^{-1}. Mean ± SD water temperature during the warm water trial was 23.5 ± 0.6 °C. Salinity ranged from 27.6‰ to 34.5‰. Mean ± SD dissolved oxygen concentration was 9.2 ± 1.5 mg L^{-1} and oxygen saturation was 128.9 ± 26.8%. pH ranged from 6.95 to 7.71. Total ammonia nitrogen (TAN) concentration ranged from 0.2-2.0 mg L^{-1}.

Fish stocking and handling procedures

All fish were sedated, weighed and their fork length was measured at the start and end of experiments using recommended doses of Aqui-S®. Equal numbers of juvenile YTK were stocked into the 1000 L tanks in each experiment (i.e. 14 fish per tank). The average weight of YTK stocked into the 16 °C trial was approximately 151 g and the average weight of fish stocked into the 24 °C trial was approximately 171 g.

Major response variables

The following performance variables were used to assess the response of YTK to different treatments using the average value of all fish from each replicate tank;

- Initial weight of fish (g) = individual weight of fish at stocking
- Final weight of fish (g) = individual weight of fish at harvest
- Specific growth rate (SGR; % d^{-1}) = [Ln(final weight) – Ln(initial weight)] / days × 100
- Condition factor K = [individual weight of fish (g) / fork length of fish $(mm)^3$] × 10^5
- Food conversion ratio (FCR) = dry basis feed intake per tank (g) / wet weight gain per tank (g)
- Relative feed intake (g kg BW^{-1} d^{-1}) = individual feed intake (g) / (GMBW / 1000) / days; where GMBW = geometric mean body weight of fish.

Digestibility of commercial feeds

The digestibility of the 9 mm diet was determined at the end of each experiment. Faecal material was collected from replicate groups of fish (n = 3 tanks) that were fed to apparent satiation either once (regime A) or twice daily (regime B) using stripping or dissection techniques.

The stripping technique was similar to that described by Booth and Pirozzi (2017). Briefly, sedated fish were netted directly from their respective tank after which the ventral surface was wiped clean. A small amount of pressure was then applied to the abdomen using the thumb and forefinger to expel urinary products. The ventral area was cleaned again before firm abdominal pressure was applied to expel faecal material from the distal intestine. Faecal matter was expelled into a clean 70 mL container. Hands were rinsed clean between the handling of different fish and care was taken to ensure that the faecal samples were not contaminated by urine or mucous. Faecal samples were immediately stored in a freezer at -17 °C. Faecal samples were generally collected about 6-8 h after the meal and fish were never stripped on consecutive days. Faecal samples from each tank were pooled and kept frozen at –17° C until a sufficient amount was obtained for chemical analysis.

Collection of faecal material using dissection was done on fish given a similar amount of time to digest their final meal. Individual tanks of fish were rapidly euthanised with an overdose of benzocaine solution after which each fish was dissected. Faecal material was expelled from the terminal or posterior section of the intestinal tract by applying gentle pressure with the thumb and forefinger. The material from all fish in each tank was pooled into a 70 mL plastic container then frozen at -17 °C.

Apparent digestibility coefficients (ADCs) for dietary dry matter (DM), nutrients and energy are calculated according to the equation described by Cho et al. (1982), with the exception that the dry matter ash content of diets and faecal samples was used as the endogenous marker;

- ADC of dry matter (%) = [1 - (concentration of ash in diet / concentration of ash in faeces)] × 100
- ADC of nutrients or energy (%) = [1 - (concentration of ash in diet / concentration of ash in faeces × concentration of nutrient or energy in faeces / concentration of nutrient or energy in diet)] ×100.

Gut transit time

The gut and intestinal transit time of food and digested matter of fish fed 9 mm pellets under regime A and B was also determined at the end of the growth study. Fish were fed their normal daily rations and sacrificed 1, 6, 9, 12, 18, 24 and 48 h after the first meal in the 24 °C experiment and at 1, 6, 9, 12, 18, 24, 48 and 72 h after the first meal in the 16 °C experiment. This was done to quantify the amount of food remaining in the stomach and in the intestine at various time points. Material recovered from stomachs and intestinal tract was oven dried to a constant weight in order to calculate stomach and intestinal contents on a dry matter basis.

Gastric (stomach) evacuation rate (GER) was estimated on fish fed a single meal at each water temperature (regime A). GER was estimated by fitting an exponential model of decay the entire data set (i.e. five fish per time point). The decay model used was $y = a \times \exp^{(-k \times x)}$, where x (elapsed hours), y (relative amount of food in gut on a dry weight basis; % body weight) and K is the estimated rate constant. The estimated half-life of the decay rate was calculated from 0.6932 / K. The plateau in each model was constrained to zero (Booth et al., 2008). Models were fit using GraphPad Prism V7.03-2017.

Chemical analysis

Commercial feeds and faecal samples were analysed for dry matter, crude protein, gross energy (bomb calorimetry), lipid and ash content, respectively. Diet and faecal samples were also analysed for acid insoluble ash (AIA) in order to estimate the apparent digestibility of the diets. However, the concentration of AIA in the commercial diet was found to be extremely low and somewhat variable. The concentration of AIA in faecal samples was also highly variable. As such the AIA content of samples could not be used as a reliable endogenous marker in calculations of apparent digestibility coefficients. Therefore, the dry matter ash content of diet and faecal samples was used as a surrogate endogenous marker. All analyses were done by CSIRO Agriculture and Food (St Lucia, QLD 4067, Australia).

Statistical procedures

Treatment groups were initially compared using one-way. Two-way and three-way ANOVA was used to investigate the interactive effects of feeding strategy (regime A, B, C or D); pellet diameter (6 mm vs 9 mm) and water temperature (16 °C vs 24 °C) on specific performance indices. The factors were considered fixed. Digestibility data was also subjected to two-way ANOVA to investigate the interactive effects of feeding regime (regime A or B) and collection method (stripping or dissection) on apparent digestibility coefficients. Alpha for ANOVA and the post-hoc multiple comparison procedure (Tukey-Kramer Test) was set at 0.05. Data was statistically analysed using NCSS-8.0.23 (Hintze, 2012) after assumptions on the normality and homogeneity of data were investigated.

Results

Proximate content of commercial feeds

The analysed proximate composition (dry matter basis) of the 6mm Huon feed was: ash = 10.5%; total lipid = 21.8%; nitrogen = 7.7%; crude protein = 48.1%; NFE = 19.7% and gross energy = 23.7 MJ kg^{-1} (CP:GE ratio 20.3 g CP MJ GE^{-1}). The analysed proximate composition (dry matter basis) of the 9 mm Huon feed was: ash = 10.4%; total lipid = 23.6%; nitrogen = 7.3%; crude protein = 45.6%; NFE = 20.4% and gross energy = 24.2 MJ kg^{-1} (CP:GE ratio 18.8 g CP MJ GE^{-1}) The minor difference in the proximate content of the two diets reflected the formulation changes discussed above. The dry matter content of the 6 mm and 9 mm feed was 93.8% and 95.9%, respectively (oven drying at 105 °C for 24 h).

Feeding strategies – 24 °C experiment

There was no difference in the average individual body weight of fish assigned to different treatments at the beginning of the 24 °C trial ($F_{7,16} = 0.90$; $P = 0.53$; one-way ANOVA; Table 3.2.4.1.2). The

average body weight of fish was 171.3 g (mean of 24 tanks). Mean performance metrics of individual treatment groups is presented in Table 3.2.4.1.2.

Comparison of treatment groups fed to apparent satiation (regimes A and B)

Two-way ANOVA was used to evaluate the effect of pellet size and feed regime on individual feed intake SGR and FCR in fish fed to apparent satiation (i.e. treatments 6A, 6B, 9A and 9B). Feed intake was significantly affected by feed regime ($F_{1,8} = 6.02$; $P = 0.039$) and pellet size ($F_{1,8} = 7.79$; $P = 0.023$), but not the interaction of terms ($F_{1,8} = 3.76$; $P = 0.088$). Fish consumed significantly more feed under regime B (589.1 g fish^{-1}; $n = 6$) than regime A (517.0 g fish^{-1}; $n = 6$) and they consumed more 9 mm pellets (594.0 g fish^{-1}; $n = 6$) than 6 mm pellets (512.1 g fish^{-1}; $n = 6$).

SGR was affected by the interaction of the fixed factors ($F_{1,8} = 7.06$; $P = 0.03$). The interaction was caused by the low SGR recorded in fish fed 6 mm pellets (1.92% d^{-1}) compared to fish fed 9 mm pellets (2.29% d^{-1}) under feed regime A. There was no great difference between the SGR of fish fed 6 mm (2.19% d^{-1}) or 9 mm (2.17% d^{-1}) pellets under regime B.

FCR was not affected by pellet size ($F_{1,8} = 2.72$; $P = 0.14$), feed regime ($F_{1,8} = 4.53$; $P = 0.066$) or the interaction of the main effects ($F_{1,8} = 0.13$; $P = 0.73$). Nonetheless, FCR tended to be lower (better) in fish fed 9 mm pellets (FCR = 1.37; $n = 6$) than 6 mm pellets (FCR = 1.48; $n = 6$) and better in fish fed once daily (FCR = 1.35; $n = 6$) as opposed to fish fed twice daily (FCR = 1.50; $n = 6$).

Comparison of pair-fed treatment groups (regimes B, C and D)

Two-way ANOVA was used to examine the interactive effects of feeding regime and pellet size on pair-fed treatment groups (i.e. regimes B, C and D). Individual feed intake was not affected by the interaction of the main effects ($F_{2,12} = 0.10$; $P = 0.90$), pellet size ($F_{1,12} = 3.72$; $P = 0.08$) or feed regime ($F_{2,8} = 0.85$; $P = 0.45$) at 24 °C (Table 3.2.4.1.2). Overall, fish fed 9 mm pellets tended to consume slightly more (592.6 g fish^{-1}; $n = 9$) than fish fed 6 mm pellets (559.4 g fish^{-1}; $n = 9$) and fish fed under regime B (589.1 g fish^{-1}; $n = 6$) consumed slightly more than fish fed under regime C (577.1 g fish^{-1}; $n = 6$) or regime D (561.7 g fish^{-1}; $n = 6$). The minor difference in the consumption of pellet sizes may reflect the fact that the satiated control groups (i.e. 6B and 9B) were decoupled; that is, each control group was used to calculate separate daily feed amounts for the 6 mm and 9 mm treatment groups, respectively. The slight decrease in feed intake of fish held on regime C and D compared to regime B may be due to the way the ration was divided.

SGR was not affected by feeding regime ($F_{2,12} = 1.41$; $P = 0.28$), pellet diameter ($F_{1,12} = 0.09$; $P = 0.77$) or the interaction of the main effects ($F_{2,12} = 0.21$; $P = 0.81$). However, SGR was numerically higher in fish fed under regime C (2.26% d^{-1}; $n = 6$) than regime B (2.18% d^{-1}; $n = 6$) or regime D (2.11% d^{-1}; $n = 6$).

FCR was not affected by feeding regime ($F_{2,12} = 3.44$; $P = 0.07$), pellet diameter ($F_{1,12} = 0.01$; $P = 0.91$) or the interaction of the main effects ($F_{2,12} = 0.56$; $P = 0.58$). Nonetheless, FCR was lower (better) in fish under regime C (FCR = 1.32; $n = 6$) than regime B (FCR = 1.50; $n = 6$) or regime D (FCR = 1.42; $n = 6$).

Digestibility of commercial feeds at 24 °C

The apparent digestibility coefficients (ADC) for each of the treatments examined in the 24 °C digestibility study are presented in Table 3.2.4.1.3. Two-way ANOVA on this data found a significant effect of collection method on all ADCs examined except lipid ADC (i.e. dry matter, protein and gross energy). However, none of the proximate or energy coefficients was significantly affected by feeding regime or the interaction of the main effects. Dry matter, protein and gross energy ADCs determined using the dissection method were all about 4-8% lower in absolute terms than if determined using the stripping method.

Gastric evacuation rate at 24 °C

The gut and intestinal transit time of YTK fed 9 mm pellets once (08:00 h) or twice daily (08:00 h and 16:00 h) is presented in Figure 3.2.4.1.1. Fish fed once daily consumed about 3% of their body weight and their stomachs were devoid of food after 36 h. The passage and clearance of food through the intestine followed a similar pattern, albeit the intestinal contents peaked between 12-18 h after feeding (Figure 3.2.4.1.1a). Fish fed twice daily also consumed about 3% of their body weight at the AM meal and a similar amount of food was found in the stomach after the PM meal. This suggests the stomach has a limited capacity and can hold no more than about 3% of body weight in this size animal. The passage and clearance of food through the intestinal passage was similar to that seen in fish fed once daily, peaking around 12-18 h. Nonetheless, the additional PM meal was cleared from the intestinal passage within 48 h. The clearance of food from the intestinal tract appears highly regulated regardless of the number of meals offered to fish.

The fitted model (mean ± SE) used to determine the GER of YTK at 24 °C was; relative stomach content (% BW) = $3.231 \pm 0.188 \times \exp^{(-0.0773 \pm 0.008 \times \text{hours})}$. The GER, as estimated by the rate constant K, was found to be 0.0773 ± 0.008% BW h^{-1}. The 95% CI for the half-life of K was 7.48-10.91 h. The R^2 for the fitted model was 0.87.

Feeding strategies – 16 °C experiment

There was no difference in the average individual body weight of fish assigned to different treatments at the beginning of the 16 °C trial ($F_{7,16} = 1.03$; $P = 0.45$; one-way ANOVA; Table 3.2.4.1.2). The average body weight of fish was 151.0 g (mean of 24 tanks). Mean performance metrics of individual treatment groups is presented in Table 3.2.4.1.4.

Comparison of treatment groups fed to apparent satiation (regimes A and B)

Two-way ANOVA was used to evaluate the interactive effects of pellet size and feed regime on individual feed intake, SGR and FCR in fish fed to apparent satiation (i.e. treatments 6A, 6B, 9A and 9B). Feed intake was significantly affected by feed regime ($F_{1,8} = 11.4$; $P = 0.009$), but not by pellet size ($F_{1,8} = 0.50$; $P = 0.50$) or the interaction of terms ($F_{1,8} = 0.15$; $P = 0.71$). Fish consumed significantly more feed under regime B (240.6 g $fish^{-1}$; $n = 6$) than regime A (215.5 g $fish^{-1}$; $n = 6$). Fish consumed a similar amount of 9mm feed (230.7 g $fish^{-1}$; $n = 6$) and 6 mm feed (225.4 g $fish^{-1}$; $n = 6$).

SGR was not significantly affected by the interaction of the fixed factors ($F_{1,8} = 0.86$; $P = 0.38$), feed regime ($F_{1,8} = 3.28$; $P = 0.11$) or pellet diameter ($F_{1,8} = 0.86$; $P = 0.38$). SGR tended to be slightly lower in fish fed 6 mm pellets once daily compared to the other treatments.

Two-way ANOVA found no effect of pellet size ($F_{1,8} = 0.03$; $P = 0.88$), feed regime ($F_{1,8} = 0.79$; $P = 0.40$) or the interaction of main effects ($F_{1,8} = 0.53$; $P = 0.49$) on FCR. The overall average FCR across the four treatment groups was 1.67:1.

Comparison of pair-fed treatment groups (regimes B, C and D)

Two-way ANOVA was used to examine the interactive effects of feeding regime and pellet size on pair-fed treatment groups (i.e. regimes B, C and D) reared at 16 °C. Individual feed intake was significantly affected by feeding regime ($F_{2,12} = 7.24$; $P = 0.009$), but not by pellet size ($F_{1,12} = 0.49$; $P = 0.50$) or the interaction of the main effects ($F_{2,8} = 0.16$; $P = 0.85$) (Table 3.2.4.1.2). Fish ate significantly more under regime B (240.6 g $fish^{-1}$; $n = 6$), than regime C (224.3 g $fish^{-1}$; $n = 6$) and regime D (225.3 g $fish^{-1}$; $n = 6$). They consumed approximately the same amount of 6 mm (228.7 g $fish^{-1}$; $n = 6$) and 9 mm pellets (231.4 g $fish^{-1}$; $n = 6$). The significant increase in consumption of feed under regime B may reflect the way the ration was divided.

SGR was not affected by feeding regime ($F_{2,12} = 1.42$; $P = 0.28$), pellet diameter ($F_{1,12} = 0.21$; $P = 0.66$) or the interaction of the main effects ($F_{2,12} = 0.43$; $P = 0.66$). However, SGR was slightly higher in fish fed under regime B (1.19% d^{-1}; $n = 6$) than regime C (1.14% d^{-1}; $n = 6$) or regime D (1.15% d^{-1}; $n = 6$).

FCR was not affected by feeding regime ($F_{2,12} = 0.01$; $P = 0.99$), pellet diameter ($F_{1,12} = 0.35$; $P = 0.56$) or the interaction of the main effects ($F_{2,12} = 0.44$; $P = 0.66$). The overall average FCR across all pair-fed treatments at 16° C was 1.69:1.

Digestibility of commercial feeds at 16 °C

The apparent digestibility coefficients (ADC) for each of the treatments examined in the 16 °C digestibility study are presented in Table 3.2.4.1.5. Two-way ANOVA found a highly significant effect of collection method on all ADCs examined (i.e. dry matter, protein, lipid and gross energy). However, none of the proximate or energy coefficients were significantly affected by feeding regime or the interaction of the main effects. Dry matter, lipid and gross energy ADCs determined using the stripping method were about 20% higher in absolute terms than the same coefficients determined using the dissection method. Protein ADCs determined using the stripping method were almost twice the magnitude of protein ADCs determined using dissection (Table 3.2.4.1.5).

Gastric evacuation rate at 16 °C

The gut and intestinal transit time of YTK fed 9 mm pellets once (08:00 h) or twice daily (08:00 h and 16:00 h) is presented in Figure 3.2.4.1.2. Fish fed once daily consumed about 3% of their body weight, but food remained in their stomachs for up to 48 h after the meal. The clearance of food from the intestine peaked between 12-24 h after feeding and some food remained in the intestine after 72 h (Figure 3.2.4.1.2a). Fish fed twice daily also consumed about 3% of their body weight at the AM meal. The clearance of food from the intestinal tract was similar to that seen in fish fed once daily, peaking around 18-24 h after the PM meal. Similarly, the food was still present in the intestinal passage after 72 h (Figure 3.2.4.1.2b).

The fitted model (mean ± SE) used to determine the GER of YTK at 16 °C was; relative stomach content (% BW) = $3.196 \pm 0.127 \times \exp^{(-0.0554 \pm 0.004 \times \text{hours})}$. The GER, as estimated by the rate constant K, was found to be 0.0554 ± 0.004% BW h^{-1}. The 95% CI for the half-life of K was 10.79-14.65 h. The R^2 for the fitted model was 0.92.

Combining data from the 16 °C and 24 °C experiments

To explore the wider ramifications of the data the effect of water temperature (16 °C versus 24 °C), feeding regime (A, B, C and D) and pellet size (6 mm and 9 mm) on fish performance was cautiously evaluated using three-way ANOVA. Only two growth performance variables were considered; SGR and FCR.

There were no significant first or second order interactions affecting the SGR of fish (all $P > 0.05$). SGR was significantly affected by two of the three main effects; namely water temperature ($F_{1,32} = 918.6$; $P < 0.0001$) and pellet size ($F_{1,32} = 4.22$; $P < 0.048$). SGR was higher in fish reared at 24 °C (2.16% d^{-1}; $n = 24$) than 16 °C (1.15% d^{-1}; $n = 24$) and higher in fish consuming 9 mm pellets (1.69% d^{-1}; n = 24) as opposed to 6 mm pellets (1.62% d^{-1}; $n = 24$). There was no significant effect of feed regime on SGR ($F_{3,32} = 1.62$; $P = 0.20$).

There were no significant first or second order interactions affecting the FCR of fish (all $P > 0.05$). FCR was significantly affected by only one of the three main effects; namely water temperature ($F_{1,32} = 70.3$; $P < 0.0001$). FCR was significantly lower (better) in fish reared at 24 °C (1.40:1; $n = 24$) than fish reared at 16 °C (1.68:1; $n = 24$). Neither pellet size ($F_{1,32} = 1.25$; $P < 0.27$) nor feed regime ($F_{3,32} = 1.62$; $P = 0.20$) significantly affected FCR.

The effect of water temperature (16 °C versus 24 °C) and collection method (stripping versus dissection) on the ADCs of dry matter, protein, lipid and gross energy was interpreted using two-way ANOVA. Feed regime was excluded from interpretation as it was previously found to have a non-significant effect on digestibility coefficients in both experiments. Results of separate two-way ANOVAs indicated that all ADCs were significantly affected by each main effect and the interaction of the main effects (all $P < 0.0024$). The interactions of the main effects for all coefficients were caused by a difference in the

magnitude of ADCs determined by stripping (higher) as opposed to dissection (lower) at 16 °C. The pooled data are presented graphically in Figure 3.2.4.1.3 and Figure 3.2.4.1.4. Despite the interactions, the figures clearly show that all ADCs based on stripping are mostly higher than those based on dissection. Furthermore, except for lipid digestibility, the ADCs of dry matter, protein and energy determined using stripping methods were remarkably similar, respectively, at both water temperatures. In contrast, there was a large discrepancy between respective ADCs determined using dissection methods at 16 °C and 24 °C.

Discussion

At present it is industry practice to feed juvenile and sub-adult YTK a morning feed followed by a top-up feed at the end of the working day. This strategy has long been practised because it was thought to maximise feed intake; the assumption being that by maximising feed intake then growth rate would be optimised. Paradoxically, the feed intake that maximises growth rate does not usually optimise FCR (Brett, 1979). Thus farmers face a choice, to feed for growth or feed conservatively for better FCR. This decision is often influenced by two factors; the prevailing cost of feed and the sale price of fish. Thus the proper delivery of feed is critical in terms of economic return. In addition, uneaten or wasted feed represents and economic loss and a burden on the environment (NRC, 2011). Lastly, other operating considerations such as labour and fuel costs may dictate a farm-based feeding plan that is contrary to the most biological appropriate feeding strategy.

This report presents data on two independent, but closely linked laboratory experiments that were done to evaluate the effect of different feeding strategies, different pellet diameters (6mm versus 9mm) and different rearing temperatures (16 °C versus 24 °C) on growth and FCR in sub-adult YTK. It also includes data on the effect of water temperature and feeding strategy on the digestibility and gut evacuation rate of a commercial diet in sub-adult YTK. The feeding regimes that were evaluated included typical commercial practises such as feeding fish once per day (regime A; single AM meal) or feeding fish twice per day (regime B; AM meal and PM meal). Two other pair-fed feeding regimes based on regime B were also assessed to determine if feeding YTK two (50:50) or three (33:33:33) equal sized meals during the day altered growth or FCR. The latter regimes were tested on YTK as there is convincing evidence in the literature that equally distributed meals may improve feed utilisation and growth in fish (Greenland, Gill, 1979). This outcome is thought to be linked to the daily peak in feeding activity, gastric capacity and nutrient demand of individual species (Bolliet et al., 2001; NRC, 2011).

Feeding to appetite once or twice per day

Not surprisingly the results indicated that fish ate more when offered two as opposed to one meal per day. This outcome was similar at both water temperatures. Fish offered a single meal each day also ate significantly more of the 9 mm pellet than the 6 mm pellet at 24 °C. The reason for the low intake of 6 mm pellets at the higher temperature appears to be related to the lower growth rate of YTK in this treatment. In contrast, there was little difference between the intake of 6 mm and 9 mm pellets in fish fed twice per day at 24 °C, and there was no difference between the intake of 6 mm and 9 mm pellets in fish at 16 °C. Lastly, neither SGR nor FCR of YTK were greatly affected by feeding regime or pellet size at each temperature, indicating fish reared in RAS systems and carefully fed to apparent satiety once per day perform as well as fish fed to apparent satiety twice per day. While feeding once per day is appropriate under carefully controlled laboratory conditions this strategy may not be practically appropriate in farming situations. Sea-cages may contain thousands of YTK and ensuring they are all fed to apparent satiation at any one time can be difficult if not impossible, especially if feeding by hand. For this reason it would be prudent to adopt a twice-daily feeding regime to ensure the majority of YTK in cages have an opportunity to consume adequate amounts of feed over a suitable duration.

Impact of meal splitting – pair-fed treatments

This part of the experiment was designed to determine if splitting the daily quantum of feed into several equal sized meals impacted growth and FCR in sub-adult YTK reared at different water temperatures

and fed different size pellets. The daily quantum of feed for each pellet size was based on the feed intake of YTK held under regime B (i.e. two meals to appetite each day). In that sense, the daily intake of dry matter, nutrients and energy offered to each tank of fish was effectively the same. This pair-feeding approach enables the benefit or otherwise of meal splitting to be examined. The primary parameters of interest were, therefore, SGR and FCR. The results indicated there was no statistical effect of feed regime (i.e. regime B. C or D) or pellet diameter on the SGR or FCR of fish reared at 16 °C or 24 °C, respectively. Therefore, it is unlikely there would be any benefit in adopting these particular strategies on-farm. Literature suggests that fish requiring multiple feeds per day generally have small stomachs; hence they lack the capacity to consume and then hold enough feed in their gastric chamber to meet nutrient demands over an extended period (NRC, 2011). This does not appear to be the case in YTK.

Combining experimental growth data from all regimes

Data from each experiment was cautiously combined in a three-way ANOVA to summarise the effects of water temperature, feeding regime and pellet size on SGR and FCR of YTK. As expected, water temperature had an overriding impact on SGR, which almost doubled in fish reared at 24 °C (2.16% d^{-1} versus 1.15% d^{-1}). However, the SGR of YTK was not affected by the chosen feeding regimes, supporting the conclusions drawn above. Interestingly, the SGR of YTK was significantly (but only slightly) higher in fish fed 9 mm pellets compared to fish fed 6 mm pellets. Although statistically different, this minor increase in SGR due to pellet size is probably not biologically relevant. Alternatively, it may reflect the minor changes in the composition of the two diets. For example the CP:DE ratio of the 9 mm diet was slightly lower (18.8 g MJ GE^{-1}) than the 6 mm pellet (20.3 g MJ GE^{-1}), reflecting the slight increase in fat and decrease in protein content of the 9 mm feed. Thus the subtle increase in energy density of the 9 mm diet may better suit the protein:energy requirements of larger YTK. Water temperature was also the overriding factor influencing FCR in YTK fed the commercial diets. On average, the FCR of fish reared at 24 °C was approximately 16.6% better than the FCR of fish reared at 16 °C.

Combining experimental data on digestibility of diets

Strong interactions were found between water temperature and collection method with respect to all proximate and energy ADCs. The interaction was caused by a difference in the magnitude of ADCs determined by stripping (higher) as opposed to dissection (lower) at 16° C. Nonetheless, it is important to acknowledge that the ADCs presented in this paper were calculated using dietary and faecal ash concentrations instead of AIA; thus data on apparent digestibility of the commercial diet should be interpreted cautiously.

Confidence in our data is improved by reference to a comparative data set presented by Miegel et al. (2010). These authors also used a dissection technique to determine the ADC's of nutrients and energy from a fishmeal-based diet fed to 0.9 kg YTK. Their feed was marked with yttrium. The ADCs were determined using digestive material collected from the anterior or posterior region of the intestinal tract during winter (12.6 °C) and summer (20.8 °C) water temperatures. Firstly, they confirmed that ADCs of dry matter did not change significantly over time (i.e. from 8 h to 48 h), implying that post-prandial sampling time did not influence resulting ADCs. Secondly, they found that ADCs for dry matter, protein, fat and energy determined using faecal matter dissected from the posterior intestine (analogous to this study), tended to be numerically higher at summer water temperatures (45.5%, 69.7%, 65.6%, 70.3%), than winter water temperatures (34.6%, 67.0%, 58.1%, 63.0%). The impact of water temperature on digestibility of nutrients and energy observed by Miegel et al. (2010) reflect those in the present study and greatly increase the confidence in our data.

Despite the statistical outcomes, our data clearly show that ADCs based on stripping are higher than those based on dissection. This indicates that a greater amount of undigested endogenous material may have indirectly contaminated the dissected samples, perhaps from the anterior section of the digestive tract. Furthermore, there was a large discrepancy between respective ADCs determined using dissection at 16 °C and 24 °C. In contrast, except for lipid digestibility, the ADCs of dry matter, protein and energy determined using stripping methods were remarkably similar, respectively, at both water temperatures. For these reasons we recommend collection of digestive material from YTK using stripping methods.

Importantly, the latter method allows the pooling of digestive samples over several days, which is likely to be a more representative of true digestibility than ADCs based on a single sample from one time point (Glencross et al., 2007). It is generally acknowledged that active collection methods such as stripping and dissection result in the underestimation of digestibility coefficients due to inadvertent contamination of faeces with undigested material (NRC, 2011). In addition, the majority of studies indicate feeding level has little impact on the digestibility of nutrients (NRC, 2011). Our results support this view insomuch as the proximate digestibility of the commercial feed used in this study was not affected by feeding either once or twice per day.

Gut and intestinal transit times and GER

The gastric evacuation rate (GER) of YTK fed a single meal at 08:00 h was found to be higher in fish reared at 24 °C (K = 0.0773% BW h^{-1}) than 16 °C (K = 0.0554% BW h^{-1}). The time taken to evacuate 50% of the ingested meal (half-life of K) at 24 °C was between 7.5-10.9 h while it took between 10.79-14.65 h to void the same amount of ingested feed at 16 °C (Figure 3.2.4.1.5). These models will prove useful in predicting suitable refeeding times for YTK at these temperatures. Both chemical and physical characteristics of the diet can influence gastric evacuation rates, however the results presented here are similar to studies on Japanses Yellowtail (*Seriola quinqueradiata*) (Watanabe et al., 2001b) and reflect data presented on the effect of winter and summer conditions on gut transit time in YTK (Miegel et al., 2010). It is somewhat more difficult to model the evacuation rate of the gastro-intestinal tract (GIT), however, in fish fed as single meal the passage of digested matter from the stomach into the GIT seemed highly regulated insofar as the disappearance of digested material from the GIT simulated the response seen in the stomach. This also appeared to be the case in fish fed twice daily, although data were more variable. This suggests GER is somewhat independent of feeding frequency and that YTK simply consume as much feed as they have evacuated when offered another meal.

Conclusions and Recommendations

The two experiments presented in this paper have demonstrated that growth rate and FCR of YTK are better in fish reared at 24 °C as opposed to 16 °C. The results also provide strong evidence that feeding sub-adult YTK a single meal to apparent satiety once per day supports acceptable growth rate and feed utilisation, irrespective of water temperature. However, industry should continue to feed at least twice daily in farm situations to ensure the average fish has the opportunity to consume enough feed to support their growth potential. There appears to be no added benefit of splitting meals into equal sized portions during the day, however, studies that investigate similar meal frequencies but where fish are fed to apparent satiation may be worthwhile. The apparent proximate digestibility of a commercial diet determined using stripping methods was relatively unaffected by water temperature. However, lipid digestibility was slightly depressed at 16 °C. This may relate to GER, the lipid content and composition of the feed or the activity of specific digestive enzymes (Bowyer et al., 2014). Ideally, more research should be done to understand the impact of water temperature on the digestibility of commercial diets and raw materials. Until better techniques are developed, such studies should be done using stripping methods (Booth and Pirozzi, 2017). The GER of sub-adult YTK is slower at 16 °C than at 24 °C. In addition, regardless of the temperatures investigated here, fish between 150-500 g appear unable to consume more than about 3% of their body weight on a dry weight basis in a single meal. Refed fish appear to consume only as much food as has been evacuated. This wide ranging study provides an extensive data set that will assist YTK farm managers improve their on-farm feeding practices. Importantly it demonstrates the biological plasticity of YTK with respect to selected feed regime and pellet size across a conservative range in environmental temperature; factors that can be manipulated to improve the economic and environmental outcomes of farm raised fish.

Findings

- Specific growth rate (SGR) and food conversion ratio (FCR) of sub-adult YTK are better in fish reared at 24 °C as opposed to 16 °C.

- The GER of sub-adult YTK is slower at 16 °C than at 24 °C.
- The apparent proximate digestibility of a commercial diet determined using stripping methods was relatively unaffected by water temperature. However, lipid digestibility of this diet was slightly depressed at 16 °C.
- This study provides strong evidence that feeding sub-adult YTK a single meal to apparent satiety once per day will support acceptable growth rate and feed utilisation, irrespective of water temperatures between 16 °C and 24 °C.
- This study has shown that under laboratory conditions acceptable growth rate and feed utilisation in sub-adult YTK can be sustained by feeding a commercial aquafeed once per day to apparent satiation. However, we recommend the YTK industry should continue to feed at least twice-daily in farm situations to ensure the average fish has the opportunity to consume enough feed to support their growth potential.
- There appears to be no benefit of splitting meals into equal sized portions during the day, however studies that investigate similar meal frequencies, but where fish are fed to apparent satiation may be worthwhile.
- The gastric evacuation rate (GER) of sub-adult YTK is slower at 16 °C than at 24 °C. In addition, fish weighing between 150-500 g appear unable to consume more than approximately 3% of their body weight on a dry weight basis in a single meal. Refed fish appear to consume only as much food as has been evacuated.
- More research should be done to understand the impact of water temperature on the digestibility of raw materials and commercial feeds. Until better techniques are developed, such studies should be done using stripping methods.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, New South Wales Department of Primary Industries (NSW DPI) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

We would also like to acknowledge the technical assistance of Ian Russell, Justin Tierney, Brooke M^{c}Cartin, Marcus Armstrong and Steve Gamble (NSW DPI) as well as John Horlick (Macquarie University). We thank David Whyte for advice on commercial feeds (formerly Huon Aquaculture) and Barney Hines and staff at CSIRO for conducting the chemical analyses on samples generated from this experiment.

References

Biswas, G., Thirunavukkarasu, A.R., Sundaray, J.K., Kailasam, M., 2010. Optimization of feeding frequency of Asian seabass (*Lates calcarifer*) fry reared in net cages under brackishwater environment. Aquaculture 305, 26-31.

Bolliet, V., Azzaydi, M., Boujard, T., 2001. Effects of feeding time on feed intake and growth. In: Houlihan, D., Boujard, T., Jobling, M. (Eds.), Food Intake in Fish. Blackwell Science, Oxford, pp. 233-249.

Booth, M., Pirozzi, I., 2017. Making sense of nonsense: using regression analysis to deal with highly variable data collected from a yellowtail kingfish (*Seriola lalandi*) digestibility experiment. Aquaculture, https://doi.org/10.1016/j.aquaculture.2017.1011.1021.

Booth, M.A., Tucker, B.J., Allan, G.L., Fielder, D.S., 2008. Effect of feeding regime and fish size on weight gain, feed intake and gastric evacuation in juvenile Australian snapper *Pagrus auratus*. Aquaculture 282, 104-110.

Bowyer, J.N., Booth, M.A., Qin, J.G., D'Antignana, T., Thomson, M.J.S., Stone, D.A.J., 2014. Temperature and dissolved oxygen influence growth and digestive enzyme activities of yellowtail kingfish *Seriola lalandi* (Valenciennes, 1833). Aquacult. Res. 45, 2010-2020.

Brett, J.R., 1979. Environmental factors and growth. in: Hoar, W.S., Randall, D.J., Brett, J.R. (Eds.), Fish Physiology, Volume 8, Bioenergetics and Growth. Academic Press, New York, pp. 599-675.

Cho, C.Y., Slinger, S.J., Bayley, H.S., 1982. Bioenergetics of salmonid fishes: energy intake, expenditure and productivity. Comparative Biochemistry and Physiology 73B, 25-41.

de la Gandara, F., Garcia-Gomez, A., Jover, M., 2002. Effect of feeding frequency on the daily oxygen consumption rhythms in young Mediterranean yellowtails (*Seriola dumerili*). Aquacult. Eng. 26, 27-39.

Dwyer, K.S., Brown, J.A., Parrish, C., Lall, S.P., 2002. Feeding frequency affects food consumption, feeding pattern and growth of juvenile yellowtail flounder (*Limanda ferruginea*). Aquaculture 213, 279-292.

Fielder, D.S., Bardsley, W.J., Allan, G.L., Pankhurst, P.M., 2002. Effect of photoperiod on growth and survival of snapper *Pagrus auratus* larvae. Aquaculture 211, 135-150.

Folkvord, A., Ottera, H., 1993. Effects of initial size distribution, day length, and feeding frequency on growth, survival, and cannibalism in juvenile Atlantic cod (*Gadus morhua* L.). Aquaculture 114, 243-260.

Glencross, B.D., Booth, M.A., Allan, G.L., 2007. A feed is only as good as its ingredients - a review of ingredient evaluation strategies for aquaculture feeds. Aquacult. Nutr. 13, 17-34.

Greenland, D.C., Gill, R.L., 1979. Multiple daily feedings with automatic feeders improve growth and feed conversion rates of Channel catfish. The Progressive Fish-Culturist 41, 151-153.

Hintze, J., 2012. NCSS 8. NCSS, LLC. Kaysville, Utah, USA. www.ncss.com. NCSS, Kaysville, Utah, USA., www.ncss.com.

Kestemont, P., Baras, E., 2001. Environmental Factors And Feed Intake: Mechanisms And Interactions. in: Houlihan, D., Boujard, T., Jobling, M. (Eds.), Food Intake in Fish. Blackwell Science, Oxford, pp. 131-156.

Lee, S.M., Hwang, U.G., Cho, S.H., 2000. Effects of feeding frequency and dietary moisture content on growth, body composition and gastric evacuation of juvenile Korean rockfish (*Sebastes schlegeli*). Aquaculture 187, 399-409.

Miegel, R.P., Pain, S.J., van Wettere, W.H.E.J., Howarth, G.S., Stone, D.A.J., 2010. Effect of water temperature on gut transit time, digestive enzyme activity and nutrient digestibility in yellowtail kingfish (*Seriola lalandi*). Aquaculture 308, 145-151.

NRC, 2011. Nutrient Requirements of Fish And Shrimp. The National Academic Press, Washington, D.C.

Riche, M., Haley, D.I., Oetker, M., Garbrecht, S., Garling, D.L., 2004. Effect of feeding frequency on gastric evacuation and the return of appetite in tilapia *Oreochromis niloticus* (l.). Aquaculture 234, 657-673.

Salama, A.J., 2008. Effects of different feeding frequency on the growth, survival and feed conversion ratio of the Asian sea bass *Lates calcarifer* juveniles reared under hypersaline seawater of the Red Sea. Aquacult. Res. 39, 561-567.

Tucker, B.J., Booth, M.A., Allan, G.L., Booth, D., Fielder, D.S., 2006. Effects of photoperiod and feeding frequency on performance of newly weaned Australian snapper *Pagrus auratus*. Aquaculture 258, 514-520.

Watanabe, K., Kuriyama, I., Satoh, K., Kiron, V., Satoh, S., Watanabe, T., 2001a. Further clarification of winter energy and protein requirements at the optimum feeding frequency for Yellowtail. Fish. Sci. 67, 90-103.

Watanabe, T., Akimoto, A., Aoki, H., Shimeno, S., 2001b. Effects of physical properties of diets on evacuation time of digesta and plasma free amino acid patterns in Yellowtail. Fish. Sci. 67, 456-460.

Yamamoto, T., Shima, T., Furuita, H., Sugita, T., Suzuki, N., 2007. Effects of feeding time, water temperature, feeding frequency and dietary composition on apparent nutrient digestibility in rainbow trout *Oncorhynchus mykiss* and common carp *Cyprinus carpio*. Fish. Sci. 73, 161-170.

Table 3.2.4.1.1. Design of two Yellowtail Kingfish feeding experiments done at different water temperatures (16 °C vs 24 °C); fish were fed one of four feeding regimes crossed with two commercial pellet diameters at each water temperature.

	Feeding strategy							
	Regime A		**Regime B**		**Regime C**		**Regime D**	
	Pellet diam.		Pellet diam.		Pellet diam.		Pellet diam.	
Water temperature	6 mm	9 mm	6 mm	9 mm	6 mm	9 mm	6 mm	9 mm
16 °C	apparent satiation; 08:00 h		apparent satiation; 08:00 h and 16:00 h		amount based on 'B'; 08:00 h and 16:00 h; 50:50 split		amount based on 'B'; 08:00 h, 12:00 h and 16:00 h; 33:33:33 split	
24 °C	apparent satiation; 08:00 h		apparent satiation; 08:00 h and 16:00 h		amount based on 'B'; 08:00 h and 16:00 h; 50:50 split		amount based on 'B'; 08:00 h, 12:00 h and 16:00 h; 33:33:33 split	

Table 3.2.4.1.2. Average performance of Yellowtail Kingfish allocated to different feeding strategies for 56 days (24 °C warm water experiment).

Treatment	**Stock weight (g fish^{-1})**	**Harvest weight (g fish^{-1})**	**SGR (% d^{-1})**	**FCR**	**K factor**	**Feed intake (g fish^{-1})**	**Feed intake (g kg BW^{-1} d^{-1})**	**CV weight (%)**
6A	172.3	505.0	1.92	1.42	1.54^{a}	448^{a}	27.1^{a}	35.3
6B	169.7	578.7	2.19	1.54	1.62^{ab}	577^{b}	32.8^{b}	41.0
6C	171.7	595.7	2.22	1.30	1.59^{ab}	555^{b}	31.0^{ab}	29.7
6D	170.3	557.3	2.11	1.40	1.56^{a}	546^{ab}	31.8^{b}	35.7
9A	172.7	623.3	2.29	1.28	1.67^{ab}	586^{b}	31.9^{b}	32.3
9B	171.0	577.7	2.17	1.45	1.67^{ab}	602^{b}	34.2^{b}	39.0
9C	170.3	619.7	2.31	1.34	1.74^{b}	599^{b}	32.9^{b}	33.7
9D	172.3	564.7	2.11	1.43	1.61^{ab}	577^{b}	33.1^{b}	37.3
SEM	*1.18*	*27.5*	*0.09*	*0.07*	*0.04*	*21.6*	*0.88*	*5.6*
F value	*0.90*	*1.90*	*2.04*	*1.53*	*3.48*	*5.3*	*6.0*	*0.44*
P value	*0.52*	*0.14*	*0.11*	*0.23*	*0.023*	*0.003*	*0.001*	*0.86*

*Data subjected to one-way ANOVA and Tukey's multiple comparison test.

Table 3.2.4.1.3. Apparent digestibility coefficients (ADC) of a 9 mm commercial diet fed to Yellowtail Kingfish either once or twice daily determined using stripping or dissection techniques (24 °C warm water experiment).

		Apparent digestibility coefficient (%)			
Regime	**Collection method**	**Dry matter**	**Crude protein**	**Lipid**	**Gross energy**
9A	Stripping	62.9^{ab}	76.6^{ab}	94.0	78.6
9B	Stripping	65.4^{b}	80.8^{b}	87.8	80.4
9A	Dissection	58.3^{a}	70.6^{a}	94.0	74.5
9B	Dissection	58.2^{a}	70.7^{a}	89.5	74.1
SEM	-	*1.32*	*1.98*	*2.73*	*1.86*
F value	-	*7.1*	*6.3*	*1.21*	*2.8*
P value	-	*0.01*	*0.02*	*0.37*	*0.11*

*Data subjected to one-way ANOVA and Tukey's multiple comparison test.

Table 3.2.4.1.4. Average performance of Yellowtail Kingfish allocated to different feeding strategies for 56 days (16 °C cool water experiment).

Treatment	**Stock weight (g fish^{-1})**	**Harvest weight (g fish^{-1})**	**SGR (% d^{-1})**	**FCR**	**K factor**	**Feed intake (g fish^{-1})**	**Feed intake (g kg BW^{-1} d^{-1})**	**CV weight (%)**
6A	151.1	277.9	1.09	1.67	1.53	211.4^{a}	18.42^{a}	14.6
6B	150.4	293.4	1.19	1.67	1.55	239.4^{b}	20.35^{ab}	13.4
6C	151.1	282.7	1.12	1.74	1.55	221.5^{ab}	19.13^{ab}	16.7
6D	151.4	288.5	1.15	1.69	1.55	225.2^{ab}	19.24^{ab}	12.2
9A	151.5	290.2	1.16	1.63	1.55	219.6^{ab}	18.69^{ab}	16.1
9B	150.3	292.8	1.19	1.70	1.58	241.8^{b}	20.58^{b}	16.5
9C	151.2	290.5	1.16	1.64	1.58	227.1^{ab}	19.36^{ab}	12.5
9D	151.0	286.8	1.14	1.67	1.51	225.5^{ab}	19.36^{ab}	10.0
SEM	*0.43*	*6.45*	*0.04*	*0.06*	*0.02*	*5.69*	*0.40*	*2.18*
F value	*1.03*	*0.67*	*0.86*	*0.32*	*2.18*	*3.10*	*3.44*	*1.20*
P value	*0.45*	*0.69*	*0.56*	*0.93*	*0.09*	*0.03*	*0.02*	*0.36*

*Data subjected to one-way ANOVA and Tukey's multiple comparison test.

Table 3.2.4.1.5. Apparent digestibility coefficients of a 9 mm commercial diet fed to Yellowtail Kingfish either once or twice daily determined using stripping or dissection techniques (16 °C warm water experiment).

		Apparent digestibility coefficient (%)			
Regime	**Collection method**	**Dry matter**	**Crude protein**	**Lipid**	**Gross energy**
9A	Stripping	65.7[b]	85.1[b]	78.8[bc]	76.6[b]
9B	Stripping	65.1[b]	83.2[b]	81.3[c]	75.6[b]
9A	Dissection	45.1[a]	45.7[a]	60.1[a]	55.1[a]
9B	Dissection	44.2[a]	43.4[a]	63.5[ab]	51.3[a]
SEM	-	*2.60*	*3.84*	*3.86*	*3.41*
F value	-	*21.2*	*35.6*	*7.6*	*15.3*
P value	-	*0.0004*	*0.0001*	*0.01*	*0.001*

*Data subjected to one-way ANOVA and Tukey's multiple comparison test.

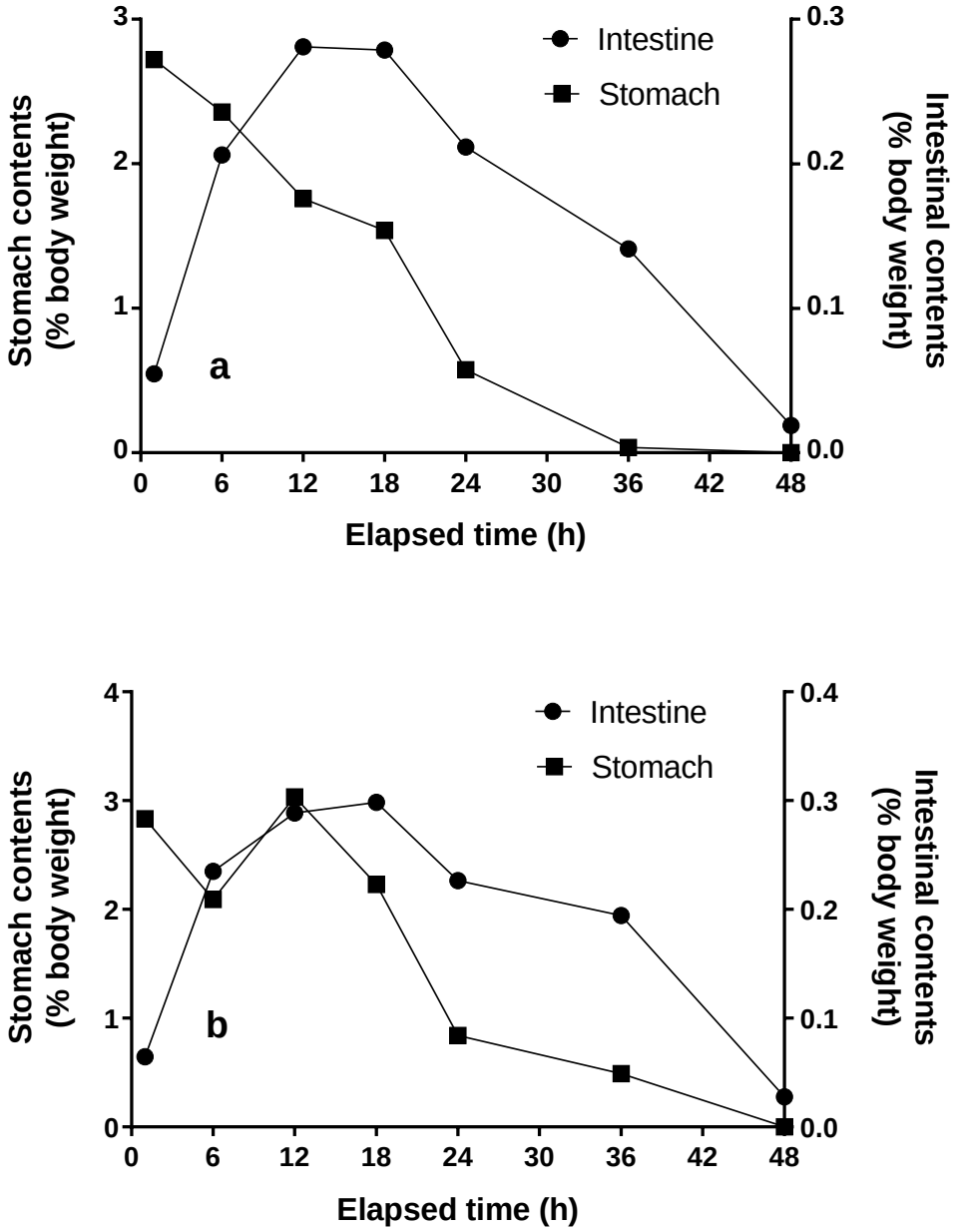


Figure 3.2.4.1.1. Gut and intestinal transit responses in Yellowtail Kingfish fed one meal (panel a; regime A), or two meals to apparent satiation (panel b; regime B) at 24 °C. Fish were fed 9 mm commercial pellets. Data points are mean of five fish.

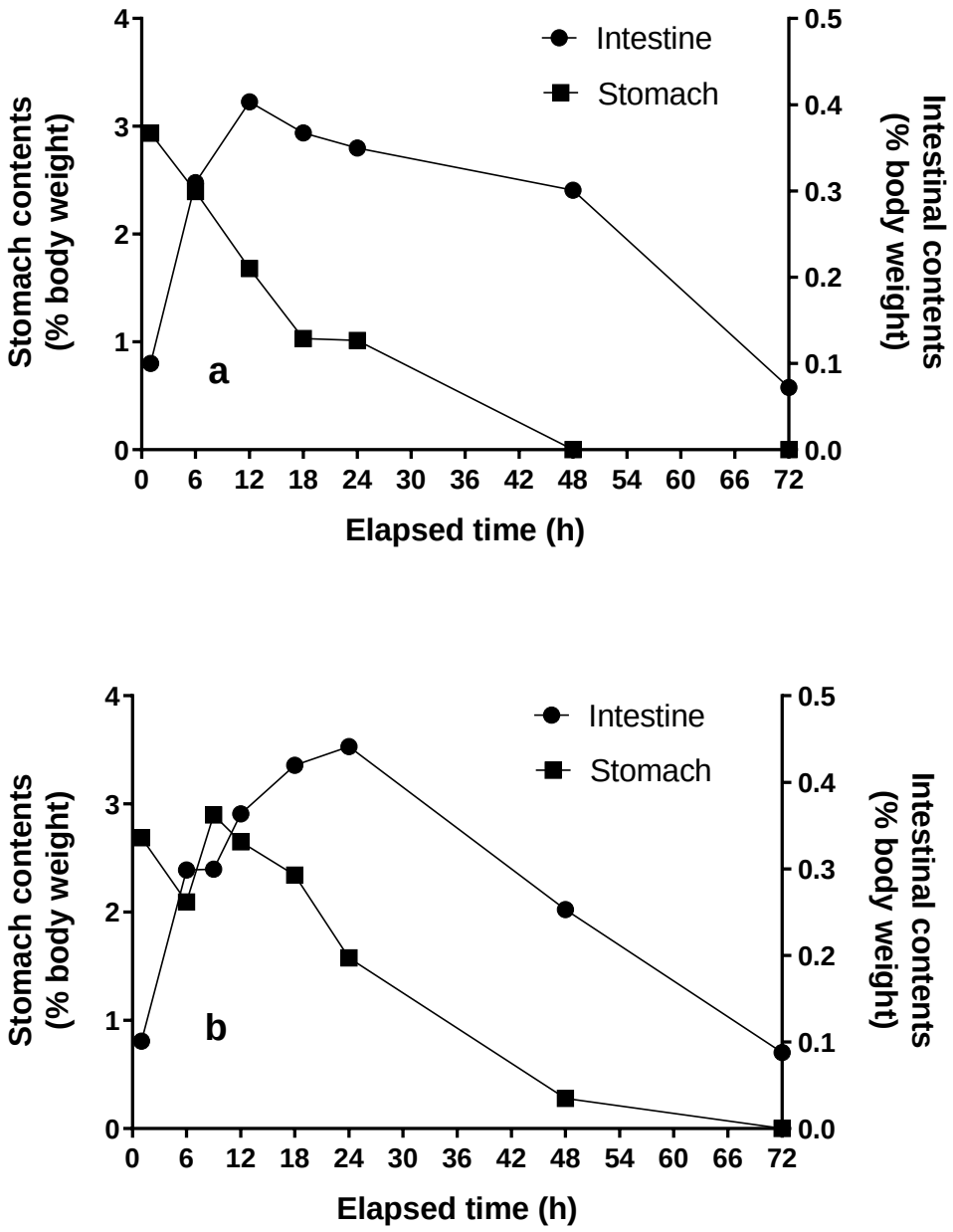


Figure 3.2.4.1.2. Gut and intestinal transit responses in Yellowtail Kingfish fed one meal (panel a; regime A), or two meals to apparent satiation (panel b; regime B) at 16 °C. Fish were fed 9 mm commercial pellets. Data points are mean of five fish.

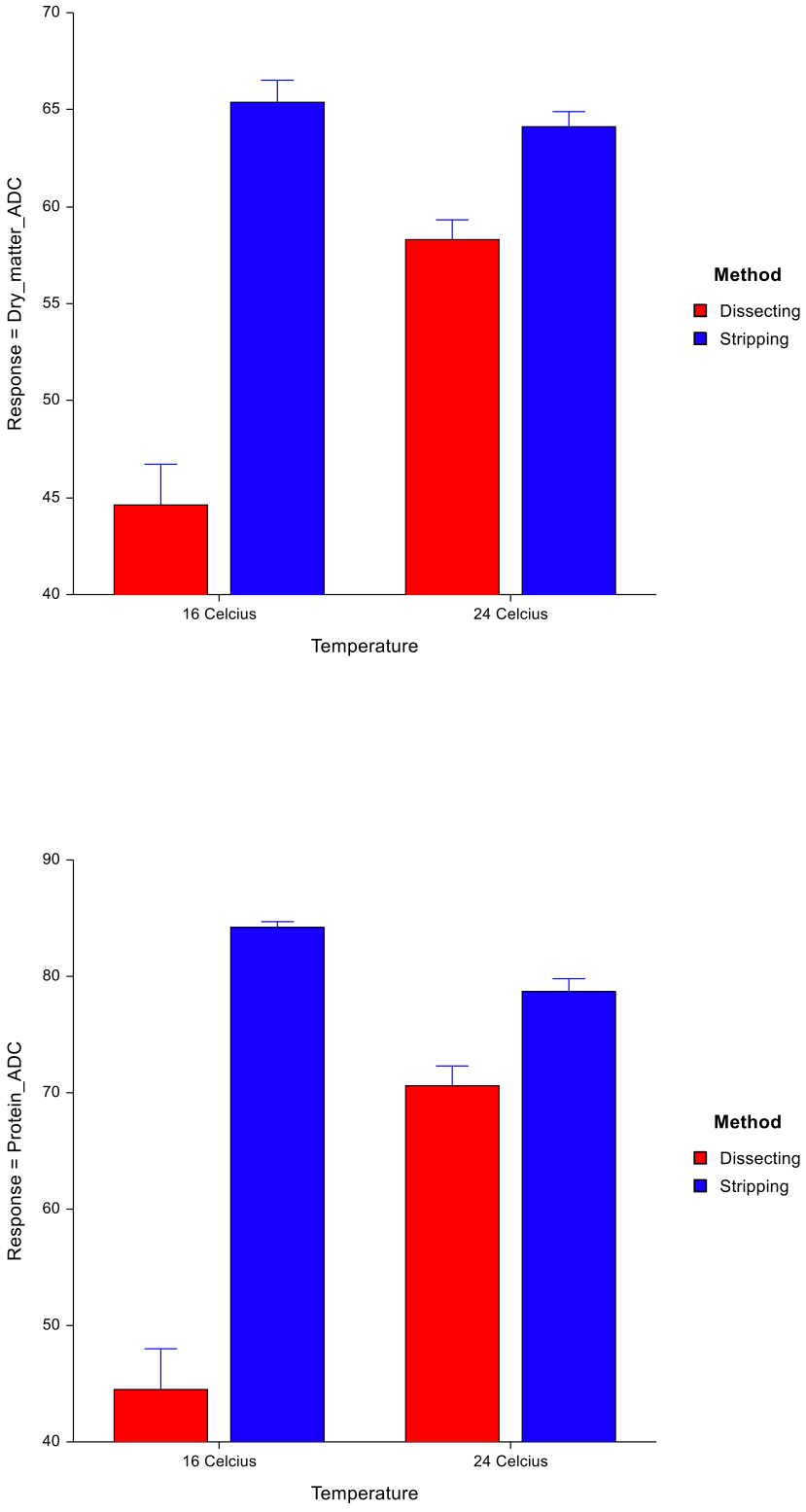


Figure 3.2.4.1.3. Comparison of dry matter (upper panel) and protein (lower panel) apparent digestibility coefficients (ADC) for Yellowtail Kingfish as affected by water temperature and faecal collection method (9 mm diet). Bars are mean ± SE of *n* = 6 values pooled over feed regime.

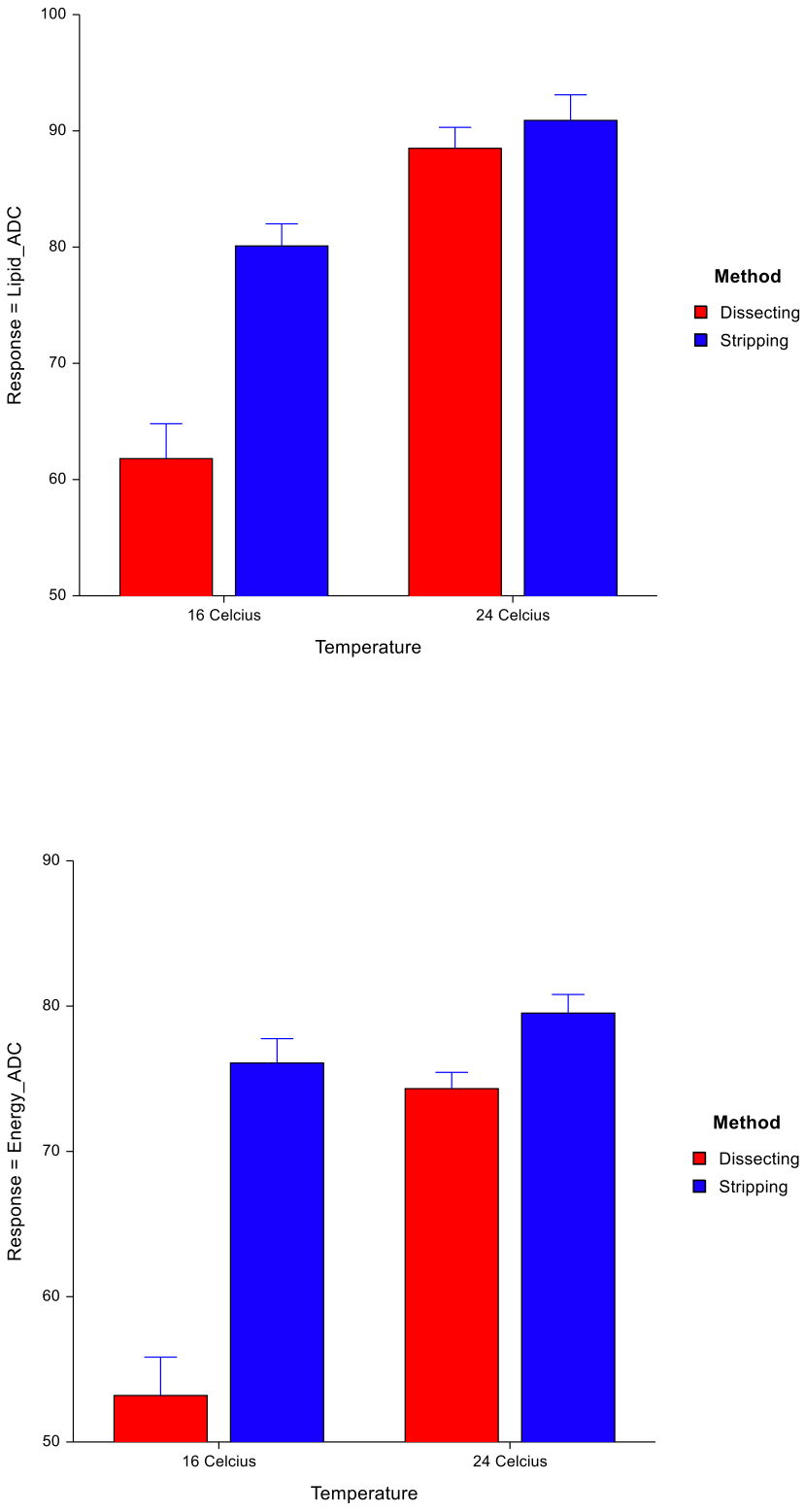


Figure 3.2.4.1.4. Comparison of lipid (upper panel) and gross energy (lower panel) apparent digestibility coefficients (ADC) for Yellowtail Kingfish as affected by water temperature and faecal collection method (9 mm diet). Bars are mean ± SE of *n* = 6 values pooled over feed regime.

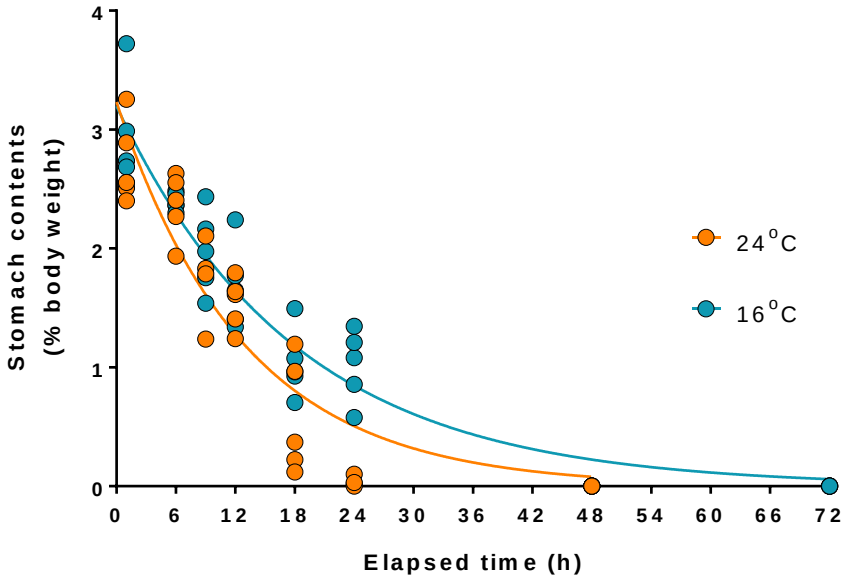


Figure 3.2.4.1.5. Gastric evacuation curves in Yellowtail Kingfish fed one meal (regime A) to apparent satiation at 16 °C or 24 °C. Fish were fed 9 mm commercial pellets. Data points are individual fish (five fish per sample point).

3.2.4.2. Manuscript - Effect of feeding strategy and diet specification on growth and feed utilisation of sub-adult Yellowtail Kingfish Seriola lalandi.

Mark Booth[a] and Igor Pirozzi[a,b]

[a] New South Wales Department of Primary Industries, Port Stephens Fisheries Research Institute, Taylors Beach 2316, NSW, Australia.

[b] College of Science and Engineering and Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville 4801, QLD, Australia.

This manuscript may be referenced as: Booth, M. and Pirozzi, I. (2019). 3.2.4.2. Manuscript - Effect of feeding strategy and diet specification on growth and feed utilisation of sub-adult Yellowtail Kingfish *Seriola lalandi*. In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027),* Adelaide, June. pp.406-426.

Abstract

A 56 day feeding experiment was done to examine the impact of feeding regime and diet specification on the performance of sub-adult Yellowtail Kingfish (*Seriola lalandi*; YTK). Three satiation feeding regimes were tested following consultation with industry. They were 1) a regular feeding regime based on feeding fish once daily 7 days per week; 2) a regime whereby fish were fed once daily 5 days per week (i.e. Monday to Friday only); and 3) a regime whereby fish were fed once daily on randomly selected days of the week (i.e. random regime). The randomised feeding regime was included in the study to mimic the missed feeding days commonly experienced in YTK farming operations due to weather or operational constraints. Both the 5 day and randomised feeding regime resulted in fish being fed approximately 70% the number of feed days experienced by the daily fed group. The three feeding regimes were crossed with two diet specifications; a standard specification diet (38.6% digestible protein (DP); 15.1 MJ digestible energy (DE) kg^{-1}) and high specification diet (46% DP; 19.2 MJ DE kg^{-1}), to create an orthogonal experiment with six treatments. Each treatment was replicated in 3 × 1000 L experiment tanks and each tank was stocked with nine fish weighing 800 g. Results indicated the SGR, FCR and condition factor of YTK fed the high specification diet were 12.2%, 22.9% and 2.8% better, respectively than the same indices measured in YTK fed the low specification diet. On average, the relative feed intake of YTK fed the high specification diet was significantly lower than fish fed the standard specification diet (12.9%). Moreover, results indicated YTK cannot upregulate their feed intake sufficiently to compensate for lower nutrient and energy intake as a result of missed feeding days or lower diet specification. Direct comparisons of performance and digestible nutrient and energy intake between YTK fed the standard and high specification diet on a daily basis suggest YTK were eating primarily to satisfy their DP requirements. These results demonstrate that the performance of sub-adult YTK is extremely sensitive to the nutrient and energy composition of aquafeeds. There is no performance benefit in feeding sub-adult YTK less than once daily to apparent satiation and they should be fed on a daily basis in order to maintain growth trajectory and optimise feed efficiency.

Introduction

New South Wales Department of Primary Industries (NSW DPI) has previously reported on the results of two feeding trials with juvenile Yellowtail Kingfish (*Seriola* lalandi; YTK) (Manuscript 3.2.4.1). These experiments demonstrated that sub-adult fish (150-500 g) are capable of consuming both 6 mm or 9 mm diameter commercial extruded pellets and that pellet size has little impact on growth rate or

FCR. The studies also indicated that under laboratory conditions, one daily meal, fed to apparent satiation is sufficient to sustain optimum growth while at the same time subtly improving FCR. These results have been provided to industry and currently represent best feeding practice for sub-adult YTK reared under controlled conditions in indoor tank facilities.

One criticism of feeding frequency trials run under controlled conditions is that they do not accurately reflect the practical and logistical problems often faced at the production scale. For example, fluctuations in water temperature, air pressure and weather conditions often affect the feeding behaviour of fish and the ability of farmers to accurately feed their stock on a daily basis. Of these events, the inability to feed stock due to inclement weather and safety concerns has the greatest effect on production performance. For example, in 2017, our partners Huon Aquaculture Group Ltd. were unable to safely feed the YTK on the NSW DPI Marine Aquaculture Research Lease (MARL) in Port Stephens for approximately 20-30% of the time due to extreme weather events, high swells or logistical issues. Similar events are experienced by the South Australian industry where sea-cage operations are located in exposed coastal locations. Under prolonged fasting or erratic feeding regimes fish reared in sea-cages will lose condition and may experience changes in body composition and record higher (worse) FCR.

Studies on other marine finfish have demonstrated positive compensatory growth with fish reared using higher specification feeds (e.g. higher energy and/or nutrient density) after periods of fasting (Cho and Heo, 2011; Lu et al., 2014). The notion of compensatory or catch-up growth is described by phenomenon whereby underfed, feed deprived or malnourished fish are returned to adequate feeding regimes usually accompanied by hyperphagia and efficient feed utilisation. This can be associated with repletion of energy stores and rapid weight gain. Often animals in the poorest condition show the greatest response; i.e. the catch-up response is inversely related to the duration of feed deprivation (Jobling, 2001). The differences between compensatory growth and catch-up-growth are subtle, but the former generally refers to a faster than usual growth rate, while the latter implies attainment of a comparable size to a control group following some form of restriction such as feed deprivation (Jobling, 2010; Hector and Nakagawa, 2012; Hitchcock, 2012). Jobling (2010) suggests "compensatory growth can occur in the absence of catch-up growth, and the simultaneous observation of compensatory growth and a recovery of body mass is a special combination of events". Further, he suggests "it is possible for growth trajectories to converge even when animals that have experienced a period of reduced growth do not display compensatory growth".

The potential of YTK to undergo compensatory of catch-up growth following limited feed restriction is appealing to the industry as it means the economic cost of feed deprivation might be offset to some extent by rapid weight gain (Gibson and Gatlin, 2001). If confirmed, it also offers the industry an opportunity to examine the deployment of their labour force (i.e. days on vs days off) and plan around the management of logistical issues such as bathing for fluke. In this final feeding frequency experiment we test the hypothesis that the performance of YTK fed restrictively (5 days per week) or sporadically (randomly) can be ameliorated by choice of feeding regime and/or the use of a high specification diet.

Methods

This study is being performed under the NSW DPI Fisheries Animal Care and Ethics (ACEC) Research Authority known as 'Aquaculture Nutrition ACEC 93/5–Port Stephens'. Care, husbandry and termination of fish were carried out according to methods outlined in 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2015).

Formulation and preparation of experimental feeds

Two test diets were formulated for this experiment; a 'standard specification diet' formulated to have 490-500 g protein kg^{-1} diet and 20-21 MJ energy kg^{-1} and a 'high specification diet' formulated to contain 580-600 g protein kg^{-1} diet and 23-24 MJ energy kg^{-1}. The diets differed in absolute protein, fat and energy content, but they had a similar protein:energy ratio of 25 g crude protein MJ gross $energy^{-1}$. The diets were also formulated to be similar in terms of their digestible protein:digestible energy ratio (DP:DE), which was estimated *a priori* to be approximately 27 g DP MJ^{-1} DE based on conservative estimates for dietary protein and energy ADCs of 60% and 65%, respectively. The formula of each diet

and treatment codes are presented in Table 3.2.4.2.1. The total amino acid content of the high specification diet was approximately 23% higher than the standard specification diet. In addition the methionine and taurine content of each diet was adjusted to reflect the results of earlier research on these nutrients (Manuscript 3.1.5.3). Cereal grains and legumes were autoclaved prior to mixing and pellet making to ensure gelatinization of starches. Both diets were made into 6 mm diameter sinking pellets on laboratory scale equipment and oven dried at < 60°C until they had a moisture content of < 7%. The measured nutrient and energy composition of each diet is presented in Table 3.2.4.2.2.

Feeding regimes

Three satiation based feeding regimes were tested in this experiment following consultation with industry. They were 1) a regular feeding approach based on feeding once daily 7 days per week (control regime); 2) a regime whereby fish were fed once daily 5 days per week (Monday to Friday only - economic regime); and 3) a feeding regime whereby fish were fed randomly during the week (random regime). The randomised feeding regime was included to represent the missed feeding days commonly experienced in farming operations due to weather or operational constraints. The randomised feeding regime also prevents fish from becoming entrained to a regular feeding pattern, thereby overcoming the opportunity of fish to synchronize their biological feeding rhythms with a predictable pattern of feed delivery (Madrid et al., 2001). The three feeding regimes were crossed with the two aforementioned diet specifications to create an orthogonal experiment having six treatments. Each treatment was replicated in 3 × 1000 L experiment tanks (18 experiment tanks in total). Fish were fed between 10:00 -11:00 h.

The randomised feeding pattern was created using a random-calendar-date-generator (www.random.org/calendar-dates/). The number of random feeding days per month was determined by randomly selecting 70% of the available dates occurring within a calendar month (all days Mon-Sun). This pattern approximately reflects the number of feeding days that are missed in a typical YTK offshore farming operation (M. Whittle, Huon Aquaculture Group Ltd., Pers. Comm., January, 2018). The random feeding pattern used in this experiment is presented in Table 3.2.4.2.3.

Fish handling and treatment

The experiment was done in a recirculating aquaculture system (RAS) fitted with 18 × 1000 L square, polyethylene tanks. The RAS was located in a greenhouse and was maintained at an optimal water temperature for YTK of 21.6 °C (Pirozzi and Booth, 2009) using reverse-cycle refrigeration units. Each tank was fitted with an air stone diffuser and a mesh screen to prevent fish escaping. Each tank was also fitted with an additional air stone that diffused industrial grade oxygen into the water column to maintain dissolved oxygen levels at > 5 mg L^{-1}. The influent flow rate to each tank was approximately 20 L min^{-1}. The RAS contained additional equipment to maintain water quality including fluidized-bed bio-filters, particle filtration (Hydrotech 501 drum filters) and foam fractionation (Aquasonic). Effluent water from the RAS was continuously removed and replaced with clean, filtered, disinfected saltwater (chlorination followed by dechlorination with sodium thiosulfate) drawn from the Tilligerry Estuary adjacent to PSFI. YTK were exposed to a natural photoperiod (i.e. summer/autumn 2018).

Sub-adult YTK were obtained from the NSW DPI - Huon MARL with the cooperation and assistance of Huon Aquaculture staff. While on-farm, and while acclimating to laboratory conditions, fish were maintained on 9 mm diameter Huon Select diet (produced by Skretting Australia, Cambridge, TAS, Australia). All fish were given several freshwater baths prior to stocking to remove residual skin and gill fluke. At stocking fish were anaesthetised (AQUI-S®), weighed and measured, then given an additional 3-5 min freshwater bath before being allocated to research tanks. Nine × 800g fish were stocked into each experiment tank on the 19/3/2018. An additional *in situ* fluke treatment (i.e. 150 ppm hydrogen peroxide for 30 min) was given to all fish on the 19/4/2018 to remove small numbers of persistent gill fluke. A random sample of five initial fish were euthanised and frozen to determine initial fish composition. Four fish were randomly selected from each tank at the end of the experiment and euthanised to determine hepatosomatic index (HSI), viscerosomatic index (VSI) and proximate composition of whole fish.

Water quality

Water quality variables were recorded on a daily basis using portable electronic instruments. Mean water temperature during the experiment was 21.1 °C with a recorded minimum and maximum of 18.6 °C and 22.9 °C, respectively. Salinity ranged from 30‰ to 33‰. Dissolved oxygen concentration remained above 7 mg L^{-1} throughout the experiment and oxygen saturation was always > 100%. pH ranged from 6.9 to 8.4 units. Total ammonia nitrogen (TAN) concentration was measured regularly using a colourmetric test kit. The mean TAN was ≤ 0.8 mg L^{-1}.

Chemical analysis

Experimental feeds, whole fish carcass (initial and harvest) and faecal samples were analysed for dry matter, crude protein, gross energy (bomb calorimetry), lipid and ash content, respectively. Diets were also analysed for amino acids. Diets and faecal material were also analysed for yttrium in order to determine apparent digestibility of the test diets. All chemical analysis was done by CSIRO Agriculture and Food (Carmody Road, St Lucia, QLD 4067, Australia).

Major response variables measured

The following performance variables were used to assess the response of YTK to different treatments using the average value of all fish from each replicate tank, unless specified otherwise;

- Initial weight of fish (g) = individual weight of fish at stocking
- Final weight of fish (g) = individual weight of fish at harvest
- Specific growth rate (SGR; % d^{-1}) = [Ln(final weight) – Ln(initial weight)] / days × 100
- Condition factor *K* = [individual weight of fish (g) / fork length of fish $(mm)^3$] × 10^5
- Food conversion ratio (FCR) = feed intake per tank (g) / wet weight gain per tank (g)
- H.S.I. = (liver weight (g) / whole weight of fish (g)) × 100
- V.S.I. = viscera weight (g) / whole weight of fish (g)) × 100; where viscera includes liver + organs + intraperitoneal fat.
- Nutrient retention efficiency (%) = digestible nutrient gain / digestible nutrient intake × 100

Apparent digestibility coefficients (ADCs) for dietary dry matter (DM), nutrients and energy are calculated according to the equation described by Cho et al. (1982), with the exception that yttrium was used as the inter marker rather than chromium;

- ADC of dry matter (%) = [1 - (concentration of Ytt in diet / concentration of Ytt in faeces)] × 100
- ADC of nutrients or energy (%) = [1 - (concentration of Ytt in diet / concentration of Ytt in faeces × concentration of nutrient or energy in faeces / concentration of nutrient or energy in diet)] × 100.

Statistical procedures

Treatment data was compared using two-way ANOVA setting the fixed factors to ‘diet type’ (standard spec. vs high spec.) and ‘feeding regime’ (5 days, 7 days and random feeding). Alpha for ANOVA and the post-hoc multiple comparison procedure (Tukey-Kramer Test) was set at 0.05. Data subjected to ANOVA was statistically analysed using NCSS-8.0.23 after assumptions related to normality and sample variance were examined (Hintze, 2012).

Results

Apparent digestibility of standard and high specification diets

One-way ANOVA indicated there was no significant difference between the high and low specification diets with respect to their crude protein, fat, ash, NFE and gross energy ADCs. In contrast, the dry matter ADC of the diets was different ($P = 0.006$), being significantly higher in the high specification diet (DM ADC = 70.0%; $n = 3$) than the standard specification diet (DM ADC = 59.1%; $n = 3$) (Table 3.2.4.2.4). As the protein ADCs of the low and high specification diets were not different, the difference in the ADCs of individual amino between diets was not compared statistically.

Multiplication of the dietary nutrients and energy contained in each diet (Table 3.2.4.2.2) by the respective ADCs determined for nutrients and energy in each diet allowed estimation of the digestible nutrient and energy content of either diet. These estimates are also presented in Table 3.2.4.2.4. Because the ADCs of most nutrients and energy were not different between diets and the crude nutrient and gross energy of the high specification diet is higher than the standard specification diet the resultant digestible nutrient and energy content of the high specification diet was also substantially higher (Table 3.2.4.2.4). However, as per the design of the experiment, the DP:DE ratio of each diet is almost the same (25.6 vs 24.0 g DP MJ DE^{-1}).

Weight gain, specific growth rate and condition factor

Individual treatment data on weight gain, SGR and condition factor is presented in Table 3.2.4.2.5 and pooled data on the results of two-factor ANOVA of these variables are presented in Tables 3.2.4.2.5a, 3.2.4.2.5b and 3.2.4.2.5c, respectively. Daily weight gain and SGR were significantly affected by feeding regime ($P < 0.05$) and diet specification ($P < 0.05$). Neither of these production indices was affected by the interaction of the main effects ($P > 0.05$). Weight gain of YTK was significantly higher in fish fed daily (8.23 g d^{-1}) than in fish fed 5 days per week (6.36 g d^{-1}) or randomly (6.51 g d^{-1}g), which were statistically similar. Weight gain was also significantly higher in fish fed the high specification diet (7.51 g d^{-1}) than in fish fed the standard specification diet (6.55 g d^{-1}) (Table 3.2.4.2.5a). Similarly, the SGR of YTK was significantly higher in fish fed daily (0.82% d^{-1}) than fish fed 5 days per week (0.66% d^{-1}) or randomly (0.67% d^{-1}), which were statistically similar. SGR was also significantly higher in fish fed the high specification diet (0.75% d^{-1}) compared to fish fed the standard specification diet (0.67% d^{-1}) (Table 3.2.4.2.5b).

The harvest condition factor (K) of YTK was not significantly affected by feeding regime ($P > 0.05$) or the interaction of the main effects ($P > 0.05$). However, the condition factor of fish was significantly affected by diet type ($P < 0.05$), being higher in fish fed the high specification feed (1.12) than in fish fed the low specification feed (1.09) (Table 3.2.4.2.5c). Although not significant, the condition factor of fish fed on a daily basis was numerically higher (1.12) than fish fed 5 days per week (1.10) or fish fed randomly (1.10) (Table 3.2.4.2.5c). The condition factor of fish on entry to the experiment was slightly higher than at harvest. Presumably this is due to a change in environmental circumstances (MARL to PSFI); a change in feeding regime (twice daily to once daily, 5 days or randomly) and diet types (commercial feed to experimental feeds).

Relative feed intake and feed conversion efficiency

Individual treatment data on relative feed intake and FCR is presented in Table 3.2.4.2.6 and pooled data on the results of two-factor ANOVA of these variables are presented in Tables 3.2.4.2.6a and 3.2.4.2.6b, respectively. Diet specification ($P < 0.05$) and feeding regime ($P < 0.05$) significantly affected relative feed intake, however there was no interaction between the fixed factors ($P > 0.05$). Relative feed intake was higher in fish fed the low specification diet (13.52 g kg BW^{-1} d^{-1}; $n = 9$) compared to the high specification diet (11.77 g kg BW^{-1} d^{-1}; $n = 9$). Relative feed intake was significantly higher in fish fed 7 days per week (14.16 g kg BW^{-1} d^{-1}; $n = 6$) than fish fed 5 days per week (11.85 g kg BW^{-1} d^{-1}) or fish fed randomly (11.92 g kg BW^{-1} d^{-1}), which were statistically similar (Table 3.2.4.2.6a).

FCR (dry matter basis) was significantly affected by diet specification ($P < 0.05$) and by a minor interaction between the main effects ($P = 0.049$). However, there was no effect of feeding regime on FCR ($P > 0.05$). The minor interaction between fixed factors was caused by a greater difference in the FCR of fish fed the low and high specification diet, respectively, under the 5 day per week regime. As the interaction was only minor, the effect of diet specification was compared cautiously. In this case the FCR of fish fed the high protein diet was significantly better (lower) (1.55:1; $n = 9$) than the FCR of fish fed the lower specification diet (2.01:1; $n = 9$). The FCRs of YTK fed daily, 5 days per week or randomly were statistically similar, being 1.73:1, 1.83:1 and 1.79:1, respectively. Nonetheless, the FCR of fish fed daily was numerically lower (better) than the FCR of fish fed 5 days per week or fed randomly (Table 3.2.4.2.6b).

Hepatosomatic and viscerosomatic indices

Individual treatment data on hepatosomatic (HSI) and viscerosomatic indices (VSI) are presented in Table 3.2.4.2.6. Both HSI and VSI were significantly affected by feeding regime ($P < 0.05$), but not by diet specification ($P > 0.05$) or the interaction of the main effects ($P > 0.05$). HSI was significantly higher in fish fed 5 days a week (1.21%; $n = 6$), than fish fed 7 days a week (1.05%; $n = 6$) and fish fed randomly (0.97%; $n = 6$), which were statistically similar. VSI was significantly higher in fish fed randomly (4.52%; $n = 6$), than for fish fed 7 days per week (3.64%; $n = 6$) and fish fed 5 days per week (3.85%; $n = 6$), which were statistically similar.

Fish composition

The dry matter, moisture, crude protein and ash composition of whole fish taken at the end of the experiment was not affected by diet specification ($P > 0.05$), feeding regime ($P > 0.05$) or the interaction of the main factors ($P > 0.05$). The fat content of YTK was significantly affected by diet specification ($P = 0.024$), but not by feeding regime ($P > 0.05$) or the interaction of main factors ($P > 0.05$). The fat content of fish fed the high specification diet was significantly higher (9.48%; $n = 9$) than the fat content of YTK fed the low specification diet (8.60%; $n = 9$). Individual treatment data on wet basis composition of whole fish is given in Table 3.2.4.2.7 and pooled data on the results of two-factor ANOVA on fat composition is presented in Table 3.2.4.2.7a.

Gross nutrient and energy intake

There was no interaction between diet type and feeding regime with respect to tests on daily crude protein, crude lipid and gross energy intake of YTK (all $P > 0.05$). Crude protein intake was not affected by diet specification ($P > 0.05$), but was affected by feeding regime ($P < 0.0001$), being significantly higher in fish fed daily as opposed to fish fed 5 days per week or randomly, which were statistically similar (Figure 3.2.4.2.1a). Crude lipid intake was significantly affected by diet specification ($P < 0.0001$) and feeding regime ($P < 0.0001$) being higher in fish fed daily as opposed to fish fed the other two regimes and higher in fish fed the high specification diet (Figure 3.2.4.2.1b). Daily gross energy intake was not affected by diet specification ($P > 0.05$), but it was affected by feeding regime ($P < 0.0001$), being about 24% higher in fish fed daily as opposed to fish fed the other two regimes, which were statistically similar (Figure 3.2.4.2.1c).

Digestible nutrient and energy intake

There was no interaction between diet specification and feeding regime with respect to tests on daily digestible protein, digestible lipid and digestible energy intake of YTK (all $P > 0.05$). Digestible protein intake was not affected by diet specification ($P > 0.05$), but was significantly affected by feeding regime ($P < 0.0001$), being significantly higher in fish fed daily as opposed to fish fed 5 days per week or randomly, which were statistically similar (Figure 3.2.4.2.1d). Digestible lipid intake was significantly affected by diet specification ($P < 0.0001$) and feeding regime ($P < 0.0001$), being higher in fish fed daily as opposed to fish fed the other two regimes and higher in fish fed the high specification diet

(Figure 3.2.4.2.1e). Digestible energy intake was affected by diet specification ($P < 0.002$) and feeding regime ($P < 0.0001$), being about 13% higher in fish the high specification diet and higher in YTK fed on a daily basis as opposed to fish fed the other two regimes, which were statistically similar (Figure 3.2.4.2.1f).

Digestible nutrient retention

Two outliers were removed from two-way assessment of means due to unusually low retention efficiencies; one from treatment C50 (random fed, std. spec. diet; Tank 11) and one from treatment B50 (5 days fed, std. spec. diet; Tank 23). Under these constraints digestible protein retention was not affected by diet specification ($P > 0.05$), feeding regime ($P > 0.05$) or the interaction of the main effects ($P > 0.05$) and averaged 27.4% (i.e. $n = 16$). Digestible fat retention was not affected by diet specification ($P > 0.05$), feeding regime ($P > 0.05$) or the interaction of the main effects ($P > 0.05$) and averaged 33.2% ($n = 16$). However, there was a reasonable amount of variation between among the fat retention efficiencies of YTK fed daily (38.9%; $n = 6$) as opposed to those fed 5 days per week (30.7%; $n = 5$) and those fed randomly (30.2%; $n = 5$). Digestible energy retention was not affected by diet specification ($P > 0.05$), feeding regime ($P > 0.05$) or the interaction of the main effects ($P > 0.05$) and averaged 29.0% (i.e. $n = 16$). Numerical variation in digestible energy retention efficiency reflected the variation seen in fat retention, being higher in fish fed daily (31.3%; $n = 6$) than in fish fed 5 days per week (26.7%; $n = 5$) or those fed on a random basis (29.1%; $n = 5$).

Discussion

SGR, FCR and condition factor of sub-adult YTK growing between 0.8-1.2 kg are improved by feeding a high specification diet containing 46.0% DP and 19.2 MJ DE kg^{-1} to apparent satiation once daily. Specifically, the SGR, FCR and condition factor of YTK fed the high specification diet were 12.2%, 22.9% and 2.8% better, respectively, than the same indices measured in YTK fed the low specification diet containing 38.6% DP and 15.1 MJ DE kg^{-1} diet. On average, the relative feed intake of YTK fed the high specification diet was significantly lower (12.9%) than fish fed the standard specification diet, presumably because YTK fed the higher specification were able to satisfy their nutrient and energy demands as a result of consuming more digestible nutrients and energy per unit of dry matter intake. As diets were ostensibly formulated and made from similar inclusions of the same suite of raw materials there is no indication that the palatability of feeds influenced this experiment, as evidenced by the feed intake data.

Digestible protein (DP) and digestible energy (DE) demands for 1 kg YTK derived from published factorial models are given to be 7.4g DP $fish^{-1}$ d^{-1} and 273kJ $fish^{-1}$ d^{-1} provided from diets supplying about 27g DP MJ DE^{-1} (Booth et al., 2010). More recent bioenergetic research (Manuscript 3.2.2.1) has indicated requirements for a 1 kg fish are approximately 6.6 g DP $fish^{-1}$ $d^{-1}$and 231 kJ $fish^{-1}$ d^{-1} at approximately 21 °C. Based on the later value for DP, an animal from the current study would have needed to consume approximately 17.1 g of the standard specification diet or 14.4 g of the high specification diet per day, respectively, to meet their DP requirement. Conversely, to meet their DE demand they would have needed to consume 15.3 g and 12.1 g of the standard and high specification diets, respectively. The mean daily feed intake of fish fed the standard (A50) and high specification (A60) diets once daily to apparent satiation were 15.2 g and 13.1 g ($n = 3$; Table 3.2.4.2.6), respectively; values that closely approximate those estimated from the most recent factorial model for similar size fish reared at approximately the same water temperature. The fact that YTK fed the standard specification diet (A50) once daily had a significantly higher feed intake than YTK fed the high specification diet (A60) suggests these fish were trying to compensate for the lower nutrient and energy density of the standard feed by increasing their intake. This trend, although not significant, was also reflected in YTK fed 5 days per week and fed randomly. Thus, fish fed the standard specification diet once per day were unable to satisfy either their DP or DE requirements for optimum growth due to sub-optimal nutrient and energy intake. This in turn negatively impacted growth rate and FCR of all fish fed the standard specification diet. In addition, the lower consumption rate of YTK fed the high specification diet implies these groups of fish could have ingested more feed but didn't, demonstrating they were likely feeding to nutrient demand. Offering YTK two meals per day to apparent satiation may have

allowed them to compensate for the nutrient and energy density of the standard specification feed (or one similar) and this strategy should be investigated.

Different feeding regimes also affected feed intake, FCR and SGR of YTK. Feeding YTK once daily to apparent satiation resulted in a 19.1% increase in relative feed intake, a 4.4% improvement in FCR and a 23.2% increase in SGR, respectively compared to the average of the other two feeding regimes. These results provide strong evidence that the major production parameters of interest with respect to farming YTK (i.e. feed intake, growth and FCR) deteriorate quickly when fish are fed restrictively, whether that be feeding in a regular systematic pattern such as 5 days per week or sporadically, such as under the random feeding regime. It should be noted that although these feed regimes were evaluated by feeding YTK to apparent satiety at each meal, offering YTK extra meals per day may result in slightly different outcomes using the same diets. For example there may be deterioration in FCR as reported in Manuscript 3.2.4.1. Nonetheless, there was no growth or FCR benefit from feeding less frequently and farms should continue to feed YTK to apparent satiation at least once daily.

The deterioration in growth performance and worsening of FCR in YTK fed randomly or 5 days per week was ultimately the result of lower feed intake in these groups of fish. Under the random and 5 days per week feed regimes YTK consumed only 81.5% and 80.4%, respectively, of the amount of feed consumed by YTK fed on a daily basis (based on dry matter intake; pooled means $n = 6$). This indicates that the fish in these groups could not maintain their growth trajectory by physically consuming enough extra feed on the days they were fed to compensate for the days they were not fed. Clearly, YTK must be fed at least once daily an adequately formulated feed to ensure they achieve their growth potential, at least when housed at the temperature used in the current study (i.e. 21.1 °C).

Of interest in this study was the potential of restrictively fed YTK to exhibit a compensatory or catch up growth response. However, the feed restrictions applied in this experiment (i.e. forcing YTK to fast for two days per week or randomly) may not have been severe enough to place the animals in a state of growth stagnation due to either prolonged feed deprivation (Skalski et al., 2005; Jobling, 2010) or the level of feed deprivation (Eroldogan et al., 2006). Thus, it not possible to determine if YTK exhibit either compensatory growth, catch-up growth or a mix of both from the data recorded in this experiment. In order to determine if YTK exhibit these responses the period of feed deprivation would need to be in the order of several weeks before fish are returned to a satiety feeding regime as demonstrated with juvenile Barramundi (*Lates calcarifer*) (Tian and Qin, 2004). In that study the authors showed that compensatory growth in Barramundi occurred in fish subjected to moderate feed restrictions (i.e. 50% and 75% satiation) within two and four weeks, respectively, after fish returned to a satiation feeding regime. However, Barramundi fed at 25% satiation for two weeks did not catch up with the satiated control fed group, even after satiation refeeding for five weeks. In a study not dissimilar to ours, juvenile Channel Catfish (*Ictalurus punctatus*) (17 g) were fed diets with variable protein (32% vs 37%) and energy content (12.5 vs 15.1 MJ DE kg^{-1}) over six weeks using either satiate feeding (control group) or groups that were fasted for three days then refed for 11 days (Gibson and Gatlin, 2001). Similar to our results, these authors found that the average cumulative feed intake was lower in the fasted groups of fish. They also found that the average cumulative weight gain of Catfish was better on the high-protein, high-energy diets, but unlike our study, cumulative weight gain was not affected by feeding regime. Feed efficiency of Catfish was significantly better on the high-protein diet, but in contrast to our study, feed efficiency was better in Catfish that had been fasted for three out of every 14 days (Gibson and Gatlin, 2001). These authors concluded that Channel Catfish can consume adequate quantities of less nutrient dense diets to adequately supply both energy and amino acids to meet their needs during the period of compensatory gain. This does not appear to be the case in YTK subjected to a similar experimental procedure; thus YTK do not exhibit a hyperphagic response, at least when limited to a single meal per day. However, we note these are very different species with quite different gut morphologies.

Apart from fat content of YTK, the wet composition of whole fish was minimally affected by the different diet specifications or feeding regimes. The average fat content of YTK proved to be significantly higher in those reared on the high specification diet compared to the standard specification diet (9.48% vs 8.60%, respectively; $n = 9$). The retention of digestible fat was statistically unaffected by experimental treatments, however, there was a high degree of variability in fat retention efficiencies among treatment groups (Table 3.2.4.2.8). The slight increase in carcass fat for fish for groups of YTK

fed the high specification diet may reflect the slight increase in digestible fat intake per unit of DP intake experienced by these groups (Figure 3.2.4.2.1 and Figure 3.2.4.2.2) (National Research Council, 2011).

Irrespective of diet specification, YTK fed once daily to apparent satiation consumed similar amounts of crude protein (Figure 3.2.4.2.1a), gross energy (Figure 3.2.4.2.1c) and DP (Figure 3.2.4.2.1d). There was slightly more DE consumed in the high specification groups than the standard specification groups (Figure 3.2.4.2.1f) and understandably there was more crude and digestible lipid consumed by fish raised on the high specification diet (Figure 3.2.4.2.1b and Figure 3.2.4.2.1e, respectively). Whether fish fed the standard specification diet were targeting additional protein, lipid or energy is difficult to ascertain. However, the lack of a significant difference between the daily crude or DP intake of YTK fed either the standard or high specification diet coupled with the variability in the daily crude or digestible intake of lipid (and to some extent energy), perhaps gives some insight into this question (Figure 3.2.4.2.2; plot of YTK fed 7 days per week). As can be seen in Figure 3.2.4.2.2 the average DP intake of YTK is 5.83 and 6.03 g fish d^{-1} for the standard and high specification diet, respectively. These values are extremely close to the DP intake required to support optimum growth in YTK as estimated from the aforementioned factorial model. In contrast, the DE intake of YTK fed the standard and high specification diets was 228.7 and 251.3 kJ d^{-1}, respectively. The latest factorial model suggests that fish of this size require approximately 231 kJ fish d^{-1} in order to optimise growth, which suggests the YTK reared on the standard specification diet were possibly not meeting or were on the threshold of meeting their energy demands; either because their protein demands had already been met or they were unable to consume enough feed during a single meal to compensate for the lower energy (fat) density if this feed.

Conclusions and Recommendations

This experiment examined the interactive effects of three satiation feeding regimes (i.e. once daily 7 days per week; once daily 5 days per week and once daily at random days each week) and two diet specifications with similar DP:DE ratio (i.e. standard specification feed having 38.6% DP and 15.1 MJ DE kg^{-1}) and high specification diet (i.e. 46% DP and 19.2 MJ DE kg^{-1}) on the performance of 1 kg YTK reared at optimal water temperature for eight weeks. With respect to the most advantageous feeding regime, the results indicate unequivocally that sub-adult YTK should be fed a high quality diet at least once daily to apparent satiation (premised on results of two-way ANOVA). Relative feed intake, SGR and FCR were all numerically better in groups of YTK fed to apparent satiation once each day and there was no biological benefit in feeding YTK 5 days per week. Improvements in these indices for fish fed 5 days and randomly throughout the week relate to simple increases in the absolute nutrient and energy intake afforded these animals as a result of consuming the high specification diet. Nonetheless, even these groups of fish were unable to increase their feed intake (i.e. nutrient and energy intake) to such an extent that it compensated for the lack of feeding opportunity. Moreover, YTK fed the standard specification diet 5 days per week or randomly performed even more poorly, presumably for the same reasons.

Direct comparisons of performance and digestible nutrient and energy intake between YTK fed the standard and high specification diet on a daily basis with recent data from bioenergetic models on YTK suggests YTK were eating primarily to satisfy their DP requirements. As a consequence, fish reared on the standard specification diet may have indirectly limited their DE intake (fat) to levels that inhibit optimal growth and feed utilisation. As such they were unable to meet their genetically programmed growth potential due to a subtle but chronic state of energy deficiency, perhaps related to a slight imbalance in the optimum DP:DE ratio of the diet for this size animal. These results demonstrate that the performance of sub-adult YTK is extremely sensitive to the nutrient and energy composition of aquafeeds.

The biological conclusions of this study are clear. However, there may be some economic benefit to be derived from feeding YTK a high specification diet to about 80% of a satiety ration 5 days per week; at least in terms of economic FCR and labour savings (i.e. FCR of A60, B60 and C60 were statistically similar; Table 3.2.4.2.6). However, these decisions also need to be made with reference to optimal growth rates of YTK, which proved to be between 15-19% higher in YTK fed the high specification diet (A60) than in YTK fed the same diet 5 days per week or randomly (i.e. B60 and C60; Table 3.2.4.2.5).

Findings

- There is no performance benefit in feeding sub-adult YTK less than once daily to apparent satiation.
- Sub-adult YTK should be fed on a daily basis in order to maintain growth trajectory and improve feed efficiency.
- Sub-adult YTK fed once daily cannot upregulate their feed intake sufficiently to compensate for lower nutrient and energy intake as a result of missed feeding days or lower diet specification.
- SGR, FCR and condition factor of sub-adult YTK growing between 0.8-1.2 kg are improved by feeding a high specification diet containing 46.0% DP and 19.2 MJ DE kg^{-1} to apparent satiation once daily.
- In this study the SGR, FCR and condition factor of YTK fed a high specification diet was 12.2%, 22.9% and 2.8% better, respectively than the same indices measured in YTK fed a lower specification diet containing 38.6% DP and 15.1 MJ DE kg^{-1} diet.
- Wherever possible the industry should aim to feed fish on a daily basis with a high quality diet.
- Minor deficits in the nutrient and energy content of aquafeeds (quality) for YTK, if known, might be overcome by feeding to apparent satiation at least twice per day (quantity).
- The growth and feed performance of sub-adult YTK is extremely sensitive the nutrient and energy composition of aquafeeds.
- Bioenergetic models for YTK are useful in interpreting data from feeding experiments.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, New South Wales Department of Primary Industries (NSW DPI) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

We would also like to acknowledge the technical assistance of Luke Vandenberg, Ian Russell, Justin Tierney, Marcus Armstrong and Steve Gamble (NSW DPI). We are extremely grateful to Ridley for providing the raw materials used in this study, especially the assistance of Dr Michael Salini and Dr Simon Tabrett. Thanks also to Barney Hines and staff at CSIRO for conducting the chemical analyses on samples generated from this experiment. We thank Steven Clarke (SARDI) for useful advice on the feeding regimes examined in this study.

References

ACEC, 2015. A Guide to Acceptable Procedures and Practices For Aquaculture and Fisheries Research - 4th Edition, 4th ed. NSW Department of Primary Industries (Fisheries) for NSW Fisheries Animal Care and Ethics Committee, Nelson Bay, NSW, Australia.

Booth, M.A., Allan, G.L., Pirozzi, I., 2010. Estimation of digestible protein and energy requirements of yellowtail kingfish *Seriola lalandi* using a factorial approach. Aquaculture 307, 247-259.

Cho, C.Y., Slinger, S.J., Bayley, H.S., 1982. Bioenergetics of salmonid fishes: energy intake, expenditure and productivity. Comparative Biochemistry and Physiology 73B, 25-41.

Cho, S.H., Heo, T.Y., 2011. Effect of dietary nutrient composition on compensatory growth of juvenile olive flounder *Paralichthys olivaceus* using different feeding regimes. Aquacult. Nutr. 17, 90-97.

Eroldogan, O.T., Kumlu, M., Kiris, G.A., Sezer, B., 2006. Compensatory growth response of *Sparus aurata* following different starvation and refeeding protocols. Aquacult. Nutr. 12, 203-210.

Gaylord, G.T., Gatlin III, D.M., 2001. Dietary protein and energy modifications to maximize compensatory growth of channel catfish (*Ictalurus punctatus*). Aquaculture 194, 337-348.

Hector, K.L., Nakagawa, S., 2012. Quantitative analysis of compensatory and catch-up growth in diverse taxa. J Anim Ecol. 81, 583-593.

Hintze, J., 2012. NCSS 8. NCSS, LLC. Kaysville, Utah, USA. NCSS, Kaysville, Utah, USA., www.ncss.com.

Hitchcock, M.J., 2012. Side effects of feed restriction and compensatory growth in fish, Zoology. University of Otago, Dunedin, New Zealand, pp. 194.

Jobling, M., 2001. Nutrient partitioning and the influence of feed composition on body composition. in: Houlihan, D., Boujard, T., Jobling, M. (Eds.), Food Intake in Fish. Blackwell Publishing, pp. 354-375.

Jobling, M., 2010. Are compensatory growth and catch-up growth two sides of the same coin? Aquaculture International 18, 501-510.

Lu, K.L., Li, X.F., Wang, L.N., Zhang, C.N., Liu, W.B., 2014. Effects of dietary nutrient composition on compensatory growth of juvenile blunt snout bream *Megalobrama amblycephala*. N. Am. J. Aquacult. 76, 55-60.

Madrid, J.A., Boujard, T., Javier Sanchez-Vazquez, F., 2001. Feeding rhythms. in: Houlihan, D., Boujard, T., Jobling, M. (Eds.), Food Intake in Fish. Blackwell Science, Oxford, pp. 189-215.

National Research Council, N., 2011. Nutrient Requirements of Fish and Shrimp. The National Academies Press.

Pirozzi, I., Booth, M.A., 2009. The routine metabolic rate of mulloway (*Argyrosomus japonicus*: Sciaenidae) and yellowtail kingfish (*Seriola lalandi*: Carangidae) acclimated to six different temperatures. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 152, 586-592.

Skalski, G.T., Picha, M.E., Gilliam, J.F., Borski, R.J., 2005. Variable intake, compensatory growth, and increased growth efficiency in fish: Models and mechanisms. Ecology 86, 1452-1462.

Tian, X.L., Qin, R.G., 2004. Effects of previous ration restriction on compensatory growth in barramundi *Lates calcarifer*. Aquaculture 235, 273-283.

Table 3.2.4.2.1. The raw material composition of the standard and high specification diets used in the feeding frequency experiment (g kg^{-1} dry matter basis).

Ingredient	**Standard spec. feed 50CP:20MJ**	**High spec. feed 60CP:24MJ**
Fishmeal	480.0	600.0
Poultry meal	70.0	40.0
Meat meal	70.0	27.0
Blood meal	15.0	78.0
Wheat flour	104.3	50.0
Fish oil	50.0	80.0
Poultry oil	50.0	80.0
Diatomaceous earth	124.0	12.6
Choline chloride (70%)	3.0	3.0
Methionine	9.7	5.4
NaH_2PO_4	5.0	5.0
Rovimix Stay-C 35	3.0	3.0
Taurine	10.0	10.0
Vit-min premix	5.0	5.0
Y_2O_3	1.0	1.0
Total	1000	1000

Table 3.2.4.2.2. Measured nutrient and energy composition of experimental diets used in feeding frequency study (g kg^{-1} dry matter basis).

Nutrient or energy (MJ kg^{-1})	**Standard spec. feed 50CP:20MJ**	**High spec. feed 60CP:24MJ**
Nitrogen	78.4	92.5
Crude protein	490.1	578.0
Total lipid	164.8	221.6
Ash	239.2	130.0
NFE	105.9	70.4
Gross energy (MJ $kg^{-1)}$	19.9	23.6
Yttrium	0.89	0.89
Alanine	27.0	33.8
Arginine	22.9	31.6
Aspartic acid	36.8	46.8
Cysteine	3.7	5.1
Glutamic acid	58.4	68.7
Glycine	31.4	33.7
Histidine	10.6	16.3
Isoleucine	15.9	18.3
Leucine	31.2	40.9
Lysine	33.4	45.3
Methionine	20.4	18.7
Phenylalanine	17.7	23.8
Proline	21.6	24.6
Serine	15.2	19.0
Taurine	13.5	14.7
Threonine	17.3	22.3
Tyrosine	12.8	16.6
Valine	19.7	26.5
Sum AA's	409.3	506.9
CP:GE ratio (g CP MJ GE^{-1})	24.6	24.5

Table 3.2.4.2.3. The randomised feeding pattern used in this experiment.

Date	Feed allocation		Date cont.	Feed allocation
19/03/2018 – stocking	na		20/04/2018	Fed
20/03/2018	Not fed		21/04/2018	Fed
21/03/2018	Fed		22/04/2018	Fed
22/03/2018	Fed		23/04/2018	Fed
23/03/2018	Fed		24/04/2018	Fed
24/03/2018	Not fed		25/04/2018	Fed
25/03/2018	Not fed		26/04/2018	Not fed
26/03/2018	Not fed		27/04/2018	Fed
27/03/2018	Not fed		28/04/2018	Fed
28/03/2018	Fed		29/04/2018	Not fed
29/03/2018	Fed		30/04/2018	Not fed
30/03/2018	Fed		1/05/2018	Fed
31/03/2018	Fed		2/05/2018	Not fed
1/04/2018	Fed		3/05/2018	Fed
2/04/2018	Fed		4/05/2018	Fed
3/04/2018	Not fed		5/05/2018	Fed
4/04/2018	Fed		6/05/2018	Fed
5/04/2018	Fed		7/05/2018	Fed
6/04/2018	Fed		8/05/2018	Fed
7/04/2018	Fed		9/05/2018	Fed
8/04/2018	Fed		10/05/2018	Fed
9/04/2018	Not fed		11/05/2018	Fed
10/04/2018	Not fed		12/05/2018	Not fed
11/04/2018	Fed		13/05/2018	Not Fed
12/04/2018	Fed		14/05/2018 - harvest	na
13/04/2018	Not fed			
14/04/2018	Fed			
15/04/2018	Fed			
16/04/2018	Fed			
17/04/2018	Not fed			
18/04/2018	Not fed			
19/04/2018	Fed			

Table 3.2.4.2.4. Apparent digestibility coefficients (ADC%) and digestible nutrient content of low and high specification diets (g kg^{-1} dry matter basis).

	Standard spec. feed 50CP:20MJ	**High spec. feed 60CP:24MJ**	**Standard spec. feed 50CP:20MJ**	**High spec. feed 60CP:24MJ**
Nutrient or energy	**ADC (%)**	**ADC (%)**	**Digestible nutrient (g kg^{-1} diet)**	**Digestible nutrient (g kg^{-1} diet)**
Dry matter	59.1±1.3	70.0±3.3*	591.2	699.7
Protein (%)	78.7±1.7	79.6±2.2	**385.9**	**460.1**
Total lipid (%)	80.8±9.4	86.6±4.9	133.1	191.8
Ash (%)	16.0±2.7	14.6±4.7	38.4	19.0
NFE	31.9±14.9	41.0±7.7	33.8	28.8
Gross energy (MJ kg^{-1})	75.8±1.8	81.2±3.1	**15.1**	**19.2**
Alanine	83.9±0.9	85.5±2.3	22.7	28.9
Arginine	95.3±1.6	97.3±0.4	21.8	30.8
Aspartic acid	71.0±0.8	76.0±1.8	26.1	35.6
Cysteine	65.7±2.1	71.2±1.1	2.4	3.7
Glutamic acid	82.9±0.3	84.8±1.3	48.4	58.3
Glycine	81.7±1.6	80.4±2.9	25.7	27.1
Histidine	77.9±4.0	84.6±2.9	8.2	13.8
Isoleucine	85.2±0.5	83.8±3.6	13.5	15.4
Leucine	88.0±0.5	86.3±2.8	27.4	35.2
Lysine	88.4±1.7	88.7±1.5	29.5	40.2
Methionine	87.4±2.6	86.8±1.8	17.8	16.2
Phenylalanine	89.1±0.7	87.3±2.7	15.8	20.8
Proline	86.5±0.7	83.9±4.0	18.7	20.7
Serine	88.4±0.9	90.6±1.3	13.4	17.2
Taurine	81.4±0.4	80.6±3.9	10.2	11.9
Threonine	85.8±0.9	83.6±2.7	14.8	18.6
Tyrosine	87.4±2.1	91.6±2.8	11.2	15.2
Valine	81.4±1.1	82.1±3.0	16.0	21.8
			DP:DE ratio = 25.6	**DP:DE ratio = 24.0**

*Dry matter ADC of low and high specification diets were significantly different (ANOVA; $P < 0.05$).

Table 3.2.4.2.5. Average stock and harvest metrics of sub-adult Yellowtail Kingfish fed different dietary treatments under different feeding regimes for 56 days*.

Treatment code	**Feed regime**	**Feed type**	**Stock weight (g)**	**Initial fork length (mm)**	**Initial condition factor *K***	**Harvest weight (g)**	**Harvest fork length (mm)**	**Harvest condition factor *K***	**SGR (% d^{-1})**
A50	7 days	Std. spec.	795.3	398.0	1.26	1240.4^{bc}	481.5	1.11	0.793^{b}
A60	7 days	High spec.	795.4	391.1	1.33	1271.8^{c}	481.5	1.14	0.837^{b}
B50	5 days	Std. spec.	799.7	393.7	1.31	1109.2^{a}	467.3	1.08	0.584^{a}
B60	5 days	High spec.	799.7	396.9	1.28	1201.9^{abc}	476.8	1.11	0.726^{ab}
C50	Random	Std. spec.	804.1	395.4	1.30	1149.4^{ab}	473.2	1.08	0.638^{a}
C60	Random	High spec.	798.8	394.2	1.31	1182.5^{abc}	472.9	1.11	0.698^{ab}
SEM	-	-	*3.10*	*2.33*	*0.02*	*22.7*	*3.4*	*0.016*	*0.030*
F value	-	-	*1.10*	*1.12*	*1.22*	*6.84*	*2.63*	*1.67*	*9.86*
P value	-	-	*0.41*	*0.40*	*0.36*	*0.003*	*0.08*	*0.22*	*0.0006*

*Data in this table has been analysed using one-way ANOVA and Tukey's post-hoc multiple comparison test.

Table 3.2.4.2.5a. Two-way ANOVA results on daily weight gain (g d^{-1}) of Yellowtail Kingfish fed different dietary treatments under feeding regimes for 56 days (mean ± SEM).

Feed regime	Standard spec. feed (50CP:20MJ)	High spec. feed (60CP:24MJ)	Marginal means
7 days	7.95	8.50	8.23 ± 0.27^{b}
5 days	5.53	7.18	6.36 ± 0.27^{a}
Random	6.17	6.85	6.51 ± 0.27^{a}
Marginal means	6.55 ± 0.22^{x}	7.51 ± 0.22^{y}	Grand mean = 7.03

Table 3.2.4.2.5b. Two-way ANOVA results on SGR (% d^{-1}) of Yellowtail Kingfish fed different dietary treatments under feeding regimes for 56 days (mean ± SEM).

Feed regime	Standard spec. feed (50CP:20MJ)	High spec. feed (60CP:24MJ)	Marginal means
7 days	0.793	0.837	0.815 ± 0.02^{b}
5 days	0.584	0.727	0.655 ± 0.02^{a}
Random	0.638	0.698	0.668 ± 0.02^{a}
Marginal means	0.672 ± 0.02^{x}	0.754 ± 0.02^{y}	Grand mean = 0.713

Table 3.2.4.2.5c. Two-way ANOVA results on condition factor *K* of Yellowtail Kingfish fed different dietary treatments under feeding regimes for 56 days (mean ± SEM).

Feed regime	Standard spec. feed (50CP:20MJ)	High spec. feed (60CP:24MJ)	Marginal means
7 days	1.11	1.13	1.12±0.011
5 days	1.08	1.11	1.10±0.011
Random	1.08	1.11	1.10±0.011
Marginal means	1.09 ± 0.009^{x}	1.12 ± 0.009^{y}	Grand mean = 1.104

Table 3.2.4.2.6. Average feed intake, survival and organ metrics for sub-adult Yellowtail Kingfish fed different dietary treatments under different feeding regimes for 56 days*.

Treatment code	**Feed regime**	**Feed type**	**Dry basis feed intake (g fish^{-1} d^{-1})**	**Relative feed intake (g kg BW^{-1} d^{-1})**	**FCR**	**Survival (%)**	**HSI (%)**	**VSI (%)**
A50	7 days	Std. spec.	15.19^{c}	15.29^{c}	1.92^{b}	96.3	1.02	3.70^{a}
A60	7 days	High spec.	13.11^{b}	13.03^{b}	1.55^{a}	88.9	1.08	3.58^{a}
B50	5 days	Std. spec.	11.83^{ab}	12.56^{ab}	2.14^{b}	88.9	1.19	3.80^{a}
B60	5 days	High spec.	10.93^{a}	11.14^{a}	1.52^{a}	100	1.23	3.90^{ab}
C50	Random	Std. spec.	12.23^{ab}	12.72^{b}	1.99^{b}	92.6	0.99	4.26^{ab}
C60	Random	High spec.	10.83^{a}	11.13^{a}	1.59^{a}	96.3	1.00	4.78^{b}
SEM	-	-	*0.43*	*0.33*	*0.05*	*4.53*	*0.07*	*0.19*
F value	-	-	*14.6*	*22.0*	*29.1*	*0.98*	*2.17*	*5.36*
P value	-	-	*<0.0001*	*<0.0001*	*0.0007*	*1.46*	*0.13*	*0.008*

*Data in this table has been analysed using one-way ANOVA and Tukey's post-hoc multiple comparison test.

Table 3.2.4.2.6a. Two-way ANOVA results on relative feed intake (g kg BW^{-1} d^{-1}) of Yellowtail Kingfish fed different dietary treatments under feeding regimes for 56 days (mean ± SEM).

Feed regime	Standard spec. feed (50CP:20MJ)	High spec. feed (60CP:24MJ)	Marginal means
7 days	15.29	13.03	14.16±0.23^{b}
5 days	12.56	11.14	11.85±0.23^{a}
Random	12.71	11.13	11.92±0.23^{a}
Marginal means	13.52±0.19^{x}	11.77±0.19^{y}	Grand mean = 12.64

Table 3.2.4.2.6b. Two-way ANOVA results on FCR (dry matter basis) of Yellowtail Kingfish fed different dietary treatments under feeding regimes for 56 days (mean±SEM).

Feed regime	Standard spec. feed (50CP:20MJ)	High spec. feed (60CP:24MJ)	Marginal means
7 days	1.92	1.55	1.73±0.03
5 days	2.14	1.52	1.83±0.03
Random	1.99	1.59	1.79±0.03
Marginal means	2.01±0.03^{x}	1.55±0.03^{y}	Grand mean = 0.713

Table 3.2.4.2.7. Average wet basis proximate composition of whole sub-adult Yellowtail Kingfish fed different dietary treatments under different feeding regimes for 56 days*.

Treatment code	**Feed regime**	**Feed type**	**Dry matter (%)**	**Moisture (%)**	**Nitrogen (%)**	**Crude protein (%)**	**Fat* (%)**	**Ash (%)**	**GE (MJ kg^{-1})**
A50	7 days	Std. spec.	34.3	65.7	3.2	20.2	9.6	3.8	8.6
A60	7 days	High spec.	34.0	66.0	3.2	19.8	9.6	3.6	8.8
B50	5 days	Std. spec.	31.7	68.3	3.2	19.9	8.2	3.7	7.8
B60	5 days	High spec.	33.8	66.2	3.2	20.1	9.6	3.6	8.6
C50	Random	Std. spec.	32.7	67.3	3.2	20.3	8.0	3.7	8.2
C60	Random	High spec.	33.1	66.9	3.2	20.0	9.2	3.6	8.5
SEM	-	-	*0.86*	*0.86*	*0.08*	*0.54*	*0.42*	*0.16*	*0.26*
F value	-	-	*1.21*	*1.21*	*0.12*	*0.12*	*3.25*	*0.25*	*1.69*
P value	-	-	*0.36*	*0.36*	*0.98*	*0.98*	*0.04*	*0.93*	*0.21*

* Data in this table has been analysed using one-way ANOVA and Tukey's post-hoc multiple comparison test. Although one-way ANOVA was significant (P = 0.0437) on the test of whole carcass fat, the Tukey's test could not separate the treatment means at the 95% confidence interval.

Table 3.2.4.2.7a. Two-way ANOVA results on whole carcass fat content (wet basis) of Yellowtail Kingfish fed different dietary treatments under feeding regimes for 56 days (mean ± SEM).

Feed regime	Standard spec. feed (50CP:20MJ)	High spec. feed (60CP:24MJ)	Marginal means
7 days	9.62	9.63	9.62±0.30
5 days	8.17	9.63	8.90±0.30
Random	8.02	9.19	8.60±0.30
Marginal means	8.60±0.24^{x}	9.48±0.24^{y}	Grand mean = 0.713

Table 3.2.4.2.8. Digestible protein, digestible fat and digestible energy retention efficiency of Yellowtail Kingfish fed different dietary treatments under different feeding regimes for 56 days*.

Treatment code	Reps	Feed regime	Feed type	Digestible protein retention (%)	Digestible fat retention (%)	Digestible energy retention (%)
A50	3	7 days	Std. spec.	27.69	41.85	30.93
A60	3	7 days	High spec	27.23	35.92	31.69
B50	2	5 days	Std. spec.	25.48	24.15	22.03
B60	3	5 days	High spec	29.10	37.23	31.33
C50	2	Random	Std. spec.	27.90	29.17	30.26
C60	3	Random	High spec	27.08	31.14	27.91
SEM	-	-	-	*2.42*	*4.07*	*2.47*
F value	-	-	-	*0.19*	*1.98*	*1.64*
P value	-	-	-	*0.96*	*0.17*	*0.26*

*Data in this table has been analysed using one-way ANOVA and Tukey's post-hoc multiple comparison test. Two outliers removed from assessment of means (Tank 11- C50; Tank 23- B50) due to unusually low retention efficiencies.

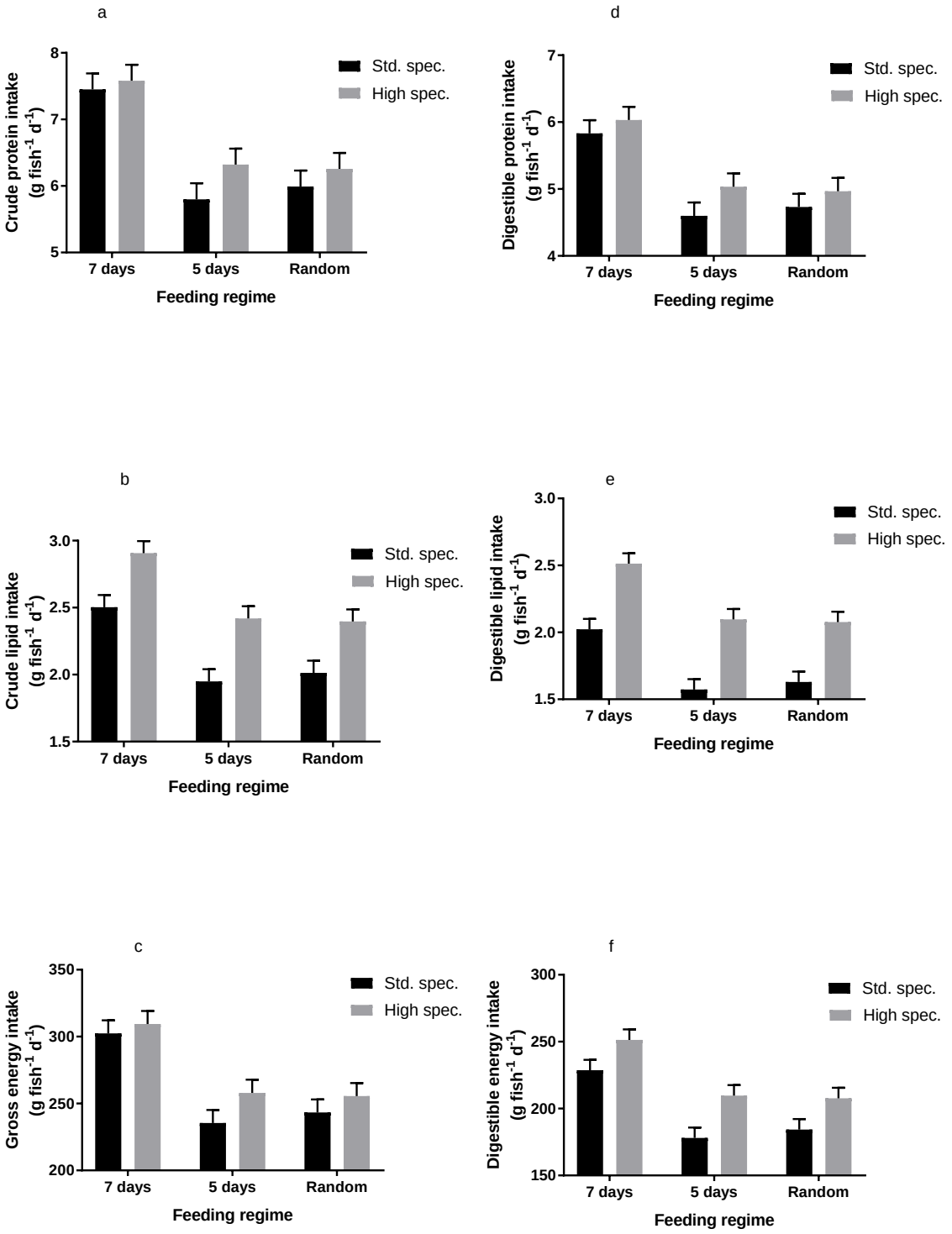


Figure 3.2.4.2.1. Average daily individual intake of crude protein (a), crude lipid (b), gross energy (c), digestible protein (d), digestible lipid (e) and digestible energy (f) in sub-adult Yellowtail Kingfish. Columns are treatment means ± pooled standard error (n = 3).

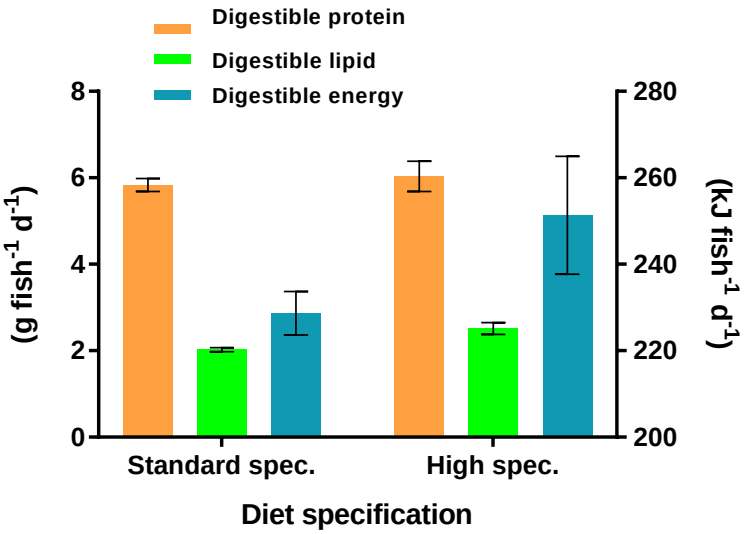


Figure 3.2.4.2.2. Comparison of digestible protein, digestible lipid and digestible energy intake in Yellowtail Kingfish fed a standard or high specification diet once daily to apparent satiation. Columns are treatment means ± SD (n = 3); Digestible protein intake (P = 0.42), digestible fat intake (P = 0.004) and digestible energy intake (P = 0.054).

3.2.5. Chapter - Field validations of newly developed feeds and feeding practices for sub-adult Yellowtail Kingfish.

3.2.5.1. Manuscript - Field evaluation of a low fishmeal diet and a diet containing fisheries by-product meal fed to juvenile Yellowtail Kingfsh Seriola lalandi.

Mark Booth[a], Luke Vandenberg[a] and Igor Pirozzi[a,b]

[a] New South Wales Department of Primary Industries, Locked Bag 1, Nelson Bay, NSW 2315
[b] College of Science and Engineering and Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville 4801, QLD, Australia.

This manuscript may be referenced as: Booth, M., Vandenberg, L. and Pirozzi, I. (2019). 3.2.5.1. Manuscript - Field evaluation of a low fishmeal diet and a diet containing fisheries by-product meal fed to juvenile Yellowtail Kingfsh *Seriola lalandi*. In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.427-448.

Abstract

This study presents encouraging results on fishmeal reduction and the use of different fishmeal sources in aquafeeds for juvenile Yellowtail Kingfish (*Seriola lalandi*; YTK) reared under fluctuating field conditions. The results of a 4 month experiment demonstrated that the dietary level of prime fishmeal can be reduced from 55% to 15% without short term productivity being affected when fishmeal reduction is offset by inclusion of other high quality proteins such as brewer's yeast, hydrolysed feather meal, poultry meal, meat meal, dehulled lupin and feed grade soy protein concentrate. The economic and environmental benefits of feeding a diet containing 15% fishmeal versus one containing 55% fishmeal were reflected in a 24% reduction in raw material costs and a 46% reduction in the fish-in fish-out ratio (FIFO) of the low fishmeal diet. The results of a 2.5 month experiment demonstrated that 30% fishery by-product meal can be used to wholly replace an equivalent amount of prime fishmeal in diets for juvenile YTK without significantly affecting short term production outcomes. The economic and environmental benefits of feeding a diet containing 30% fishery by-product meal versus one containing 30% prime fishmeal were reflected in a 4.5% reduction in raw material costs and a 45% reduction in the FIFO of the fishery by-product meal diet. These results confirm there is enormous scope in not only the choice of alternative protein sources for YTK but also a high degree of formulation plasticity. In addition, the incremental changes in body weight of YTK during experiments closely matched the predicted body weight of YTK according to an updated temperature-dependent growth model developed for this species by NSW DPI. These models will be beneficial for benchmarking growth in laboratories and field situations.

Introduction

At the inception of the K4P project, NSW DPI and Huon Aquaculture Group made plans to conduct two research based field trials on the yet to be developed NSW DPI offshore Marine Aquaculture Research Lease (MARL; -32°39';152°17.0'). These field trials were conceived with the expectation that the MARL would be constructed, operational and stocked with Yellowtail Kingfish (YTK) that could be used to run simple, but large scale experiments. These trials were purposely planned for the end of the K4P project, specifically so as much new project information on the nutrition and feeding of YTK could be included in the design of experiments. Unfortunately, there have been several well documented set-backs on the MARL during the latter stages of the K4P project which have precluded

any field based studies being done at this location. Therefore, the MARL experiments have been relocated to a large outdoor pond facility at PSFI.

The original MARL experiments had several objectives. One was to track the seasonal growth and FCR of a single cohort of YTK fed one of Huon's bespoke feeds for YTK. This particular formulation was highly conservative, being high in fishmeal and fish oil. It was purposely formulated to ensure the growth rate of YTK was not limited by raw material selection or nutrient and energy density. A diet such as this is expensive; however it was instituted to enable the initial performance characteristics of farmed YTK reared under MARL conditions to be established; information which did not exist at that time. A second objective was to 'benchmark' the performance of YTK fed the bespoke diet against the performance of YTK fed either modified versions of the bespoke feed or other experimental diets, such as those designed to evaluate reduced levels of fishmeal or fish oil. A third aim was to use both environmental and growth data from the MARL to validate and improve NSW DPIs current bioenergetic model for YTK. At the inception of the project the model was primarily based on data from laboratory trials (Booth et al., 2010).

The PSFI pond experiments have similar objectives to our original goals and were designed to assess performance of YTK in a field-based rather than a laboratory-based situation. These objectives were 1) to compare the performance of YTK fed a diet that was similar to the bespoke Huon diet to YTK fed a very low fishmeal diet; 2) to evaluate the impact of using fishery by-product meal as an alternative to prime fishmeal in diets for YTK and 3) to compare the growth of fish to that predicted by the latest iteration of the NSW DPI temperature-growth model. The trials were designed to incorporate our recent advances in understanding of choline, taurine and methionine requirements, digestibility of raw materials and appropriate feeding strategies.

Methods

Meeting the objectives

Meeting the first objective required the formulation of two diets that were similar in nutrient and energy content but that differed significantly in their fishmeal content. Two fishmeal levels were established; 55% and 15%. The reduction of fishmeal from 55% to 15% required the use of several alternative raw materials to ensure protein and energy content remained high. These included brewer's yeast (Tacon, Auclair, 2010), feather meal (Bureau, 2010), poultry offal meal (Saadiah et al., 2011; Booth et al., 2017), soy protein concentrate and dehulled lupins (Petterson, 2000).

Meeting the second objective required the substitution of prime fishmeal with fishery by-product meal. This endeavour was relatively straightforward in terms of meeting formulation constraints and permitted the testing of a diet containing 30% prime fishmeal against a diet containing 30% fishery by-product meal or a diet containing and equal blend of these raw materials. Fishery by-product meals are proving to be promising options in aquafeeds for high order pelagic species, especially by-product meals such a tuna meal (Herpandi et al., 2011; Siddik et al., 2018). The tuna canning industry uses less than 35% of the whole fish, with the remaining portion used for production of low-value fishmeal, fertilizers or fishmeal hydrolysates (Herpandi et al., 2011; Oncul et al., 2018). In addition, fishery by-product meals are becoming widely adopted in many animal production industries due to their perceived sustainability and the lowering of fish-in fish-out ratios. One drawback with low value fishery by-product meal is that it may be relatively high in ash as a consequence of bones and scales; however these meals contain many of the intangible properties found in normal fishmeal sources (Siddik et al., 2018).

The third objective was addressed by comparing the changing weight of YTK stocked into the pond experiments against estimates derived from a recent version of the PSFI temperature-dependent growth model for YTK.

Outdoor pond system and management

Two field-based experiments were done in a single, rectangular outdoor pond (0.5 ML) located at PSFI. The pond was approximately 15 m wide × 20 m long and approximately 2 m deep and lined with black PVC. Similar research trials with fish have been done in the same pond at PSFI and the research has been published (Booth et al., 2012).

A modular PVC pontoon (Ultimate Modular Dock UMD; http://superiorjetties.com/umd-modules/) was installed lengthwise in the pond. Twelve specially constructed floating cages were then attached to the pontoon with a gap of approximately 0.5 m between each cage. Six cages were attached on each side of the pontoon. Each set of cages formed one experimental system. Cage frames were circular (2 m diameter × 1.5 m deep) and made from high grade stainless steel (10 mm). The side was encased in 15 mm oyster mesh whereas the base and top of each cage was enclosed with high density nylon mesh (20 mm). Four 300 mm polystyrene floats (MAE220; www.roadtechmarine.com.au) were secured to the frame of each cage to make it float. The base of each cage was approximately 1.1 m from the pond surface. The submerged volume of each cage was approximately 3.5 m^3.

An oxygen delivery manifold was installed along the pontoon to provide dissolved oxygen to each cage (BOC industrial grade oxygen; MAN15). A single high pressure oxygen diffuser was installed at the base of each cage and the flow of oxygen to each cage was regulated by an individual flow-meter. Oxygen was diffused into each cage over the entire experiment to ensure levels remained above > 6 mg L^{-1} at all times, especially during the photosynthetic dark cycle when the respiration rate of the resident algal biomass in the pond increased.

The pond was fed with unfiltered estuarine water from the Tilligerry Creek adjacent PSFI. Water was pumped to the pond inlet via a large reticulation system (150 mm diameter PVC pipe) and exited from an internal sump located at the opposite end of the pond. Water flow through the pond was maintained at a rate of approximately 288 kL day^{-1} up to the 5-11-2018. Elevation in ambient air temperature at the beginning of November 2018 increased the water temperature of the pond to critical levels approaching 28.5 °C. For this reason the influent flow rate was increased to approximately 345 kL d^{-1} in order to reduce the pond temperature to an acceptable level. Water quality (pH, temperature, dissolved oxygen, salinity) was monitored on a daily basis using electronic meters and a temperature logger was installed at 1.0 m depth midway along the pontoon to record diurnal fluctuations in water temperature (Tinytag Explorer V 4.5.127; Gemini Data Loggers (UK) Ltd.).

Biofouling was removed from cages as necessary, but fish were generally not disturbed more frequently than once per month for this procedure. As far as practicable, this task was incorporated into weight check procedures. Biofouling was removed after fish were temporarily relocated to a 1000 L tank located on the bank of the pond. This tank was fitted with a dissolved oxygen diffuser and contained a low level of anaesthetic (Aqui-S). Cages were then taken from the pontoon to an area adjacent to the pond and high pressure washed to remove fouling organisms and algae. The cage was then reinstated and the fish returned. This procedure usually took no more than 15-20 min per cage. The area directly beneath the cages was also vacuumed siphoned from time to time to remove the build-up of settled organic material.

A small 200 L floating cage of sentinel fish was installed in the pond to monitor the presence of skin or gill fluke. These fish were located away from the experiment cages and fed a commercial diet. One or two sentinel fish were removed on a weekly basis for inspection under a stereo dissecting microscope. Experimental fish were also visually inspected for fluke during all weight check procedures.

Stocking procedures and feeding regime

Prior to entering the experiment, fish were held in a 25 kL recirculating aquaculture system (RAS) at PSFI and fed a commercial aquafeed produced exclusively for Huon Aquaculture Group by Skretting Australia (Huon 3 mm). Preceding each stocking event a subsample of fish was taken in order to determine the weight distribution within the cohort. The cohort was then lightly sedated before small groups were moved to a 200 L vessel containing a stronger dose of anaesthetic and an oxygen stone

(Aqui-S). Once sedated fish were graded and then individually weighed and measured. Small groups of fish were then sequentially transferred to the cages in 20 L buckets ensuring that each run was placed in a separate cage. Each cage was stocked with 35 juvenile YTK which were progeny of wild caught broodstock held at PSFI.

Experiment 1 was stocked on the 19-7-2018. The average weight (g), fork length (mm) and condition factor (*K*) of fish at placement was 77.5 g, 179.4 mm and 1.34, respectively.

Experiment 2 was stocked on the 6-9-2018. The average weight (g), fork length (mm) and condition factor (*K*) of fish at placement was 96.9 g, 195.5 mm and 1.30, respectively.

Fish in both experiments were fed to apparent satiation once daily between 11:30 h-12:30 h and intake was recorded on a daily basis. The time of feeding coincided with the cyclic increase in water temperature that occurred in the pond each day. In addition, cages were fed their respective diets in a randomised fashion during the experiments to reduce the chance of anticipatory feeding behaviour being established. All diets were stored in a freezer between feeding events (-17 °C).

Experimental diets and manufacture

All test diets examined in this study were based on the crude protein, crude fat and gross energy content of the bespoke YTK diets being used by Huon Aquaculture on the NSW DPI - Huon MARL. The formulations and estimated nutrient and energy composition of the two diets used in Experiment 1 are presented in Table 3.2.5.1.1 and Table 3.2.5.1.2, respectively, whereas, the formulations and estimated nutrient and energy composition of the three diets used in Experiment 2 are presented in Table 3.2.5.1.3 and Table 3.2.5.1.4, respectively. Estimated nutrient and energy composition of all diets is based on prior chemical analysis of raw materials and least cost formulation software (WinFeed 2.8 release 3; WindFeed (UK) Ltd., Cambridge University, U.K.). The raw material cost of each diet was based on the spot pricing of raw materials provided by reliable commentators such as Hammersmith Marketing Ltd (http://hammersmithltd.blogspot.com/). As such the estimated cost of each feed in this report may not reflect current commodity pricing. However, the estimated data do provide a means of comparing the relative difference in the raw material cost of each formula at a moment in time.

Prior to pellet making all raw materials were ground in a high speed turbine powder mill fitted with a 0.8 mm sieve (Model EFWB30B; Ernest Fleming Machinery and Equipment Pty Lt., Lane Cove West, 2066, NSW, Australia). Wheat flour was autoclaved for 2 min at 121 °C prior to inclusion in the dry mash. The raw materials and supplements were then dry mixed in a 120 L stainless steel vertical mixer (Flamingo 120 L; Ernest Fleming Machinery and Equipment Pty Lt., Lane Cove West, 2066, NSW, Australia), before the addition of oils and a suitable amount of fresh water. The damp mash was then pressed into 6 mm or 9 mm diameter pellets using a stainless steel meat mincer (Dadaux TX-82; Barnco Sales, Ashfield 2131, NSW Australia). The damp pellets were dried for two days at approximately 50 °C in a specially constructed dehydrator until the dry matter content of pellets was ≥ 95%.

The majority of raw materials used experiments were provided by Ridley (Robart Court, Narangba 4504, QLD, Australia), apart from brewer's yeast which was obtained from Farmers Warehouse (M^{c}Dougal's Hill, Singleton 2330, NSW, Australia). All feeds were stored frozen at -17 °C during the experiment. The scale of the experiments necessitated the manufacture of several batches of each diet. This required the use of updated batches of raw materials from Ridley. Minor adjustments were therefore made to the original formulations to correct for changes in the moisture content of raw materials.

Production characteristics

The following response variables were determined;

- Initial weight of fish (g) = individual weight of fish at stocking
- Final weight of fish (g) = individual weight of fish at harvest

- Specific growth rate (% d^{-1}) = [Ln(final weight) - Ln(initial weight)] / days × 100
- Condition factor *K* = [individual weight of fish (g) / fork length of fish $(mm)^3$] × 10^5
- Food conversion ratio (FCR) = feed intake per tank (g) / wet weight gain per tank (g)

Apparent digestibility of diets

The digestibility of the diets from each experiment was determined in two separate indoor trials at PSFI. Small groups of fish (10 per tank; ≈130 g body weight) were held in 200 L circular tanks that formed part of a laboratory-scale recirculating aquaculture system (RAS). The water temperature of the RAS was controlled to 20 ± 2 °C which was the intermediate temperature recorded in the outdoor pond. Faecal material was collected from replicate groups of fish (*n* = 3 tanks per treatment) using stripping techniques similar to that described by (Booth and Pirozzi, 2017). Briefly, sedated fish (AQUI-S®) were individually netted from their respective tank and the ventral surface was wiped clean. A small amount of pressure was then applied to the abdomen using the thumb and forefinger to expel urinary products. The ventral area was cleaned again before firm abdominal pressure was applied to expel faecal material from the distal intestine. Faecal matter was expelled into a clean 70 mL container. Hands were rinsed clean between the handling of different fish and care was taken to ensure that the faecal samples were not contaminated by urine or mucous. Faecal samples were immediately stored in a freezer at -17 °C. Faecal samples were generally collected about 16h after the last meal and fish were never stripped on consecutive days. Faecal samples from each tank were pooled and kept frozen at –17 °C until a sufficient amount was obtained for chemical analysis.

Apparent digestibility coefficients (ADCs) for dietary dry matter (DM), nutrients and energy are calculated according to the equation described by (Cho et al., 1982), with the exception that yttrium was used as the endogenous marker;

- ADC of dry matter (%) = [1 - (Ytt in diet / Ytt in faeces)] × 100
- ADC of nutrients or energy (%) = [1 - (Ytt in diet / Ytt in faeces × concentration of nutrient or energy in faeces / concentration of nutrient or energy in diet)] × 100.

The digestible nutrient and energy content of each diet was calculated by multiplying the nutrient or gross energy content of the diet by its respective apparent digestibility coefficient.

Temperature-growth model

A recent version of the NSW DPI temperature growth model for YTK was used to compare the actual versus predicted weight gain of fish in Experiment 1 and 2. The model is being constantly updated as new data is available, but at present it takes the form of;

Weight gain (g $fish^{-1}$ day^{-1}) = (a + b × T + c × T^2 + d × T^3) × BW^e: where T = water temperature, BW = body weight (g) and the other model parameters are as follows (see inserted table below);

	Parameter				
Model	a	b	c	d	e
PSFI MB V5	0.7600	-0.1931	0.0149	-0.0003	0.4839

A graphical representation of the model is presented in Figure 3.2.5.1.3 showing the predicted daily gain of fish weighing from 50 g to 1 kg exposed to water temperatures between 9 °C and 30 °C.

Fish-in fish-out ratio (FIFO)

The fish-in fish-out ratio (FIFO) of each experimental diet was calculated based on the assumptions and equation presented by Terpstra (2015), which accounts for the combined inclusion of wild derived fishmeal and fish oil in diets;

FIFO = FCR × 0.75 × 0.5 × [(% fish meal in feed / 22.5) + ((% fish oil in feed – 0.08 × % fish meal in feed) / 5)]

where the FIFO ratio is expressed in reduction fish equivalents and FCR is the feed conversion ratio. The yield of reduction fish is about 22.5% fish meal and 5.0% fish oil. The factor 0.75 takes into account that about 25% of the fishmeal and fish oil is nowadays produced from fish slaughter byproducts and the factor 0.08 takes into account that fish meal contains 8% fish oil (Terpstra, 2015). Fishery by-product meal such as the one examined in this study is excluded from the calculation.

Chemical analysis

Experimental feeds and faecal samples were analysed for dry matter, crude protein, gross energy (bomb calorimetry), lipid and ash content, respectively. Diet and faecal samples were also analysed for yttrium (Ytt) in order to estimate the apparent digestibility of the diets. All concluding analyses were done by CSIRO (Agriculture and Food, Carmody Road, St Lucia, QLD 4067, Australia), whereas preliminary analysis of raw materials was done by Upscience Lab Solutions (Tinh Binh Duong, Vietnam; https://www.upscience-labs.com/).

Statistical analysis

Treatment groups were compared using one-way ANOVA. Alpha for ANOVA and the post-hoc multiple comparison procedure (Tukey-Kramer Test) was set at 0.05. Data was statistically analysed using NCSS-8.0.23 (Hintze, 2012) after assumptions related to normality and homogeneity of variances were investigated.

Results

General observations

All groups of fish readily accepted the experimental feeds and there was no sign of feed rejection; indicating the palatability of all feeds was acceptable. Mean, minimum and maximum water quality parameters recorded during daylight hours between the 19-7-2018 to the 15-11-2018 are presented below (see inserted table below). Dissolved oxygen levels remained high during the trial, partly due to the addition of industrial oxygen into each cage and partly due to the production of dissolved oxygen by resident algae. Both pH and salinity were very stable. Water temperature ranged from 11.6 °C to 28.5 °C during the same period (Figure 3.2.5.1.1).

	pH	**Dissolve oxygen (mg L^{-1})**	**Saturation (%)**	**Salinity (‰)**	**Total ammonia (mg L^{-1})**
mean	7.81	12.80	132.05	32.45	0.02
max	8.57	18.26	199.00	34.70	0.20
min	7.28	7.24	68.14	28.10	0.00

Fish from both experiments remained healthy during the trial. There was no visual sign of skin or gill fluke in either the sentinel fish or the experimental fish.

There were only three mortalities between the stocking events and the 15-11-2018; two fish from cage 6 (104 g BW and 197 mm FL on 29-8-2018; 376 g BW and 320 mm FL on 4-11-2018) and one fish from cage 10 (362 g BW and 310 mm FL on 5-11-2018). The last two mortalities occurred within a day of each other and they coincided with the highest water temperatures recorded in the pond (28.5 °C).

Experiment 1 – Fishmeal reduction

Incremental changes in cage biomass, feed intake and FCR of fish in Experiment 1 are presented in Table 3.2.5.1.5. A graphical representation of individual weight gain can be found in Figure 3.2.5.1.2. There was no significant difference between dietary treatments at different sample points with respect to cage biomass and FCR. Feed intake was also unaffected by dietary treatment, apart from the comparison made on the 4-9-2018, where the feed intake of fish offered the fishmeal control diet was significantly higher (1551 g cage^{-1}) than those offered the low fishmeal option (1469 g cage^{-1}; Table 3.2.5.1.6). Food conversion ratio (FCR) improved over time in Experiment 1, decreasing from about 1.41-1.48:1 to 0.95-0.99:1 at the last weight check (25-10-2018; Table 3.2.5.1.5). The FCR of both diets increased from 1.0:1 to 1.16:1 during the last stage of Experiment 1. The higher FCRs (worse) at the beginning and end of Experiment 1 are likely related to the cold and warm water temperature periods experienced by the fish during this part of study (Figure 3.2.5.1.1).

The apparent digestible nutrient and energy coefficients of diets from Experiment 1 are presented in Table 3.2.5.1.2. The high fishmeal diet was significantly ($P < 0.05$) more digestible for dry matter protein and fat. The digestible energy content was similar between the diets.

Experiment 2 – Fishmeal origin

Incremental changes in cage biomass, feed intake and FCR of fish in Experiment 2 are presented in Table 3.2.5.1.6. A graphical representation of individual weight gain can be found in Figure 3.2.5.1.3. After 70 days there was no significant difference among the biomass, FCR or SGR of YTK fed diets containing prime fishmeal, a fishery by-product meal or a blend of these two fishmeal sources. As for Experiment 1, the FCR of fish in Experiment 2 increased slightly (worsened) after the first weight assessment. This minor increase is likely related to the elevated water temperature occurring in that period (Figure 3.2.5.1.1).

The apparent digestible nutrient and energy coefficients of diets from Experiment 2 are presented in Table 3.2.5.1.4. There was no significant difference ($P > 0.05$) among the diets when considering the digestibility of dry matter, protein, fat and energy.

Predicted versus actual weight gain of YTK

The measured versus predicted weights of YTK from Experiment 1 and Experiment 2 are presented in Figure 3.2.5.1.5 and Figure 3.2.5.1.6, respectively. There was close agreement between the actual weight values and those predicted by the temperature dependent growth model (PSFI MB V5). At the end of the Experiment 1 there was a difference of 35 g between the actual and predicted average weight of fish, the model value (711 g) being slightly lower than the overall average value (746 g). At the end of Experiment 2 there was a difference of 54 g between the actual and predicted average weight of fish, the model value (612 g) being slightly lower than the overall average value (667 g). The average standard deviation and coefficient of variation (CV) of individual fish weight at the end of Experiment 1 was 101.3 g and 13.5%, respectively. Therefore, the model predictions were within one standard deviation of the average weight of fish in both experiments (Figure 3.2.5.1.5 and Figure 3.2.5.1.6).

Simple economic assessment of diets and FIFO ratio

The estimated raw material cost of the high and low fishmeal diets was AUD$1685 t^{-1} and AUD$1284 t^{-1}, respectively (Table 3.2.5.1.1). The difference in the estimated cost of the two diets is approximately $401 t^{-1}, which represents a 23.8% saving on the total cost of raw materials used in the high fishmeal formula.

The estimated raw material cost of the prime fishmeal, fishery by-product meal and blended fishmeal diet was AUD$1418 t^{-1} and AUD$1354 t^{-1} and AUD$1385 t^{-1}, respectively (Table 3.2.5.1.3). The difference in the estimated cost of the prime fishmeal diet and the other diets was AUD$64 t^{-1} and AUD$33 t^{-1}, representing a saving of 4.5% and 2.3%, respectively.

A simple economic appraisal of all diets was done based on the following assumptions: the population of fish in cages was normally distributed with a mean ± SD of 746.3 ± 100.3 g (Figure 3.2.5.1.7); 50,000 fish were stocked into each cage; survival was 100% and the raw material cost and overall FCR of each feed is as presented in Table 3.2.5.1.7. The cost to produce 1 tonne of fish was calculated by multiplying the respective raw material cost of each feed by the overall FCR obtained for each feed during the experiment. This amount was multiplied by the estimated biomass of 50,000 fish (i.e. 37.32 tonnes) in order to calculate the raw material cost to produce this weight of YTK (Table 3.2.5.1.7). Under this scenario use of the low fishmeal diet realised a saving of 25.2% over the use of the high fishmeal diet. There was less difference among the raw material costs of production for the prime, fishery by-product meal and blended fishmeal diets. However, the raw material cost of production for these diets was still 13.6% lower than the high fishmeal diet (Table 3.2.5.1.7).

Fish-in fish-out ratios for each diet are also tabulated in Table 3.2.5.1.7. The highest FIFO of 2.08 was calculated for the 55% prime fishmeal diet used in Experiment 1. The lowest FIFO of 0.59 was calculated for the diet containing 30% fishery by-product meal and a low amount of fish oil (6.91%). The FIFO calculated for the blended fishmeal diet was also below 1 due to the fact the amount of wild derived fishmeal (15%) and fish oil (6.75%) in this diet was very low. The slight increase in the FIFO of the low fish meal diet used in Experiment 1, even though it also contained 15% wild derived fishmeal is due to the relatively higher inclusion of fish oil (11%) in this particular diet (Table 3.2.5.1.7).

Discussion

This study presents encouraging results on fishmeal reduction and the use of different fishmeal sources in aquafeeds for juvenile YTK reared under fluctuating field conditions. The results confirm that the dietary level of prime fishmeal can be reduced from 55% to 15% without short term productivity being affected when fishmeal reduction is offset by inclusion of other high quality proteins such as brewer's yeast, hydrolysed feather meal, poultry meal, meat meal, dehulled lupin and feed grade soy protein concentrate. This demonstrates there is enormous scope in not only the choice of alternative protein sources for YTK but also a high degree of formulation flexibility, provided minimum nutrient specifications are met. The results also demonstrate that a fishery by-product meal can be used in isolation or combined with prime fishmeal in diets for juvenile fish without significantly affecting short term production outcomes. This confirms the utility of lower value fishery by-product meal in diets for rapidly growing juvenile YTK.

The results from Experiment 2 on fishery by-product meal support conclusions found by colleagues using a similar ingredient fed to large YTK at SARDI (Bansemer et al., 2018; see Manuscript 3.1.3.1). Their long term (252 days) tank based study under ambient conditions compared the performance of large fish fed a prime quality fishmeal diet (30% FM inclusion) to several diets containing blends of prime quality fishmeal and fishery by-product meal or prime quality fishmeal combined with poultry meal or soy protein concentrate (see Chapter 3.1.3.1). These authors' found no significant differences among any diet with respect to growth rate (SGR), feed conversion ratio, haematology or plasma biochemistry. The synergies and similarities in these two project studies should increase confidence in the use of fishery by-product meal during the juvenile (50 g - 1.0 kg) and adult stages (> 1.0 kg) of YTK production.

Fishery waste by-product meals have been evaluated in other species. For example, the use of tuna by-product meal to replace about 50% of premium grade sardine derived fishmeal proved effective in Spotted Rose Snapper (*Lutjanus guttatus*) (Hernández et al., 2014) and fermented tuna by-product meal was useful in replacing up to 50% Chilean fishmeal in test diets for Olive Flounder (*Paralichthys olivaceus*) (Oncul et al., 2018). However, both the aforementioned studies were run for only short periods (8 weeks) using very small animals < 11 g initial weight. A more recent study investigating the use of tuna hydrolysate and fermented tuna hydrolysate to replace fishmeal in diets for small Barramundi (*Lates calcarifer*) examined far higher replacement levels than the previous two studies; ≈ 60% of the diet (Siddik et al., 2018). In contrast to the previous studies on tuna meal the later authors' found a decrease in feed intake and growth rate and a worsening in FCR of Barramundi fed diets containing tuna hydrolysate and fermented tuna hydrolysate compared to the fishmeal control. To explain these responses they hypothesised that the presence of excessive numbers of short chain peptides and free amino acids in the by-product meals may have saturated the peptide transport mechanism of fish. Alternatively, they also hypothesised that feed intake on the hydrolysate treatments may have been depressed due to the presence of bitter elements caused by hydrophobic amino acids residues created during the hydrolysis process. None of these issues appear to have affected the performance of YTK in the present study in terms of performance and feed intake after 70 and 110 days, as neither of these responses proved to be significantly different to the prime fishmeal control (Table 3.2.5.1.6). Indeed, the feed intake of fish offered the by-product meal diet was numerically higher than that of the prime control or the blended fishmeal diet.

Significant economic savings can be made by reducing the amount of wild derived fishmeal and fish oil in YTK diets. This claim is clearly supported by the difference in the raw material cost of the prime fishmeal formula and the low fish meal formula tested in Experiment 1 (i.e. approximately 24%). Additionally, significant environmental savings can also be made as evidenced by the lower FIFO ratio of the low fishmeal diet (1.12) and indeed the FIFO ratio of the diet based wholly on fishery by-product meal (0.59) or the diet based on a blend of prime fishmeal and fishery by-product meal (0.83) (Table 3.2.5.1.7). However, these production and environmental savings can only be realised as long as the biological and economic FCR of fish fed low fishmeal and fish oil diets are comparable to the FCRs of fish fed traditional carnivorous diets such as the high fishmeal control used in Experiment 1 or the prime fishmeal control used in Experiment 2 (e.g. diets containing 55% or 30% wild derived fishmeal, respectively). This proved to be the case in both the experiments presented in this chapter where the FCR of all diets ranged narrowly between 1.04:1 and 1.14:1. As such there was a 46% decrease in the FIFO ratio of the low fishmeal diet compared to the high fishmeal control in Experiment 1 and a 45% and 22% reduction, respectively, in the FIFO ratio of fish fed the diet containing fishery by-product meal or the diet based on a blend of fishmeal products in Experiment 2. The digestibility of the low fishmeal diet in Experiment 1 was generally poorer than the high fishmeal diet, which was to be expected. There is therefore a trade-off to consider; while significant reductions in the FIFO ratio can be achieved, waste output including nitrogen will potentially increase, depending on the dietary protein source.

With respect to the latest version of the NSW DPI temperature growth model for YTK, there was good agreement between the estimated and actual growth (body weight) of YTK in both experiments. This result greatly increases the confidence in the model, especially as the experiments were done under real world conditions and over a large range of water temperatures (e.g. 11 °C to 28.5 °C). Nonetheless, the temperature-dependent growth model is not populated with actual farm data, either from NSW or South Australia. Incorporation of data from commercial YTK farms would greatly improve the temperature-dependent growth model, benefiting researchers and farm managers alike. However, like all models it will need to be continually updated and refined to reflect changes in feeding practices, genetics and abiotic factors. It should also be noted that the model used here estimates growth of the average animal and implies that fish are fed daily to apparent satiation. The experiences gained on the NSW DPI - Huon MARL over the last three years and from other parts of the industry have shown that feeding is often interrupted on-farm due to poor weather conditions and bathing activities. These types of events interrupt feed intake and subsequently alter the growth and or health of the animal. The impacts of these events will thus limit growth potential, or at least reset it each time a perturbation is experienced by the fish. At present these effects on-farm productivity are poorly understood. However, models such as the

one used in this report can provide great insight into production losses caused by perturbations as they can be used to monitor the divergence between the model outcome and the actual outcome measured on the farm.

Conclusions and Recommendations

This study presents encouraging results on fishmeal reduction and the use of different fishmeal sources in aquafeeds for juvenile YTK reared under fluctuating field conditions. The results demonstrated that the dietary level of prime fishmeal can be reduced from 55% to 15% without short term productivity being affected when fishmeal reduction is offset by inclusion of other high quality proteins. The economic (measured as reduction in raw material cost) and environmental benefits of feeding a low fishmeal diet were reflected in a 24% reduction in raw material cost and a 46% reduction in the FIFO of the low fishmeal diet, respectively. Results from Experiment 2 demonstrated that 30% fishery by-product meal can be used to wholly replace an equivalent amount of prime fishmeal in diets for juvenile YTK without significantly affecting short term production outcomes. While there was little economic benefit (measured as reduction in raw material cost) in using 30% fishery by-product meal to replace an equivalent amount of prime fishmeal in diets for YTK, there was a 45% reduction in the FIFO of the fishery by-product meal diet. These results confirm there is enormous scope in not only the choice of alternative protein sources for YTK but also a high degree of formulation flexibility. These results have been obtained from field experiments run under ambient conditions in an outdoor pond at PSFI. While robust, the results need to be validated in a farm-based location to confirm these diets produce similar outcomes under large-scale production conditions. The incremental changes in body weight of YTK during experiments closely matched the predicted body weight of YTK according to an NSW DPI updated temperature-dependent growth model for this species. Models such as these will require constant updating; however they will remain highly beneficial for benchmarking growth in laboratories and field situations. We recommend follow up research to test other alternative protein sources for YTK and field experiments that test even lower amounts of dietary fishmeal.

Findings

- There is enormous potential to reduce the level of wild derived fishmeal in diets for juvenile - sub-adult YTK (< 1.0 kg body weight) using other suitably selected, high quality raw materials.
- Fishery by-product meal is a suitable alternative to prime quality fishmeal in carefully formulated diets for juvenile - sub-adult YTK (< 1.0 kg body weight).
- Raw material costs and FIFO ratios can be substantially reduced in carefully formulated aquafeeds for juvenile – sub-adult YTK (< 1.0 kg body weight).
- New temperature-dependent growth models for juvenile - sub-adult YTK are highly predictive.
- Very low, prime fishmeal diets such as the one tested in this report (≤ 15%) should be trialled on larger YTK under field conditions.
- Adoption of similar diets to those used in this study may improve economic outcomes as well as the environmental ‘blue footprint of Australian YTK farmers.

  Australian YTK farmers should provide high quality farm data on weight gain and variation in abiotic factors to help continually revise and improve growth and bioenergetic models for this species.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, New South Wales Department of Primary Industries (NSW DPI) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

Special thanks to Ian Russell, Marcus Armstrong, Dylan Nash and Steve Gamble for technical assistance (NSW DPI Fisheries). Special thanks to Ian Powell, Richard Popplewell and Lindsay Coates for assistance with outdoor pond infrastructure and Port Stephens Machining for manufacturing the stainless steel cage frames. We also acknowledge the assistance Craig Jones for installation of outdoor electrical equipment and Paul Coughin Plumbing for installation of the high pressure oxygen system. We acknowledge Barney Hines and his team for undertaking the biochemical analysis on feedstuffs, feeds and faecal material (CSIRO). We are extremely grateful to Ridley for providing the raw materials used in this study, especially the assistance of Dr Michael Salini and Simon Tabrett.

References

Bansemer, M.S., Stone, D.A.J., Skordas, P., Nankervis, L., Salini, M., 2018. Reducing dietary wild derived fish meal inclusion levels in production diets for large Yellowtail Kingfish (*Seriola lalandi*; >1.5 kg). DAWR Milestone Report 6, 25-44.

Booth, M., Pirozzi, I., 2017. Making sense of nonsense: using regression analysis to deal with highly variable data collected from a yellowtail kingfish (*Seriola lalandi*) digestibility experiment. Aquaculture 485, 39-48.

Booth, M., Smullen, R., Salini, M., Lewis, M., 2017. Utilisation of poultry meal by Barramundi *Lates calcarifer*: premium grade poultry meal can replace fishmeal in nutritionally balanced aquafeeds for Barramundi. NSW DPI Fisheries, pp. 44.

Booth, M.A., Allan, G.L., Anderson, A.J., 2012. Influence of poultry meal, meat meal or soybean meal inclusion on weight gain and production characteristics of Australian snapper *Pagrus auratus*. Aquacult. Int. 20, 99-115.

Bureau, D.P., 2010. Feather meal, Render, pp. 14-17.

Cho, C.Y., Slinger, S.J., Bayley, H.S., 1982. Bioenergetics of salmonid fishes: energy intake, expenditure and productivity. Comparative Biochemistry and Physiology 73B, 25-41.

Hernández, C., Hardy, R.W., Contreras-Rojas, D., López-Molina, B., González-Rodríguez, B., Domínguez-Jimenez, P., 2014. Evaluation of tuna by-product meal as a protein source in feeds for juvenile spotted rose snapper *Lutjanus guttatus*. Aquacult. Nutr. 20, 574-582.

Herpandi, N.H., Rosma, A., Wan Nadiah, W.A., 2011. The tuna fishing industry: A new outlook on fish protein hydrolysates. Compehensive Reviews in Food Science and Food Safety 10, 195-207.

Oncul, F.O., Aya, F.A., Hamidoghli, A., Won, S., Lee, G., Han, K.R., Bai, S.C., 2018. Effects of the dietary fermented tuna by-product meal on growth, blood parameters, nonspecific immune response, and disease resistance in juvenile Olive Flounder, *Paralichthys olivaceus*. J. World Aquacult. Soc. 0, doi: 10.1111/jwas.

Petterson, D.S., 2000. The Use of Lupins in Feeding Systems - Review. Asian Australas. J. Anim. Sci. 13, 861-882.

Saadiah, I., Abol-Munafi, A.M., Che Utama, C.M., 2011. Replacement of fishmeal in cobia (*Rachycentron canadum*) diets using poultry by-product meal. Aquacult. Int. 19, 637-648.

Siddik, M.A.B., Howieson, J., Ilham, I., Fotedar, R., 2018. Growth, biochemical response and liver health of juvenile barramundi (*Lates calcarifer*) fed fermented and non-fermented tuna hydrolysate as fishmeal protein replacement ingredients. PeerJ. 6, e4870.

Tacon, P., Auclair, E., 2010. Yeast in aquaculture from nutrition to well-being. AQUA Culture Asia Pacific Magazine, July/August 2010, 2022-2024.

Terpstra, A.H.M., 2015. The Use of Fish Meal and Fish Oil in Aquaculture and Calculation of the Fish-In-Fish-Out (FIFO) Ratio. Universitate Vadensi, The Netherlands, pp. 19.

Table 3.2.5.1.1. Formula of diets prepared for Experiment 1 (fishmeal reduction).

Ingredients (%)	**High fishmeal**	**PSFI Low fishmeal**
Prime fishmeal	55.00	15.00
Brewer's yeast	0.00	11.00
Feather meal	0.00	11.00
Poultry meal	0.00	5.00
Meat meal	0.00	1.00
Corn gluten	0.00	5.00
Wheat gluten	1.00	6.00
Dehulled lupin	1.00	11.00
SPC - Selecta	11.00	13.90
Fish oil	14.00	11.00
Poultry oil	3.00	4.00
Wheat flour	11.90	0.00
Starch	1.00	1.85
Choline chloride (70%)	0.30	0.30
Rovimix Stay-C	0.05	0.30
Vit-min premix	0.30	0.30
NaH_2PO_4	0.30	0.50
Lysine	0.00	0.97
Methionine	0.66	1.00
Taurine	0.29	0.68
Protexin	0.10	0.10
Y_20_3	0.10	0.10
Total (%)	100.00	100.00
Estimated raw material cost of diet ($AUD tonne^{-1})	$1685	$1284

Table 3.2.5.1.2. Estimated nutrient and energy composition of diets used in Experiment 1 (dry matter basis).

Nutrient (%)	**High fishmeal**	**PSFI Low fishmeal**
Crude protein	56.13	55.48
Crude fat	19.39	18.63
Ash	8.72	6.16
NFE	15.75	19.73
Gross energy (MJ kg^{-1})	24.09	23.92
CP:GE ratio (g CP MJ GE^{-1})	22.15	23.53
Amino Acids		
Alanine	3.27	2.78
Arginine	3.64	3.11
Aspartic Acid	4.75	4.46
Cysteine	0.52	0.87
Glutamic Acid	8.15	8.95
Glycine	4.16	3.15
Histidine	1.53	0.89
Isoleucine	2.36	2.15
Leucine	4.14	4.07
Lysine	3.18	3.38
Methionine	2.17	1.55
Phenylalanine	2.60	2.42
Proline	2.89	3.55
Serine	2.54	3.17
Taurine	0.90	0.66
Threonine	2.29	2.03
Tyrosine	1.89	1.71
Valine	2.63	2.52
Apparent Digestibility Coefficient		
Dry matter (%)	0.596	0.518
Crude protein (%)	0.822	0.732
Crude fat (%)	0.809	0.456
Gross energy (MJ kg^{-1})	0.742	0.736

Table 3.2.5.1.3. Formula of diets prepared for Experiment 2 (fishmeal origin).

Ingredient (%)	**Prime fishmeal**	**Fishmeal by-product**	**Blended fishmeal sources**
Prime fishmeal	30.00	0.00	15.00
Recycled fishmeal (tuna)	0.00	30.00	15.00
Blood meal	1.02	5.99	3.50
Meat meal	7.99	2.07	5.03
Poultry meal	14.28	14.98	14.63
Lupin meal	11.81	12.56	12.19
SPC - Selecta	14.31	14.98	14.64
Fish oil	6.59	6.91	6.75
Poultry oil	6.59	6.91	6.75
Waxy maize starch	3.73	2.00	2.87
Choline chloride (70%)	0.30	0.30	0.30
Lysine	0.40	0.12	0.26
Methionine	1.13	1.12	1.12
Taurine	0.50	0.71	0.60
Vit-min premix	0.50	0.50	0.50
Rovimix Stay-C	0.05	0.05	0.05
NaH_2PO_4	0.50	0.50	0.50
Protexin®	0.10	0.10	0.10
Pro(N8)ure®	0.10	0.10	0.10
Y_20_3	0.10	0.10	0.10
Total (%)	100.00	100.00	100.00
Estimated raw material cost ($AUD tonne^{-1})	$1418	$1354	$1385

Table 3.2.5.1.4. Estimated nutrient and energy composition of diets used in Experiment 2 (dry matter basis).

Nutrient (%)	**Prime fishmeal**	**Fishmeal by-product**	**Blended fishmeal sources**
Crude protein	54.87	53.97	53.93
Crude fat	16.94	20.91	19.44
Ash	12.06	11.81	11.90
NFE	16.13	13.31	14.72
Gross energy (MJ kg^{-1})	22.66	23.43	23.02
CP:GE ratio (g CP MJ GE^{-1})	24.21	23.04	23.43
Amino Acids			
Alanine	3.14	3.10	3.06
Arginine	2.79	2.65	2.95
Aspartic Acid	4.84	5.13	5.01
Cysteine	0.41	0.42	0.43
Glutamic Acid	7.81	7.59	7.75
Glycine	3.90	3.60	3.86
Histidine	1.03	1.40	1.32
Isoleucine	2.02	1.90	1.92
Leucine	3.66	3.95	3.84
Lysine	3.75	3.59	3.45
Methionine	1.98	1.83	1.90
Phenylalanine	2.11	2.35	2.27
Proline	2.66	2.56	2.65
Serine	2.17	2.58	2.22
Taurine	0.71	0.80	0.76
Threonine	1.89	2.05	1.91
Tyrosine	1.63	1.63	1.64
Valine	2.27	2.58	2.40
Apparent Digestibility Coefficient			
Dry matter (%)	0.540	0.580	0.478
Crude protein (%)	0.800	0.777	0.737
Crude fat (%)	0.669	0.664	0.654
Gross energy (MJ kg^{-1})	0.742	0.716	0.707

Table 3.2.5.1.5. Gross changes in cage biomass, feed intake* and FCR* of fish in Experiment 1.

Biomass	Date	High fishmeal	PSFI Low fishmeal	SE	*F-value*	*P-value*
Stock biomass (g)	19-07-18	2703	2720	8.37	2.14	0.217
Biomass (g cage^{-1})	10-08-18	3422	3351	43.7	1.33	0.312
Biomass (g cage^{-1})	04-09-18	4638	4496	67.33	2.25	0.208
Biomass (g cage^{-1})	03-10-18	9911	9437	200.8	2.79	0.170
Biomass (g cage^{-1})	25-10-18	18152	17894	396.8	0.21	0.669
Biomass (g cage^{-1})	12-11-18	26288	25455	653.14	0.813	0.418
Feed intake (g cage^{-1})	10-08-18	1018	925	24.50	7.10	0.056
Feed intake (g cage^{-1})	04-09-18	1551^{b}	1469^{a}	15.18	14.46	0.019
Feed intake (g cage^{-1})	03-10-18	4776	4506	92.14	4.27	0.108
Feed intake (g cage^{-1})	25-10-18	8214	8029	213.16	0.375	0.573
Feed intake (g cage^{-1})	12-11-18	9296	8743	245.03	2.62	0.180
FCR cage^{-1}	10-08-18	1.41	1.48	0.05	0.804	0.421
FCR cage^{-1}	04-09-18	1.29	1.28	0.059	0.002	0.970
FCR cage^{-1}	03-10-18	0.91	0.91	0.012	0.036	0.859
FCR cage^{-1}	25-10-18	0.99	0.95	0.014	6.428	0.065
FCR cage^{-1}	12-11-18	1.16	1.16	0.058	0.000	1.000
Total gain (g cage^{-1})	12-11-18	23585	22735	657.11	0.837	0.412
Overall SGR (% d^{-1})	12-11-18	1.96	1.93	0.023	0.982	0.422
Total intake (g cage^{-1})	12-11-18	24854	23664	480.15	3.07	0.212
Overall FCR	12-11-18	1.06	1.04	0.018	0.43	0.547
Condition factor *K*	12-11-18	1.53	1.48	0.020	3.905	0.119
CV individual wt (%)	12-11-18	15.03	12.05	1.12	3.56	0.132

*Feed intake and FCR presented on a dry matter basis; average dry matter of feeds was 96.0%.

Table 3.2.5.1.6. Gross changes in cage biomass, feed intake and FCR of fish in Experiment 2.

Parameter	Date	Prime fishmeal	Fishmeal by-product	Blended fishmeal sources	SE	*F-value*	*P-value*
Stock biomass (g)	06-09-18	3427	3366	3387	22.78	1.82	0.303
Biomass cage^{-1}	12-10-18	10172	10042	9914	65.35	3.89	0.146
Biomass cage^{-1}	15-11-18	23776	23171	22758	301.38	2.88	0.199
Feed intake cage^{-1}	12-10-18	6320	6453	6083	66.64	7.910	0.064
Feed intake cage^{-1}	15-11-18	15664	16108	15256	247.28	2.969	0.194
FCR cage^{-1}	12-10-18	0.94^{a}	0.97^{b}	0.93^{a}	0.002	84.42	0.002
FCR cage^{-1}	15-11-18	1.15	1.23	1.19	0.026	2.00	0.279
Total gain (g cage^{-1})	15-11-18	20350	19805	19372	295.63	2.75	0.209
Overall SGR (% d^{-1})	15-11-18	2.77	2.76	2.74	0.023	0.271	0.779
Total intake (g cage^{-1})	15-11-18	21983	22561	21339	290.49	4.43	0.127
Overall FCR	15-11-18	1.08	1.14	1.10	0.016	3.45	0.167

*Feed intake and FCR presented on a dry matter basis; average dry matter of feeds was 95.6%.

Table 3.2.5.1.7. Basic economic and FIFO assessment of test feeds from Experiment 1 and Experiment 2.

Test diet	Cost of raw materials ($ tonne^{-1})	FCR	Cost to produce 1 tonne fish*	Biomass of 50,000 fish‡ (tonne)	Cost of raw materials to produce D¥	FIFO ratio#
	A	B	C	D	E	
Experiment 1						
High fishmeal	$1,685	1.06	$1,786	37.315	$66,648	2.08
Low fishmeal	$1,284	1.04	$1,335	37.315	$49,829	1.12
Experiment 2						
Prime fishmeal	$1,418	1.08	$1,531	37.315	$57,146	1.07
Fishmeal byproduct	$1,354	1.14	$1,544	37.315	$57,598	0.59
Fishmeal blends	$1,385	1.10	$1,524	37.315	$56,849	0.83

*C = A × B
‡ Estimated biomass of 50,000 fish based on normal distribution of fish in Experiment 1 (see Figure 3.2.5.1.7).
¥ E = C × D
FIFO calculated using equation presented in Terpstra (2015).

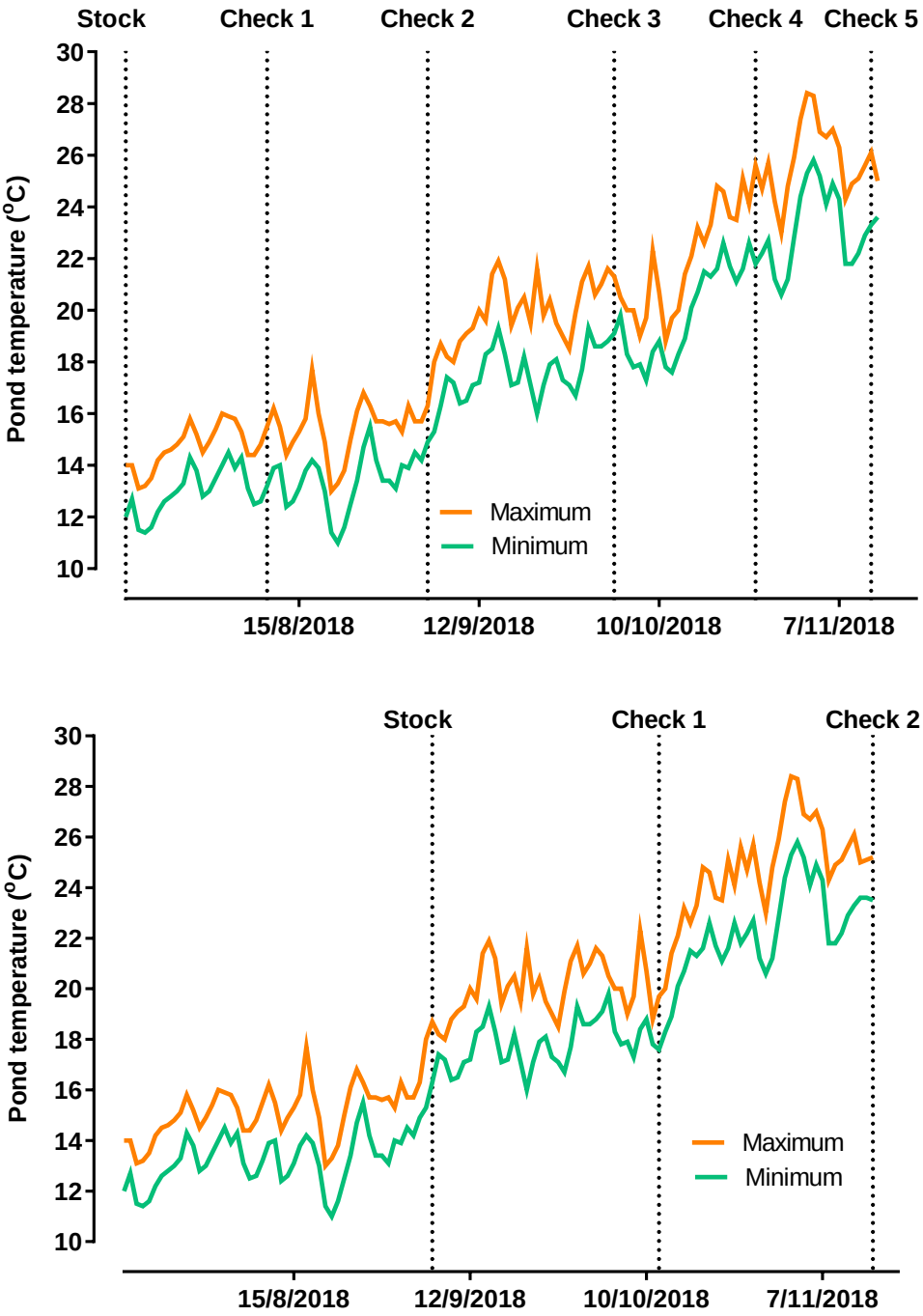


Figure 3.2.5.1.1. Daily minima and maxima water temperature recorded in the outdoor pond at PSFI. Data are from 19 July 2018 to 15 November 2018. Drop lines indicate weight checks done on fish in Experiment 1 (upper panel) and Experiment 2 (lower panel).

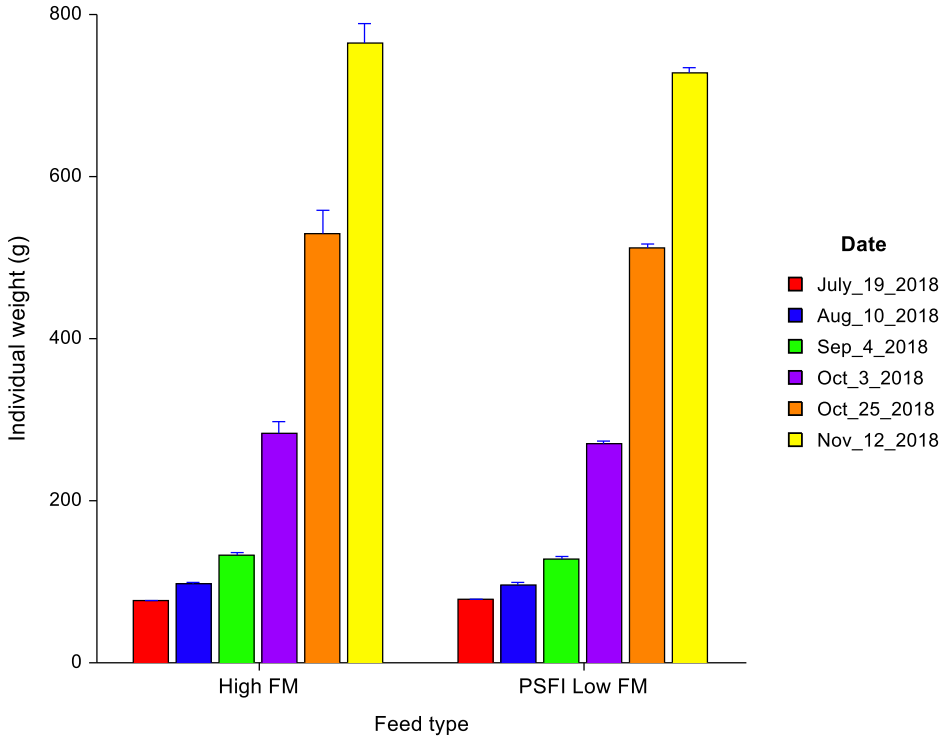


Figure 3.2.5.1.2. Incremental weight of juvenile Yellowtail Kingfish from pond Experiment 1 reared on a high fishmeal or low fishmeal diet from 19th July to 12th November 2018 (bars represent mean ± SD).

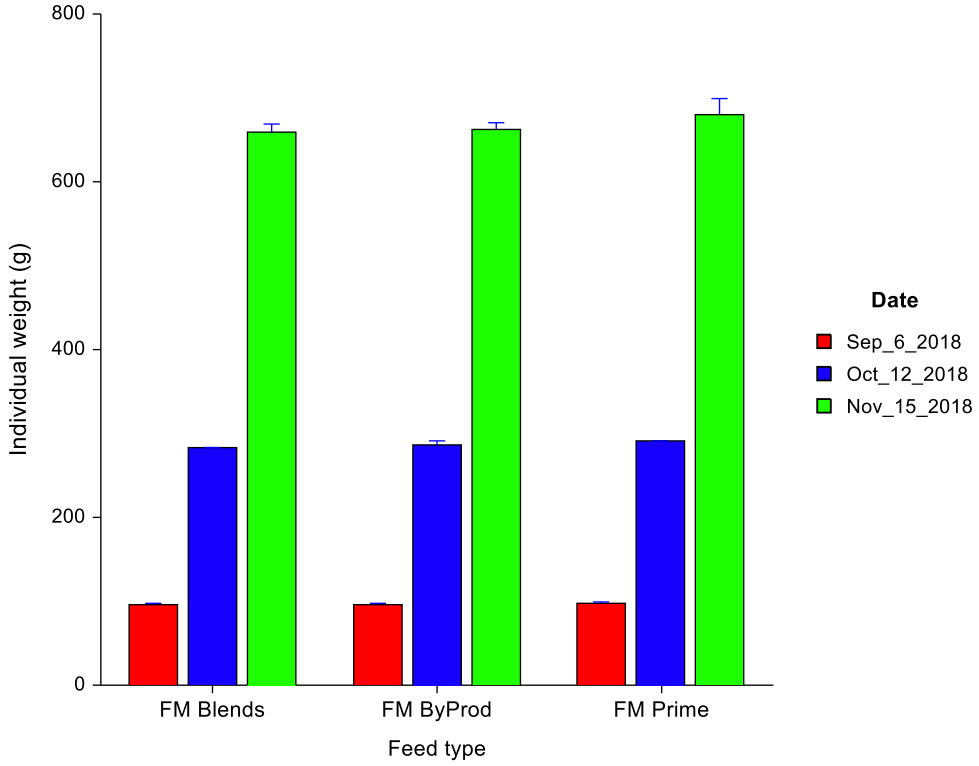


Figure 3.2.5.1.3. Incremental weight of juvenile Yellowtail Kingfish from pond Experiment 2 reared on a diet containing prime fishmeal, fishmeal by-product meal or a blend of fishmeal sources from 6th Sept to 15th November 2018 (bars represent mean ± SD).

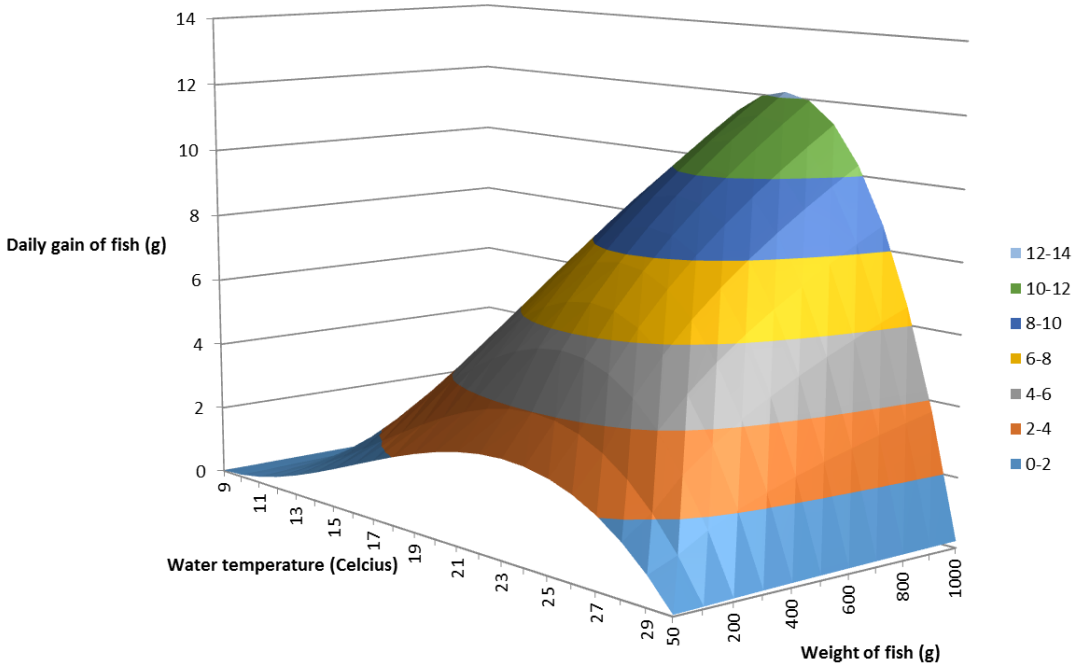


Figure 3.2.5.1.4. Graphical representation of the temperature-dependent growth model for Yellowtail Kingfish (PSFI MB V5).

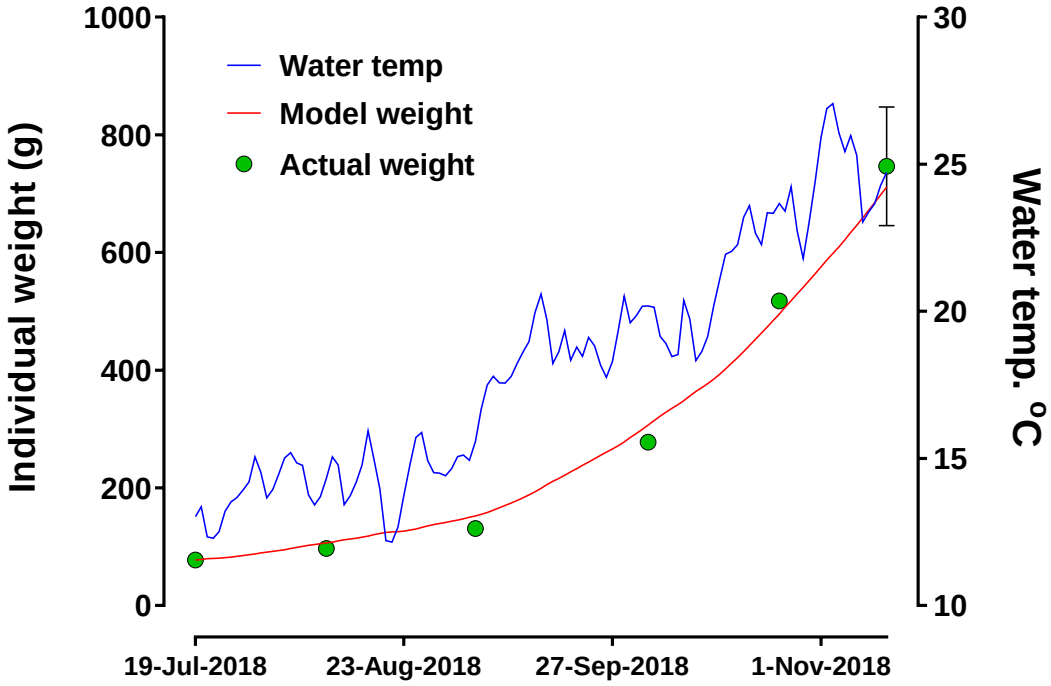


Figure 3.2.5.1.5. Average weight of Yellowtail Kingfish (*n* = 6 cages) in pond Experiment 1 versus predicted weight of Yellowtail Kingfish. Predicted weight of fish is based on a growth-temperature model (PSFI MB V5). Harvest data presented as mean ± SD.

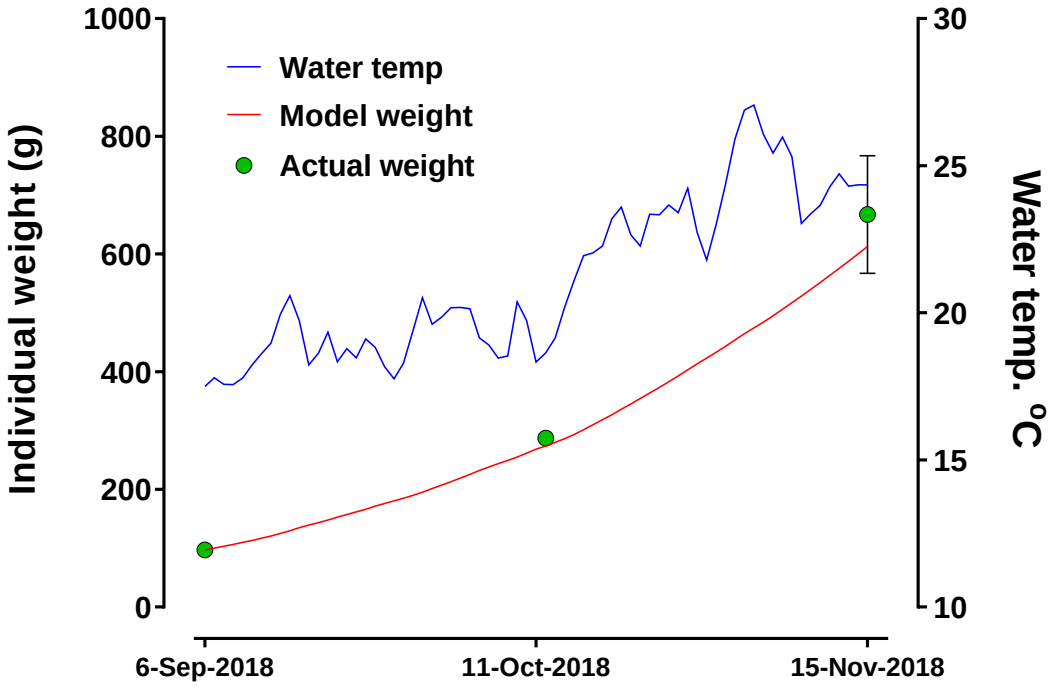


Figure 3.2.5.1.6. Average weight of Yellowtail Kingfish (*n* = 6 cages) in pond Experiment 2 versus predicted weight of Yellowtail Kingfish. Predicted weight of fish is based on a growth-temperature model (PSFI MB V5). Final data point presented as mean ± SD.

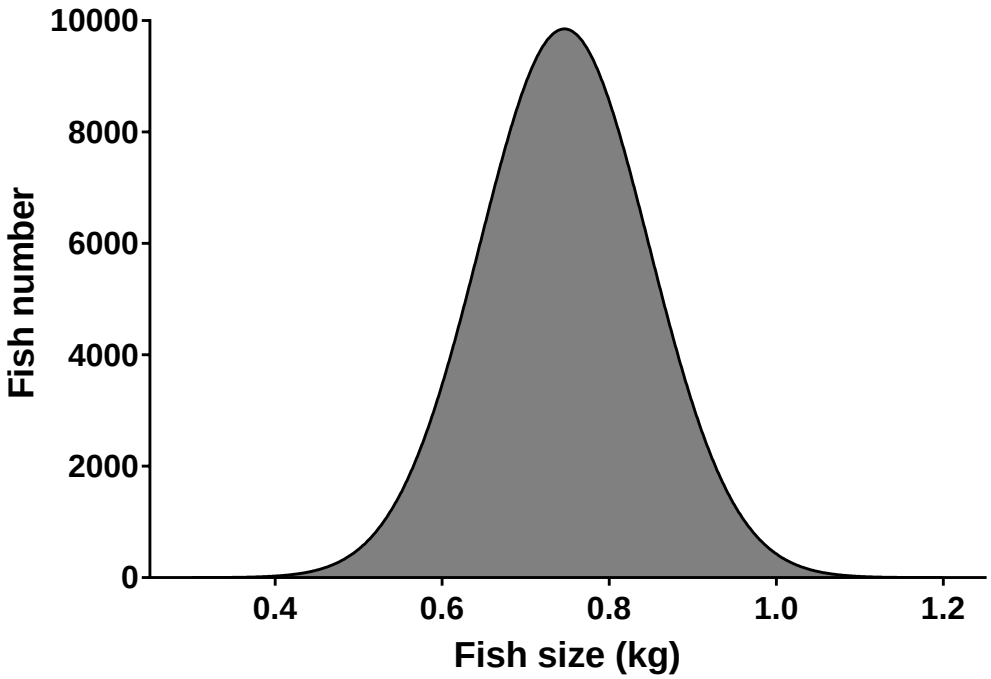


Figure 3.2.5.1.7. Normal distribution of Yellowtail Kingfish from pond Experiment 1 extrapolated to 50,000 fish; mean ± SD of distribution = 746.3 ± 101.3 g; CV = 13.6%. Assuming 100% survival the predicted biomass = 37.315 tonnes.

3.2.6. Chapter - Optimising feeding strategies that boost reproductive output for Yellowtail Kingfish broodstock.

3.2.6.1. Manuscript - Impact of changing the diet of Yellowtail Kingfish (Seriola lalandi) broodstock on fecundity, egg quality and heredity.

Mark Booth [a], Luke Cheviot [a], Brendan Findlay [a], Wayne Knibb [b] and D. Stewart Fielder [a]

[a] New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach, NSW, 2316, Australia

[b] Sunshine Coast University, Locked Bag 4, Maroochydore, QLD 4558

This manuscript may be referenced as: Booth, M., Cheviot, L., Findlay, B., Knibb, W., and Fielder, S. (2019). 3.2.6.1. Manuscript - Impact of changing the diet of Yellowtail Kingfish *Seriola lalandi* broodstock on fecundity, egg quality and heredity (Output 3b). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.449-472.

Abstract

Broodstock feeding and nutrition is one of the least studied and most poorly understood areas in aquaculture and research into broodstock husbandry, care and wellbeing is often neglected due to the focus of farmers and industries on the grow-out stage. However the implications of farming progeny of poorly maintained and nourished broodstock are profound, having negative ramifications across the nursery and production cycle. Historically, marine broodstock have been maintained on a diet of natural prey such as sardines and squid. These foods are often highly variable in composition and can also be vectors for the introduction of pathogens into hatcheries. Modern and specialised proprietary broodstock feeds are available to industry, however, the efficacy of these feeds in terms of broodstock health, reproductive output and quality of offspring is largely unknown. This long term experiment addresses some fundamental questions regarding the feeding of Yellowtail Kingfish (*Seriola lalandi*; YTK) broodstock. It was designed to compare the reproductive output and egg quality of wild and first generation (F1) broodstock fed a ration of Australian Sardines (*Sardinops sagax;* Sardines) and Atlantic Atlantic Squid (*Doryteuthis pealeii*; Squid) to those fed a ration of pelletised feeds. The results indicated that feeding broodstock Sardines and Squid is better than the use of two commercial feed sources in terms of maintaining high levels of fecundity and hatching rates in wild and F1 YTK broodstock at the New South Wales Department of Primary Industries (NSW DPI) Port Stephens Fisheries Institute (PSFI). No major differences were found in the proximate quality of eggs or their morphology with respect to feeding regime, however, there were clear differences in the FAME composition of eggs that could be inferred from diet type. Furthermore, the enumeration of genetic data indicated there were greater numbers of offspring groups identified in the wild and F1 broodstock fed Sardines and Squid compared to those fed pelletised feeds. Collectively, these results confirm that natural feeds (i.e. Sardine and Squid) support better hatchery outcomes in broodstock housed at PSFI and these feeds will continue to be used as part of the "best-practice" broodstock management at this hatchery for the present time.

Introduction

The nutrition provided to broodstock is critical in ensuring they are healthy and produce the highest quality milt, eggs and larvae. Production of high quality larvae from healthy broodstock enhances

hatchery and production outcomes (Snyder and Zeigler, 2013). However, research on the nutritional requirements of most broodstock is lacking. For this reason the NRC (2011) has recently advocated for more research on broodstock nutrition, with an emphasis on specialised species-specific diets (Migaud et al., 2013). Marine broodstock has traditionally been fed natural foods which are thought to approximate the type of prey the species would normally consume in the wild. It is commonplace to use frozen squid and oily fish such as pilchards to feed Yellowtail Kingfish (*Seriola* lalandi; YTK) broodstock (Kolkovski, 2005). However the quality and nutrient composition of these products is often unknown because they can vary depending on where and when they are caught (season) and how they are stored to name but a few factors. In contrast commercial aquafeeds, whether they be formulated for hatchery, grow-out or broodstock are often more stable and predictable in nutrient composition.

The Marine Fish Hatchery at New South Wales Department of Primary Industries (NSW DPI) Port Stephens Fisheries Institute (PSFI) uses natural feeds for their YTK broodstock following recommendations made by Fielder and Heasman (2011); predominantly high grade whole Atlantic Squid (*Doryteuthis pealeii*; Squid) and Australian Sardine (*Sardinops sagax*; Sardines). In general, the YTK juveniles produced by NSW DPI have historically been used in research trials investigating changes in rearing protocols (Fielder et al., 2010) or advancing the understanding of nutritional requirements (Booth et al., 2010). Clean Seas Seafood has a more advanced broodstock program than NSW DPI due to the age of their business and the integrated nature of their hatchery and commercial grow-out facilities. Clean Seas Seafood broodstock are fed a bespoke commercial 'broodstock diet' fortified with vitamin-C, vitamin-E, taurine and astaxanthin. The use of the commercial feed with their YTK broodstock has apparently had no adverse consequences in terms of reproductive output (Craig Foster - Clean Seas Seafood; *email communication* 15.12.15; 9 mm diameter pellet Pelagica diet produced by Ridley). However, scientific evidence that commercial broodstock feeds can replace natural feeding regimes in YTK broodstock without compromising the reproductive output of these animals is lacking (Fielder and Heasman, 2011).

There are good reasons to use manufactured feeds in hatcheries. For one reason their use should reduce the biosecurity risk to broodstock and offspring posed by using natural foods which can act as vectors for the accidental introduction of diseases (Watanabe and Vassallo-Agius, 2003). Use of natural feeds in modern recirculating aquaculture systems can also lead to deterioration in water quality resulting in sub-standard environments and additional labour costs (Morais et al., 2014). Manipulation of nutrient quality and density as well as effective inclusion of feed supplements (e.g. vitamins, minerals, astaxanthin and attractants) is also problematic when using natural food sources as opposed to formulated feeds. Operating hatcheries is expensive and the cost of feeding can account for a large proportion of the operating budget. For this reason there may be good economic reasons to move from natural to commercial feeds, however, the cost-benefit of doing so would need to be assessed in terms of hatchery output or outcomes versus potential savings.

The composition of broodstock diets has direct implications for the quality of offspring. The potential impact of diet on the essential fatty acid (EFA) profile of eggs and even larvae is particularly critical (Verakunpiriya et al., 1996; Agius et al., 2001; NRC, 2011). For this reason it is imperative that broodstock diets have adequate EFA and other nutrients (e.g. vit. A [Furuita et al., 2003] cited on p 197 in NRC, 2011; vit E [several sources cited on p 199 NRC, 2011]; astaxanthin [Watanabe and Vassallo-Agius, 2003]; and taurine [Matsunari et al., 2006]) that positively affect the composition of developing eggs. New and emerging technologies have linked broodstock health and reproductive success not only to basic nutrition but also to nutrigenomic and epigenetic mechanisms. Epigenetic changes may be passed onto subsequent generations having implications for the longer term genetic management of broodstock and breeding programs (Cabrita et al., 2014). The multitude of exogenous and reproductive factors that can influence the development of high quality fingerlings are discussed at length by Migaud et al. (2013).

NSW DPI and industry partner Huon Aquaculture are evaluating a YTK enterprise in marine waters off the coast of Port Stephens NSW, Australia. Recently thousands of juvenile YTK produced at PSFI have been stocked into offshore sea-cages on NSW DPI's Marine Aquaculture Research Lease (MARL). As the industry develops, there will be increasing pressure on the broodstock underpinning the supply of these fingerlings, meaning it is imperative we understand how manipulation of broodstock nutrition: a)

impacts on reproductive output; and b) impacts on the economic outcomes of the hatchery. Knowledge of these impacts will guide NSW DPI in current best-practice protocols for reliable year-round production of YTK (Fielder and Heasman, 2011; Migaud et al., 2013) and provide Huon Aquaculture with important data for the future operation of their own hatchery.

The aim of this experiment was to determine if changing YTK broodstock from a natural 'best practice' feeding regime of Sardines and Squid to a commercial pellet feeding regime affected reproductive output and egg quality. Reproductive output was measured in several ways including fertilization rate and enumeration of eggs and hatched eggs. Morphometric indices such as egg size and oil droplet size were also assessed. In addition, the chemical composition of eggs was examined to determine if feeding regime affected nutrient or FAME content. The YTK broodstock at PSFI have been genotyped, therefore the genetic diversity within different tanks was inferred using DNA extracted from eggs and PCR amplification in an attempt to relate these outcomes to diet selection.

Methods

This study was performed under the NSW DPI Fisheries Animal Care and Ethics (ACEC) Research Authority known as 'Aquaculture Nutrition ACEC 93/5–Port Stephens'. Care and husbandry of fish was carried out according to methods outlined in 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2015).

YTK broodstock

The PSFI broodstock facility at PSFI houses YTK broodstock in four independent 25 kL recirculating systems (RAS). Each RAS consists of a main holding tank, a 200 L sump for collecting eggs, a rotating drum screen filter (20 um; Hydrotech 501), a rotating biological contactor (biofilter) and reverse cycle refrigeration unit used to control water temperature (targeting 16 °C). Tanks are fitted with lids that contain a fluorescent light to control photoperiod. Water is constantly circulated through the RAS via a 2.1 kW centrifugal pump (300-400 L min^{-1}). Each system is fed with high quality, filtered estuarine water drawn from the intersection of Fenningham's Island Creek and Tilligerry Creek, constantly exchanged to ensure optimal water quality parameters are maintained. Tanks are siphoned weekly to remove build-up of organic material. Broodstock at PSFI are essentially managed under the best-practice regimes advocated for YTK by Fielder and Heasman (2011).

Each tank contained different numbers of broodstock. Two tanks contained wild broodstock and two tanks contained offspring of wild broodstock from the PSFI hatchery (i.e. F1 generation), however, the F1 offspring are not related to any of the current parent stock and all F1 animals are siblings (Knibb et al., 2017). Basic tank variables are presented in Table 3.2.6.1.1. Prior to commencing the experiment all fish were being fed a mixed ration of Sardines and Squid, once daily on Monday, Wednesday and Friday of each week.

Feeding regimes and spawning cycles

At the commencement of the experiment two broodstock tanks were allocated to the new feeding regime (i.e. pellet regime; Pelagica diet + Breed-M diet) and two tanks were maintained on the current best-practice feeding regime (i.e. natural regime; Sardines + Squid). Fish were fed to apparent satiation once on Monday, Wednesday and Friday of each week at approximately 13:00 h. This feeding frequency was in keeping with current hatchery practices at PSFI and similar to that used to feed YTK broodstock at Challenger Institute (Rob Michael - Challenger Institute WA; *email communication* 9.3.16). Uneaten feed was counted and recorded on a daily basis.

In order to evaluate the effect of feeding regime we adopted a three monthly spawning cycle, aiming for four synchronised spawning events in 12 months. Cycles were;

- 23.3.16 to 5.6.16 (74 days)

- 6/6/16 to 4/9/16 (90 days)
- 5/9/16 to 4/12/16 (90 days
- 5/12/16 to 12/3/17 (97 days)

The last week of each cycle was dedicated exclusively to egg collection and enumeration. Fish were not fed during that week.

All tanks of broodstock were induced to spawn naturally in the last week of each cycle by increasing the water temperature from 16 °C to 22 °C within 24 to 48 h. Spawning typically occurred three to four days after thermal manipulation (Fielder and Heasman, 2011).

Selection of commercial feeds

The commercial feeds chosen for this trial were based on feedback from industry collaborators and feed companies. Based on the advice from Clean Seas Seafood (see above), a Pelagica 15 mm diameter floating pellet was selected (Ridley Pelagica Float 15 mm; run# B616394, product code 08437). In addition a proprietary broodstock preparation produced by INVE Aquaculture known as 'Fish Breed-M' (www.inveaquaculture.com) was selected based in part on advice received from Ridley and the fact that our collaborators in Western Australia were also using this product to feed their YTK broodstock (Gavin Partridge - Challenger Institute WA; email communication 1/3/16). The INVE product has also been trialled on Atlantic Halibut (*Hippoglossus hippoglossus*) broodstock, which indicated Breed-M contained high amounts of squid meal and krill meal (Brown, 2009).

INVE Aquaculture makes the following claims regrading this product. Fish Breed-M is claimed to incorporate highly refined and digestible raw materials. The powder is usually mixed with water in order to make a stable moist-paste or moist-sausage with around 50% moisture. It is an imported product and it is expensive, retailing for approximately \$38 kg^{-1} exclusive of GST (INVE, 2015).

The measured proximate and amino acid composition of the four feed types as well as the approximate retail cost of each feed type is presented in Table 3.2.6.1.2. The measured fatty acid categories of the pelletised and natural feed sources are presented in Table 3.2.6.1.3 and Table 3.2.6.1.4, respectively. The mineral composition of Pelagica and Breed-M is presented in Table 3.2.6.1.5.

Feeding strategy and behaviour of broodstock

Broodstock tanks assigned to the natural feeding strategy were given Squid and Sardines, respectively on alternate feeding days. This approach allowed the absolute amount of each natural food-type to be quantified and also prevented fish selectively feeding on either prey type. We encountered no problems with feeding the broodstock tanks allocated to the natural feeding strategy at any time during the trial (i.e. Tank 1 and Tank 6).

A pellet fed regime was designed such that fish allocated to this treatment were given the Pelagica diet for eight weeks followed by the Breed-M diet for four weeks. The soft Pelagica pellets were fed from the start of each spawning cycle and the soft Breed-M pellets in the final four weeks leading up to each induction. However, we experienced immediate problems with this approach because the wild-type and F1 fish rejected the 15 mm floating pellets (Tank 5 and Tank 8). Although the F1 tank seemed more interested in feeding on the floating pellets than the wild-type tank, it became obvious these groups were not going to consume the floating pellets in their original form. We overcame this issue by grinding the Pelagica 15 mm pellets into a fine powder, mixing it with drinking water and reforming it into moist sausages. The same pelleting procedure was also used to form the Breed-M powder into moist sausages (as per the manufacturer's recommendations).

Four kilogram batches of Pelagica or Breed-M were mixed on an as-is basis with 2.8 L and 2.6 L of water, respectively, in a commercial mixer (Hobart Mixer; Troy Pty. Ltd., Ohio,USA). The wet mash was then extruded through a mincer fitted with a 20 mm diameter sausage funnel (Barnco Australia Pty. Ltd., Leichhardt, NSW, Australia). Pellets were cut to a length of about 80 mm weighing about 36 g, wrapped in cling wrap to prevent them dehydrating, packed into sealed plastic boxes and stored in a

refrigerator or frozen until used. The Pelagica and Breed-M sausages had a moisture content of 42% and 39%, respectively, were negatively buoyant and sank slowly in seawater.

The F1 tank readily accepted the new soft pellets. However, the wild-type broodstock in Tank 8 took about 1.5 cycles to become comfortable eating the pelleted sausages. Small amounts of Squid had to be used in the first few weeks to induce a strong feeding response among all individuals in this tank. Remarkably, this was achieved by placing a very small piece of Squid (usually only a tentacle) in the end of the sausage. This approach was discontinued once the fish had been successfully weaned onto the soft pellets.

Due to the low fecundity of fish fed pelletised feeds in Tank 5 and Tank 8, fish on these treatments were returned to the natural feeding regime in spawning cycle 4. This was done ostensibly to determine if a switch back to the "best practice" approach impacted spawning outcomes.

Egg collection and larviculture

Collection, preparation and enumeration of eggs as well as larval rearing of YTK were done according to best-practice protocols described by Fielder and Heasman (2011).

Genetic diversity of offspring

Eggs were submitted to University of Sunshine Coast (USC) for pedigree testing using similar methods to those described by Knibb et al. (2014).

Chemical analysis

Chemical analysis of feed samples was done by either NSW DPI Feed Quality Service Laboratory (Wagga Wagga, NSW, 2650, Australia) or the CSIRO Agriculture and Food Laboratory (St Lucia, QLD, 4067, Australia). Amino acid analysis of Pelagic and Breed-M was done by the Australian Proteome Analysis Facility Ltd (Macquarie University, Sydney, NSW, 2109, Australia).

Statistics

Of major interest in this qualitative study was the effect of feed type on spawning outcomes. As such some of the data was cautiously interpreted using ANOVA, having $n = 2$ reps per feeding regime; acknowledging that the origin of the wild fish groups were different (at least with respect to age). As both groups were caught from the same geographic location (Port Stephens) they are likely to be related. Statistical analyses were performed using NCSS Professional Version 8.0.23 (Hintze, 2012).

Results

Feed intake

The dry basis feed intake of different broodstock tanks is presented in Table 3.2.6.1.6. It is important to note that these values are influenced by the standing biomass of broodstock in each tank (see Table 3.2.6.1.1) and that in some cases a minor amount of feed was not consumed on some days. Overall feed intake was higher in fish allocated to the pellet regime than fish given natural food sources during the first three cycles. This probably reflects the fact that the all fish were eating roughly the same volume of feed on a per weight basis, but that those on the natural feeds were consuming less dry matter. Wild broodstock originally allocated to pelletised feeds (Tank 8) but switched back to natural feeds in cycle 4 maintained a similar amount of dry matter intake by consuming large amounts of Sardines and Squid; hyperphagia. In contrast, the F1 group returned to the natural feed regime consumed considerable less dry matter in cycle 4.

Fecundity and hatch rate

The number of spawing events recorded from each tank per cycle is presented graphically in Figure 3.2.6.1.1a. Overall fecundity, measured as the sum of viable and non-viable eggs collected from each tank per cycle is presented in Figure 3.2.6.1.1b. Figure 3.2.6.1.1c presents the total fecundity data of all tanks summarised and then categorised by feed type (natural versus pelletised diets). Note that Tank 5 and Tank 8 were returnded to natural feed sources in cycle 4. Apart from a very minor single spawn in cycle 1 (< 12,000 eggs total), Tank 8 did not spawn again until being returned to natural feeds in cycle 4, where it spawned five times and produced more than 400,000 eggs. Collectively, the broodstock held on the natural feed regime or changed back to the natural feed regime in cycle 4 produced a total of approximately 7.4 million eggs over 4 cycles, whereas fish fed the pellet regime produced about 0.75 million eggs. The fecundity of the main breeding tank at PSFI (Tank 1) decreased steadily from cycle 2, indicating the frequecny of spawining might have influenced the fecundity of fish in this tank.

Figure 3.2.6.1.2a presents the fecundity data in terms of viable eggs from all tanks summarised and then categorised by feed type (natural versus pelletised diets). Note that Tank 5 and Tank 8 were returnded to natural feed sources in cycle 4. The fertilisation rate recorded from each tank during each cycle is presented in Figure 3.2.6.1.2b while the hatching rate is presented in Figure 3.2.6.1.2c. The number of larvae successfully hatched from viable (ozonated) eggs derived from broodstock fed natural feeds was approximately 3,064,645 compared to 411,178 from broodstock fed pellet regimes.

Egg morphology

Figure 3.2.6.1.3a presents the diameter of viable eggs (mean ± SD) collected from each tank during each cycle. Figure 3.2.6.1.3b presents the oil droplet size of viable eggs collected from each tank during each cycle. Note that Tank 5 and Tank 8 were returnded to natural feed sources in cycle 4. Tank 8 did not spawn until cycle 4, after being returned to natural feed. There was very little difference among the average morphology of viable eggs with respect to diameter and oil droplet size.

Egg composition

The proximate and energy composition of viable eggs is presented in Table 3.2.6.1.7. A two-way ANOVA was employed to evaluate the interaction between spawning cycle (factor A; cycle 1, 2, and 3 only) and feed type (factor B; feed type, fresh or pellet) Data from cycle 4 was excluded from two-way ANOVA as all fish had been returned to Sardines and Squid. Results indicated there was no significant interaction between the main effects or of each main effect on ash, protein, energy and NFE content of viable eggs (all $P > 0.05$). A significant interaction ($P = 0.029$) was found between the main effects for lipid content of eggs, but neither of the main effects was significant (both $P > 0.05$). The interaction was caused primarily by the decline in lipid content of eggs collected from fish fed Sardine and Squid over cycles 1 to 3 (Tank 1 and Tank 6), while the lipid content of eggs collected from Tank 5 tended to increase.

The individual identified fatty acid composition of eggs is presented in Figure 3.2.6.1.4. The average total amount of fatty acids identified in eggs, pooled across cycles, summed to 652.3, 627.8, 613.0 and 580.6 mg g^{-1} lipid for Tank 1, Tank 5, Tank 6 and Tank 8, respectively. The content of most individual fatty acids was similar among tanks. All egg groups were high in C16:0 (hexadecanoic), C17:0 (heptadecanoic) , C18:1n-9c (oleic) and particularly C22:6n-3 (DHA) (Figure 3.2.6.1.4). The amount of C18:2n-6c (linoleic) recorded from eggs collected from Tank 5 (F1, mostly pellet fed), was far higher than other tank samples and consistently high at each cycle. A comparison of the major fatty acids grouped by feed type is presented in Figure 3.2.6.1.5 and comparisons of DHA and EPA content is presented in Figure 3.2.6.1.6. The DHA content of eggs collected from broodstock fed Sardines and Squid was significantly higher than in eggs collected from fish fed pelletised feed (Figure 3.2.6.1.6). The linoleic content of eggs collected from broodstock fed pelletised feed was significantly higher than eggs collected from broodstock fed Sardines and Squid (Figure 3.2.6.1.5) (this result was mostly driven by Tank 5).

Genetic diversity of offspring

Eggs collected from broodstock tanks during cycle 1 and cycle 2 were linked to individual parent stock within each tank. This allowed identification of the individuals that were making a contribution to different spawning events. However, although broodstock had been genotyped, the sex of the animals was unknown. Enumeration of the data indicated there were greater numbers of offspring groups identified in the wild (Tank 1) and F1 (Tank 6) broodstock fed Sardines and Squid than in the wild (Tank 8) and F1 (Tank 5) broodstock fed pelletised feeds (Table 3.2.6.1.8). When the data was compared using feed type as the fixed factor, ANOVA found a highly significant difference ($F_{1,2}$ = 50.89; P = 0.019) between the mean number of offspring groups identified in the natural fed group (138.0 ± 2.8) as opposed to the pellet fed group (26.5 ± 21.9).

Discussion

We found that feeding broodstock Sardines and Squid is better than the use of two commercial feed sources in terms of maintaining high levels of fecundity and hatching rates in wild and F1 YTK broodstock at PSFI. No major differences were found in the proximate quality of eggs or their morphology with respect to feeding regime, however there were clear differences in the FAME composition of eggs that could be inferred from diet type. Furthermore, the enumeration of genetic data indicated there were greater numbers of offspring groups identified in the wild and F1 broodstock fed Sardines and Squid than in the wild and F1 broodstock fed pelletised feeds. Collectively, these results confirm that natural feeds (i.e. Sardine and Squid) support better hatchery outcomes in broodstock housed at PSFI and these feeds will continue to be used as part of the "best-practice" broodstock management at this hatchery for the present time.

There is still much conjecture regarding the use of commercial feeds for marine broodstock. Most research to date indicates feeding broodstock on natural, fresh food stuffs results in better hatchery outcomes, but this field of research is still quite new and the number of exogenous and endogenous factors that can potentially affect the results of manipulative experiments is large (Izquierdo et al., 2001). This means interpretation of results must be done cautiously.

Broodstock offered the pelletised diets generally consumed more dry matter, protein and energy (dry matter basis) per kilogram of body weight (estimated) than fish kept on the natural feeding regime. For example, the F1 fish were of similar size in Tank 5 and Tank 6, yet the fish in Tank 5 were estimated to be consuming about 2.2 g protein kg BW^{-1} d^{-1} while the fish in Tank 6 about 1.8 g kg BW^{-1} d^{-1}. Although it is not clear at this stage, the difference between the protein:energy intake ratio of naturally fed versus pellet fed broodstock may have implications for the number of eggs produced and the hatching rate of larvae. For example, a low-protein, high calorie diet caused a reduction in reproductive performance of Red Sea Bream (*Pagrus major*) and reductions in protein content coupled with an increase in carbohydrate content of the diet reduced egg viability in Seabass (*Dicentrarchus labrax*) (Izquierdo et al., 2001).

Other differences in the nutrient composition and content of the feed types may have influenced reproductive success. Of major interest are the differences in the fat and fatty acid profiles of the different feed types (Izquierdo et al., 2001). Many fish species preferentially utilize lipids to provide energy for growth, but they are also a vital source of essential fatty acids (EFA) required for the development of cell membranes vital for successful larval development (Sargent et al., 2002). It is therefore, important that the correct EFA are provided (in excess of requirement levels) to broodstock to allow the accumulation of sufficient energy-providing fatty acids and EFA from their diet to not only support somatic growth but to deliver the essential long-chain polyunsaturated fatty acids (LC-PUFA) that are required for successful gamete, egg and subsequent larval production (Migaud et al., 2013).

Examination of the dry basis linoleic acid content (C18:2n-6) of the Breed-M and Pelagica diet indicates they both contain very high levels of this fatty acid; presumably indicative of some plant based meals or oils in the formulations. The high level of oleic acid (C18:1n-9) in the Pelagica feed also supports this hypothesis. On a dry matter basis, the Pelagica diet was also lower in EPA and DHA than the either

Breed-M, Sardines or Squid. The high amount of linoleic acid (C18:2n-6) in the pelletised feeds resulted in a lower ratio of n3:n6 fatty acids compared to Sardines and Squid; of the feed types Squid had the highest n3:n6 ratio. Among natural ingredients, cuttlefish, squid and krill are recognized as valuable components of broodstock diets. The protein component of cuttlefish and squid together with their optimal concentration of highly unsaturated fatty acids (HUFA) appear to be responsible for their positive effect on reproductive performance in marine fish (Izquierdo et al., 2001). Many marine fish studies have demonstrated that egg fatty acid composition is also affected by broodstock diet, especially the level of lipid in the diet and presence of n-3 LC- HUFA, most significantly the level of DHA (Izquierdo et al., 2015). This was reflected in the FAME analysis of eggs from the present study, which were all high in DHA compared to EPA. DHA appeared to be well conserved across tanks, but it was found to be significantly higher in the groups reared predominantly on Sardines and Squid compared to those reared on pelletised feeds. In contrast, the oleic and linoleic content of eggs derived from pellet fed broodstock was higher while the EPA content of the same eggs was lower. Again this points to a shift in the FAME content of eggs as a direct consequence of the selected feed types.

Egg quality criteria such as fertilisation rate, hatching rate and early survival have also been positively correlated with increased n-3 LC-PUFA and arachidonic acid (ARA) content in eggs (NRC, 2011; Migaud et al., 2013; Peng et al., 2015). For example, elevation of lipid levels in broodstock diets increased both growth and survival of newly hatched Rabbitfish (*Siganus guttatus*) larvae and in several other marine species increases in DHA of broodstock diets enhanced growth of first feeding larvae (several authors cited in Izquierdo et al., 2015). The arachidonic content of eggs in this study were similar, however, there was a slight numerical increase in the arachidonic content of eggs collected from broodstock fed Sardine and Squid. There is some evidence that excess dietary EFA can also have negative effects on reproductive performance in broodstock such as Gilthead Seabream (*Sparus aurata*). This may be due to the effect of high dietary n-3 HUFA on the brain-pituitary-gonad endocrine axis, as both EPA and DHA have been found to reduce the steroidogenic action of gonadotropin in the ovary of teleost fish (Mecure and Van Der Kraak, 1995 cited in Izquierdo et al., 2001). Eventual examination of the fatty acid composition of eggs and further evaluation of actual EFA intake of YTK broodstock during this experiment will allow the impact of feeding regime to be correlated with reproductive output.

Taurine has been implicated in improving the ovarian maturation rate in Japanese Yellowtail (*Seriola quinqueradiata*). A level of 1.2% of the diet (dry basis) has been recommended (Matsunari et al., 2006). Both pelletised diets used in this experiment had a similar taurine content (1.7% dry basis) and provided at least the recommended level of taurine to broodstock, even when considered on as as-fed basis. The dry matter taurine content of Squid (4.1%) was five times higher than that of Sardine (0.82%). These values fall to 0.89% (Squid) and 0.23% (Sardine) when considered on an as-fed basis. As spawning outcomes in our experiment appear to be better on the natural feeding regime, it is unlikely the taurine content of either Sardine or Squid is limiting reproductive performance.

Our feeding regimes were based on best-practice protocols established for YTK held at PSFI (Fielder and Heasman, 2011). Under this strategy YTK broodstock are fed once to apparent satiation three times weekly (Mon - Wed - Fri) at approximately 13:00 h. Wild caught YTK spawn spontaneously in captivity after 1 or 2 years of domestication. In our hatchery, year-round, on-demand spawning (ad-hoc) has been achieved in single tanks containing domesticated wild-fish using truncated photo-therms. However we have less data on the use of this strategy with F1 fish or indeed the impact that regular and repeated manipulation of the photo-therm has on reproductive success of wild or F1 broodstock. Indeed, the choice of feed type, feeding strategy and the spawning frequency required by the hatchery must take account of the biological factors which control gonad development, oogenesis, spermatogenesis, duration of vitellogenetic cycle and oocyte maturation. These physical and biological factors will need to be synchronised if reproductive output is to be optimised and evaluation of these interactions could form the basis of the next YTK broodstock experiment. The fecundity of the main breeding tank at PSFI (Tank 1) decreased steadily from cycle 2 indicating the frequecny of spawning might have influenced the fecundity of fish in this tank through time.

Based on the enumeration of viable eggs it would appear that the natural feeding regime was better than the soft-pelletised regime in terms of fecundity (i.e. output of viable fertilised eggs prior to ozonation

and transfer to incubators). This result translates into a greater number of viable hatched-larvae (i.e. post ozonation) being available for transfer to incubators. Although not fully explored, the transfer of wild fish in Tank 8 back to a natural feeding regime in cycle 4 resulted in spawning outcomes that were similar to other spawning tanks in terms of number of spawns, fecundity and fertilisation rate. This may indicate that fish of wild origin require a lengthy period of acclimation to artificial diets before they will spawn naturally. This outcome is worthy of further investigation.

Conclusions and Recommendations

Based on the results of this 12 month trial the "best-practice" feeding regime for YTK broodstock at PSFI remains the use of natural foods such as Sardines and high quality Squid. Wild and F1 fish allocated to dry commercial feeds would not accept standard 15 mm diameter floating pellets. Wild fish took almost 50 days to be weaned onto the moist pellets, whereas, F1 broodstock weaned immediately. This response has implications for broodstock management and breeding programs reliant on wild fish stocks. Further broodstock experiments should consider ways to improve the consumption of pelletised feeds by wild fish as their use will reduce the biosecurity risks associated with using natural feeds in hatchery environments. In addition, the short and repetitive spawning cycles adopted for this experiment might need to be reconsidered in future trials, or indeed as part of commercial relationships, as they may have placed undue reproductive stress on the animals and or their ability to recover both physically and sexually.

Findings

- Our results indicate that for the broodstock housed at PSFI a natural feeding regime remains 'best-practice' in terms of fecundity and egg quality (i.e. higher levels of DHA).
- Diet selection will affect the fatty acid composition of eggs and by extension the ontogenetic development of larvae and hatchlings.
- Other commercial or proprietary formulations should be examined in order to reduce exposure of hatcheries to biosecurity risks.
- These results have given greater certainty about the hatchery management of broodstock at PSFI.
- The data gathered in this experiment will be helpful in planning additional broodstock experiments.
- These results will be useful in planning commercial YTK hatchery operations for Huon Aquaculture and other industry partners.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds. We would like to thank all

technical staff at the PSFI Marine Fish Hatchery for assisting in this experiment and unnamed staff at USC for assisting in genetic testing of eggs.

References

ACEC, 2015. A Guide to Acceptable Procedures and Practices For Aquaculture and Fisheries Research - 4th Edition, 4th ed. NSW Department of Primary Industries (Fisheries) for NSW Fisheries Animal Care and Ethics Committee, Nelson Bay, NSW, Australia.

Agius, R.V., Watanabe, T., Satoh, S., Kiron, V., Imaizumi, H., Yamazaki, T., Kawano, K., 2001. Supplementation of paprika as a carotenoid source in soft-dry pellets for broodstock yellowtall *Seriola quinqueradiata* (Temminck & Schlegel). Aquacult. Res. 32, 263-272.

Booth, M.A., Pirozzi, I., Allan, G.L., Fielder, D.S., 2010. Diet Development: temperate species, Aquafin CRC Project 1B5: Feed Technology For Temperate Fish Species; Final Report to Aquafin CRC & FRDC, Project No. 2004/220, Volume 2. Industry & Investment NSW, Cronulla, Sydney, pp. 376.

Brown, N., 2009. Development of broodstock diets for the Atlantic halibut *Hippoglossus hippoglossus*, Final Report to NRAC Grant # 2001-38500-10691 & # 2002-38500-12056. University of Maine, Center for Cooperative Aquaculture Research, Franklin, Maine, pp. 51.

Cabrita, E., S.Martínez-Páramo, Gavaia, P.J., Riesco, M.F., Valcarce, D.G., Sarasquete, C., Herráez, M.P., Robles, V., 2014. Factors enhancing fish spermquality and emerging tools for sperm analysis. Aquaculture 432, 389-401.

Fielder, D.S., Ballagh, D., Booth, M.A., Pankhurst, P.M., Becker, C.E., Allan, G.L., 2010. Feeding Strategies: temperate species, Aquafin CRC Project 1B5: Feed Technology For Temperate Fish Species; Final Report to Aquafin CRC & FRDC, Project No. 2004/220, Volume 1. Industry & Investment NSW, Cronulla, Sydney, pp. 140.

Fielder, D.S., Heasman, M., 2011. Hatchery manual for the production of Australian Bass, Mulloway and Yellowtail Kingfish. Industry & Investment NSW & Aquafin CRC.

Hintze, J., 2012. NCSS 8. NCSS, LLC. Kaysville, Utah, USA. NCSS, Kaysville, Utah, USA., www.ncss.com.

INVE, 2015. Fish broodstock diets - Technical Card BRDSTCK-EN-0415. INVE Aquaculture, pp. 4.

Izquierdo, M.S., Fernández-Palacios, H., Tacon, A.G.J., 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197, 25-42.

Izquierdo, M.S., Turkmen, S., D.Montero, Zamorano, M.J., Afonso, J.M., Karalazos, V., Fernández-Palacios, H., 2015. Nutritional programming through broodstock diets to improve utilization of very low fishmeal and fish oil diets in gilthead sea bream. Aquaculture 449, 18-26.

Knibb, W., Elizure, A., Miller, A., 2014. Broodstock and genetic managment of Yellowtail kingfsh, Final Report on Project 2010/768. Australian Seafood Cooperative Research Centre, pp. 83.

Knibb, W., Fielder, S., O'Connor, W., Nguyen, N., Whyte, D., 2017. Kingfish genetics: genetic planning and operations, FRDC.

Kolkovski, S., 2005. Evaluation of Yellowtail Kingfish (*Seriola lalandi*) grow out in land-based systems and broodstock management, A Final Report to the Aquaculture Development Council and the Department of Fisheries Western Australia. Department of Fisheries – Research Division, Western Australian Fisheries and Marine Research Laboratories, North Beach, Western Australia, pp. 64.

Matsunari, H., Hamada, K., Mushiake, K., Takeuchi, T., 2006. Effects of taurine levels in broodstock diet on reproductive performance of yellowtail *Seriola quinqueradiata*. Fish. Sci. 72, 955-960.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herraez, M.P., Carrillo, M., 2013. Gamete quality and broodstock management in temperate fish. Reviews in Aquaculture 5, S194-S223.

Morais, S., Mendes, A.C., Castanheira, M.F., Coutinho, J., Bandarra, N., Dias, J., Conceicao, L.E.C., Pousao-Ferreira, P., 2014. New formulated diets for *Solea senegalensis* broodstock: Effects of parental nutrition on biosynthesis of long-chain polyunsaturated fatty acids and performance of early larval stages and juvenile fish. Aquaculture 432, 374-382.

NRC, 2011. Nutrient Requirements of Fish And Shrimp. The National Academic Press, Washington, D.C.

Peng, S., Gao, Q., Shi, Z., Zhang, C., Wang, J., Yin, F., Zhang, Y., 2015. Effect of dietary n-3 LC-PUFAs on plasma vitellogenin, sex steroids, and ovarian steroidogenesis during vitellogenesis in female silver pomfret (*Pampus argenteus*) broodstock. Aquaculture 444, 93-98.

Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The Lipids. in: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition, 3rd Edition. Academic Press, San Diego, CA., pp. 181-257.

Snyder, S., Zeigler, T.R., 2013. Broostock nutrition management enhances reporduction, profits. Global Aquaculture Advocate, 32-33.

Verakunpiriya, V., Watanabe, T., Mushiake, K., Kiron, V., Satoh, S., Takeuchi, T., 1996. Effect of broodstock diets on the chemical components of milt and eggs produced by Yellowtail. Fish. Sci. 62, 610-619.

Watanabe, T., Vassallo-Agius, R., 2003. Broodstock nutrition research on marine finfish in Japan. Aquaculture 227, 35-61.

Table 3.2.6.1.1. Description and status of each broodstock tank at PSFI during the feeding experiment.

Variable	**Tank 1**	**Tank 5**	**Tank 6**	**Tank 8**
Fish type	Wild-type - older	F1	F1	Wild-type - new
Number of fish	7	5	8	9
Est. size of fish (kg)	25-26	12-13	10-11	10-12
Est. biomass (kg)*	182	65	88	108
Feeding regime	Natural regime	Pellet regime	Natural regime	Pellet regime

*Estimated biomass at start of experiment.

Table 3.2.6.1.2. Typical nutrient composition of feed types used in experiment (dry matter basis; g kg^{-1} or MJ kg^{-1}).

	Broodstock feed			
Parameter	**Breed-M***	**Pelagica***	***Sardines* (*Sardinops sagax*)****	**Squid (*Doryteuthis pealeii*)****
Dry Matter (%)	1000.0	1000.0	1000.0	1000.0
Moisture content (%)	0.0	0.0	0.0	0.0
Ash (%)	141.1	80.3	142.1	100.0
Total nitrogen (%)	108.5	93.6	104.4	120.2
Crude protein (%)	678.3	585.2	652.2	751.4
Total Lipid (%)	145.3	154.3	194.0	93.9
Carbohydrate (%)	35.4	180.1	11.7	54.8
Gross energy (MJ Kg^{-1})	23.5	24.1	23.2	22.4
CP:GE ratio	28.9	24.3	28.1	33.6
Alanine	30.7	31.8	38.3	28.4
Arginine	34.5	31.7	38.0	42.8
Aspartic acid	46.0	45.6	52.3	104.7
Cysteine	-	-	8.2	7.9
Glutamic acid	102.7	73.2	90.0	91.2
Glycine	33.2	33.9	38.6	14.4
Histidine	13.7	17.9	33.5	29.3
Hydroxyproline	4.2	5.5	1.8	2.3
Isoleucine	25.7	17.7	29.6	27.9
Leucine	42.9	45.9	51.7	43.7
Lysine	38.2	34.2	55.6	42.8
Methionine	16.8	9.3	15.4	14.0
Phenylalanine	25.1	26.3	27.2	22.8
Proline	34.5	31.0	26.3	27.4
Serine	24.9	27.8	24.8	25.1
Taurine	17.2	17.1	8.2	41.4
Threonine	23.4	22.8	29.2	27.0
Tryptophan	-	-	2.2	5.1
Tyrosine	19.6	14.4	21.5	17.2
Valine	30.2	34.3	33.2	25.6
Sum of AAs (excl. tryp.)	563.6	520.4	625.7	640.9
Retail cost \$ kg^{-1} (circa 2016-17)	40.28	3.33	18.64	42.47

*NSW DPI Wagga Wagga Laboratory; Lab **CSIRO Laboratory.

Table 3.2.6.1.3. Fatty acid composition of commercial feed sources used in experiment (g kg^{-1} total fatty acid identified).

Category	Breed-M*	Pelagica*
∑ **SFA**	35.67	42.23
∑ **MUFA**	27.50	56.92
∑ **n-6 PUFA**	22.58	19.51
∑ **n-3 PUFA**	35.98	13.05
Ratio n-3:n-6	1.59	0.67
Total FAME identified	122.63	132.87

*NSW DPI Wagga Wagga Laboratory;

Table 3.2.6.1.4. Fatty acid composition of natural feed sources used in experiment (g kg^{-1} dry matter).

Category	Sardines *(Sardinops sagax)***	Squid (*Doryteuthis pealeii*)**
∑ **SFA**	84.11	16.26
∑ **MUFA**	27.33	4.50
∑ **n-6 PUFA**	8.24	1.69
∑ **n-3 PUFA**	36.77	24.39
Ratio n-3:n-6	4.46	14.43
Total FAME identified	193.2	46.83

**CSIRO Laboratory.

Table 3.2.6.1.5. Mineral composition of Breed-M and Pelagica diet determined using neutron activation analysis (analysis by ANSTO).

Element (unit)	**Breed-M**	**Pelagica 15mm**
Na (%)	0.88 ± 0.06	0.448 ± 0.027
Mg (mg kg^{-1})	1950 ± 150	1340 ± 120
Al (mg kg^{-1})	58 ± 14	44 ± 16
Cl (%)	1.14 ± 0.08	0.571 ± 0.035
K (%)	0.78 ± 0.06	0.606 ± 0.040
Ca (mg kg^{-1})	1.83 ± 0.13	1.52 ± 0.11
Sc (ng g^{-1})	10.5 ± 1.0	7.4 ± 0.9
V (mg kg^{-1})	0.44 ± 0.16	< 0.4
Cr (mg kg^{-1})	0.95 ± 0.22	0.57 ± 0.18
Mn (mg kg^{-1})	19.4 ± 1.3	55.7 ± 3.4
Fe (mg kg^{-1})	990 ± 70	594 ± 37
Co (ng g^{-1})	82 ± 18	1.33±0.09
Zn (mg kg^{-1})	117 ± 8	168 ± 10
As (mg kg^{-1})	3.62 ± 0.49	1.49 ± 0.25
Se (mg kg^{-1})	2.34 ± 0.25	1.80 ± 0.20
Br (mg kg^{-1})	51.5 ± 3.1	21.0 ± 1.3
Rb (mg kg^{-1})	2.6 ± 0.6	7.9 ± 0.9
Sr (mg kg^{-1})	55 ± 10	42 ± 9
Ag (ng g^{-1})	< 150	< 200
Sb (ng g^{-1})	86 ± 20	94 ± 26
Cs (ng g^{-1})	52 ± 22	49 ± 16

Table 3.2.6.1.6. Total amount of feed offered to broodstock during each spawning cycle (kg tank^{-1} dry matter basis).

Natural feed sources				**Pelletised feed sources**			
Tank 1 - wild	Sardines	Squid	**Row total**	**Tank 5 – F1**	Pelagica	Breed-M	**Row total**
Cycle 1	19.70	15.20	**34.91**	Cycle 1	10.63	8.22	18.84
Cycle 2	24.55	17.73	**42.28**	Cycle 2	12.29	6.76	19.06
Cycle 3	22.68	22.54	**45.22**	Cycle 3	22.20	7.30	29.50
Cycle 4	32.84	21.79	**54.63**	Cycle 4*	*6.88*[¥]	*4.32*[‡]	*11.20*
Column total	**99.77**	**77.26**	**177.04**	**Column total**	**51.99**	**26.60**	**78.60**
Tank 6 – F1				**Tank 8 – new wild**			
Cycle 1	7.86	7.21	15.07	Cycle 1	9.11	10.79	19.91
Cycle 2	9.76	11.39	21.15	Cycle 2	19.20	19.14	38.34
Cycle 3	11.28	10.27	21.55	Cycle 3	34.07	16.66	50.73
Cycle 4	14.16	8.67	22.83	Cycle 4*	*34.01*[¥]	*26.17*[‡]	*60.18*
Column total	43.07	37.54	80.60	**Column total**	**96.40**	**72.75**	**169.15**

** Pellet fed broodstock in Tank 5 and Tank 6 were changed to natural feed sources in cycle 4 ([¥] Sardine; [‡] Squid).

Table 3.2.6.1.7. Proximate composition of viable eggs collected from brood stock tanks during the experiment (dry mater basis; g $100g^{-1}$ or MJ kg^{-1}).

		Proximate category**				
Cycle	**Tank**	**Ash**	**Protein**	**Lipid**	**NFE**	**Energy**
1	1	24.95	47.97	14.00	13.08	19.27
1	5	24.76	49.11	13.15	12.99	19.04
1	6	28.69	46.20	14.97	10.13	18.45
1	8*	32.94	39.43	13.59	14.04	16.41
2	1	25.87	46.35	13.78	14.00	19.12
2	5	24.15	47.70	14.73	13.42	19.18
2	6	23.87	49.11	14.01	13.02	19.51
3	1	26.38	46.62	13.33	13.67	18.95
3	5	24.85	47.34	14.91	12.90	18.97
3	6	24.04	49.00	13.60	13.35	19.00
4	1	27.06	44.93	12.54	15.46	17.24
4	5	24.86	46.49	14.46	14.19	17.27
4	6	22.47	49.12	14.02	14.38	19.86
4	8	24.88	47.86	14.22	13.04	19.05

*Minor spawn in cycle 1

**CSIRO Laboratory; note that all broodstock were fed on natural feeds in cycle 4.

Table 3.2.6.1.8. Number of offspring groups identified from spawning events (cycle 1 and cycle 2) in Experiment 1*.

		Sample date											
Tank	**Regime**	**3/6/16**	**5/6/16**	**14/6/16**	**22/6/16**	**30/8/16**	**1/9/16**	**2/9/16**	**3/9/16**	**4/9/16**	**5/9/16**	**7/9/16**	**Sum**
1	Wild + natural						20	28	28	28	24	12	**140**
5	F1 + pellet	4	4					4		27		3	**42**
6	F1 + natural			28		28	28		28		24		**136**
8	Wild + pellet				11								**11**

*Analysis by the University of Sunshine Coast.

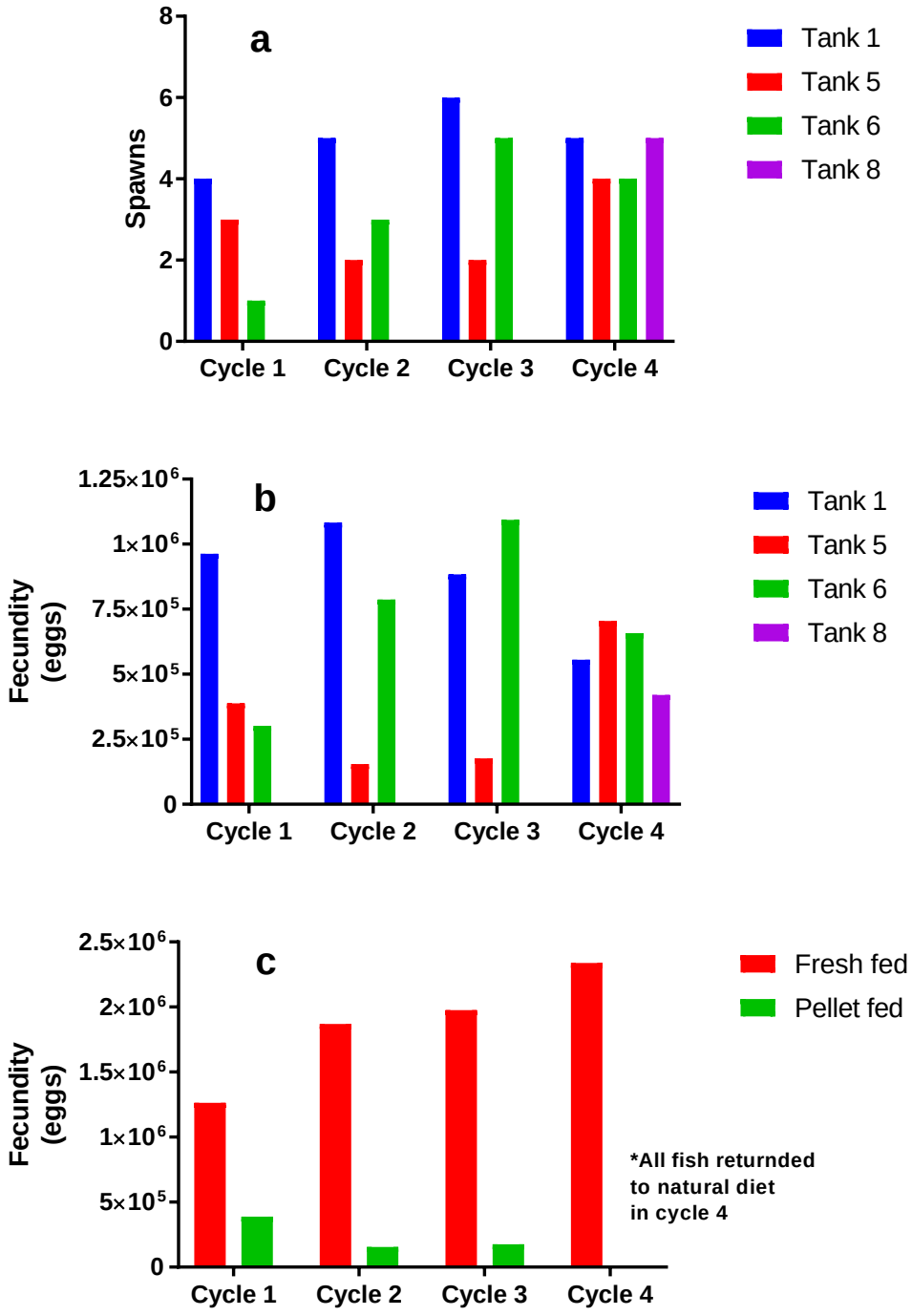


Figure 3.2.6.1.1. (a) number of spawning events recorded in each tank during each cycle; (b) total number of eggs (viable + non-viable eggs) collected from each tank during each cycle; (c) total number of eggs (viable + non-viable eggs) collected from tanks categorised by feed type.
*Note: Tank 5 and Tank 8 were returnded to natural feed sources in cycle 4. Tank 8 (wild caught) did not spawn until cycle 4, after being returned to natural feed.

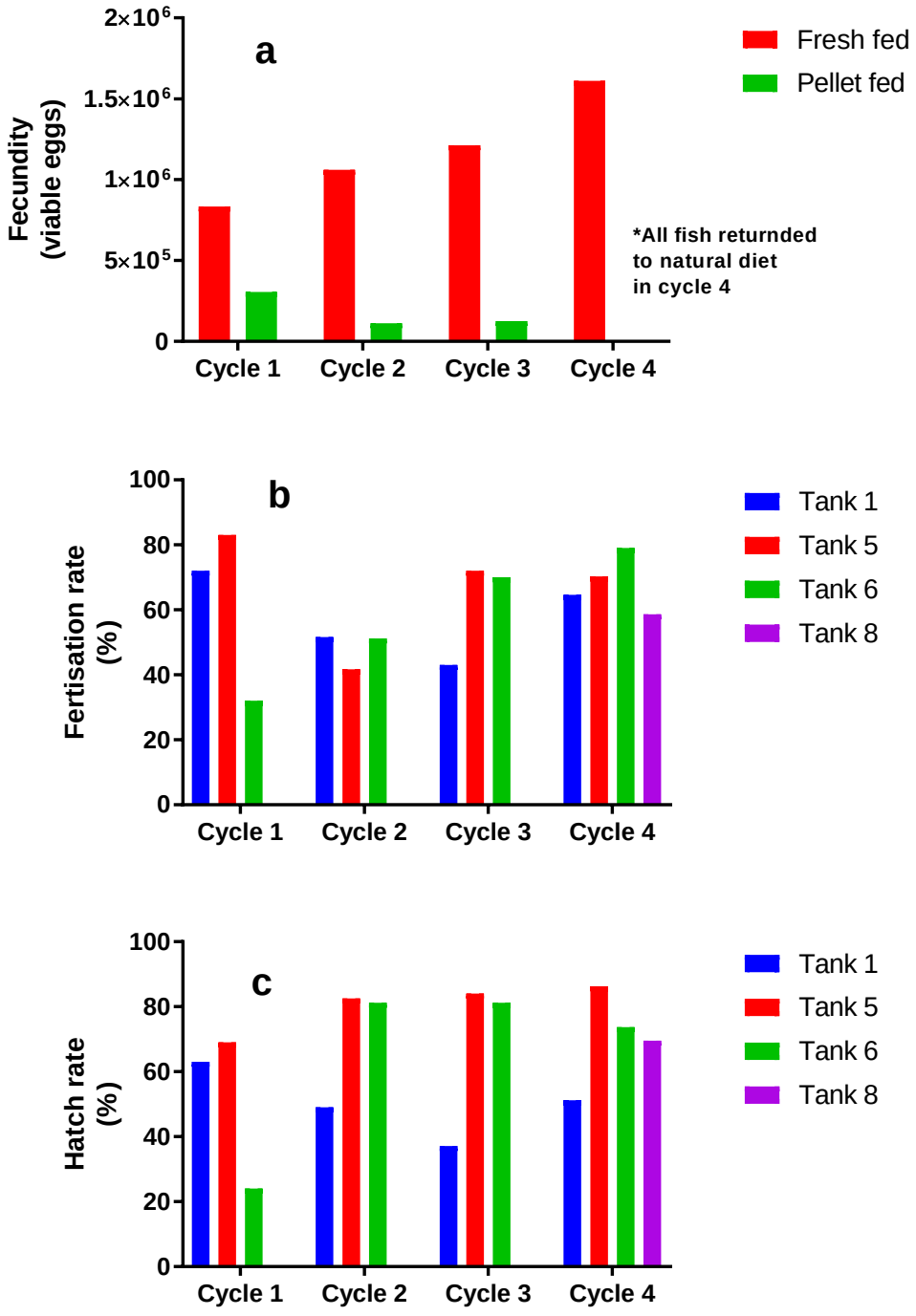


Figure 3.2.6.1.2. (a) total number of viable eggs collected from each tank during each cycle; (b) fertiilsation rate (viable eggs / total eggs × 100) of each tank during each cycle; (c) hatching rate of viable eggs post-ozonation.
*Note: Tank 5 and Tank 8 were returnded to natural feed sources in cycle 4. Tank 8 did not spawn until cycle 4, after being returned to natural feed.

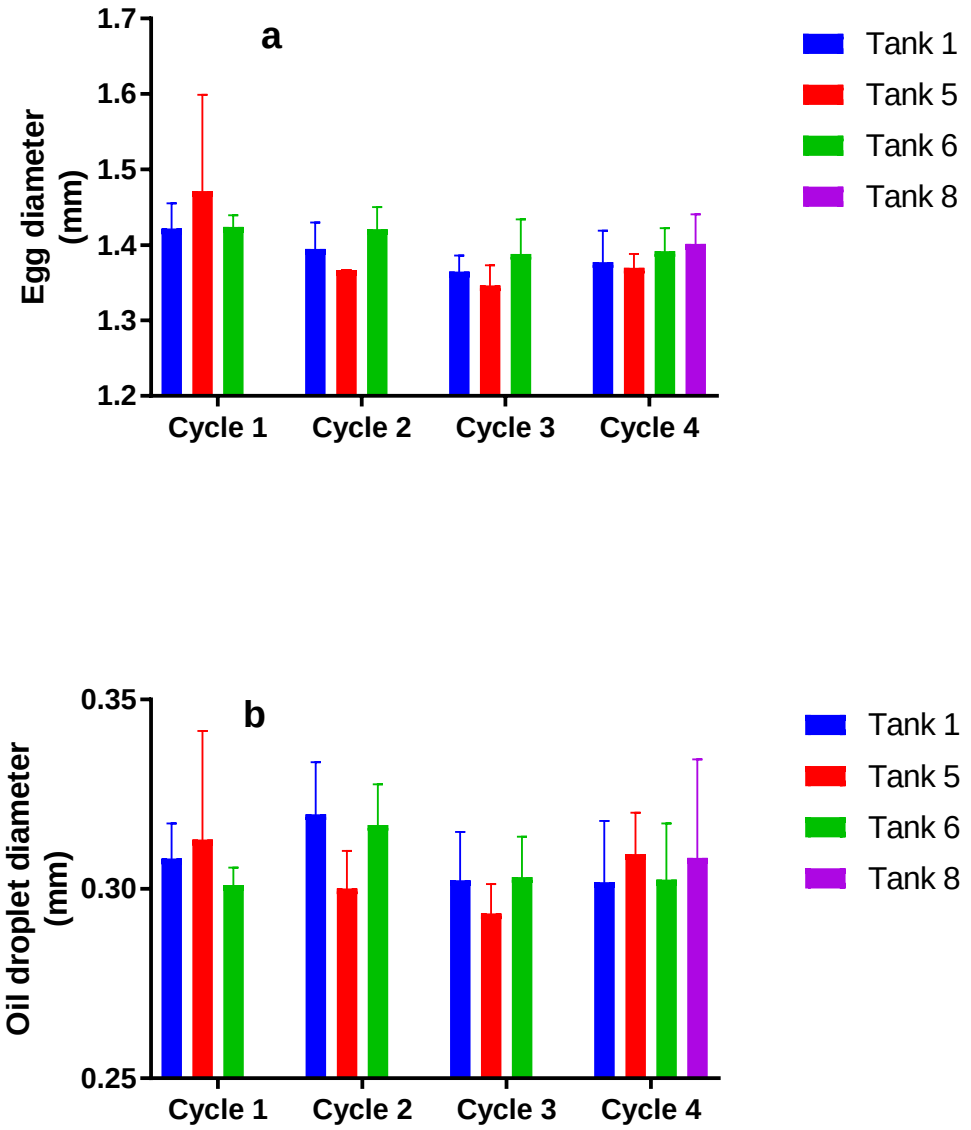


Figure 3.2.6.1.3. (a) mean±SD diameter of viable eggs collected from each tank during each cycle; (b) mean±SD diameter of oil droplet size in eggs collected from each tank during each cycle.
*Note: Tank 5 and Tank 8 were returnded to natural feed sources in cycle 4. Tank 8 did not spawn until cycle 4, after being returned to natural feed.

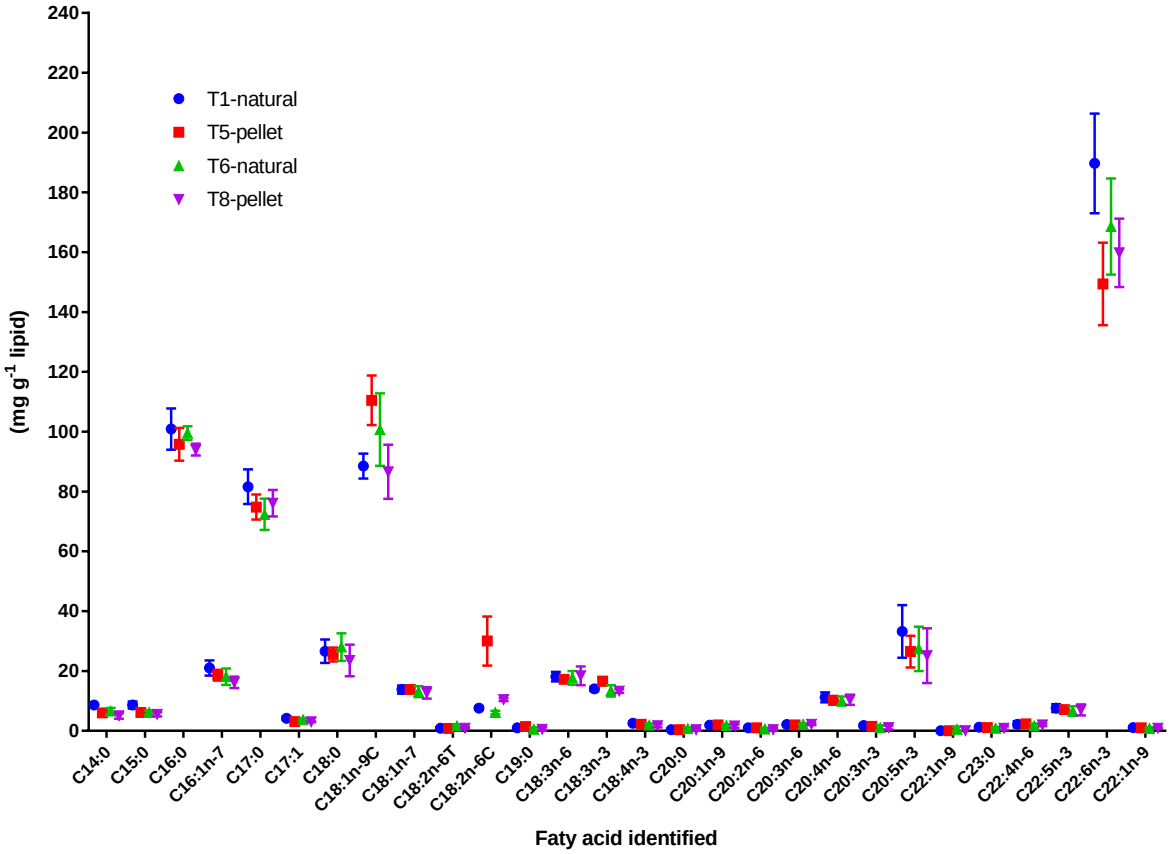


Figure 3.2.6.1.4. Fatty acids identified in viable eggs collected from individual brood stock tanks (dry matter basis; as mg g^{-1} lipid). Tank data is averaged over 4 spawning cycles for Tank 1, Tank 5 and Tank 6 and 2 spawning events in Tank 8. FAME analysis by CSIRO Laboratory.

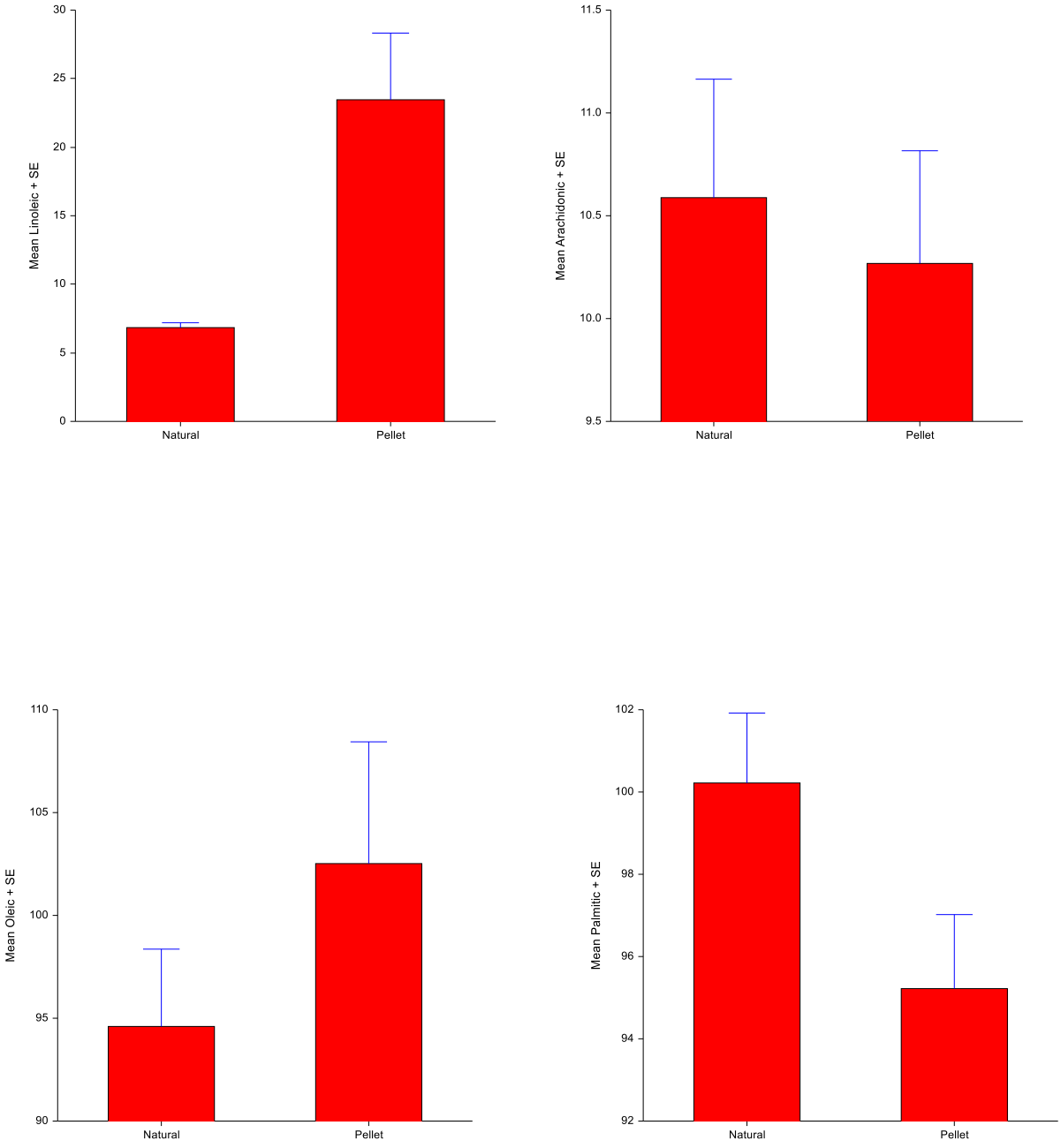


Figure 3.2.6.1.5. Comparison of linoleic (*P = 0.0018; top left*), arachidonic (*P* > 0.05; top right), oleic (*P* > 0.05; bottom left) and palmitic (*P* > 0.05; bottom right) acids found in egg samples taken from broodstock fed Sardines and Squid (natural) versus broodstock fed pelletised feeds. Data are grouped over all cycles.

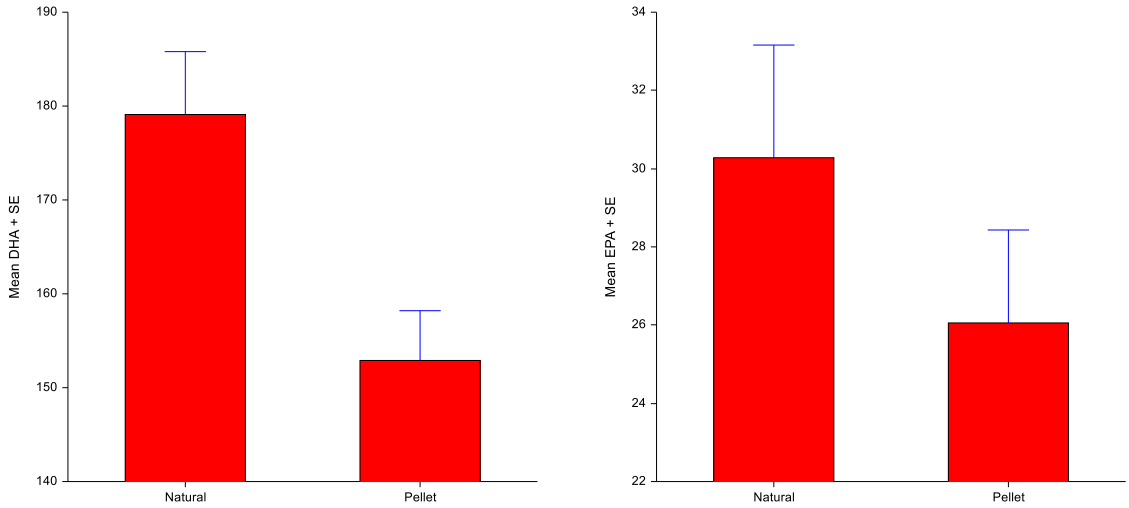


Figure 3.2.6.1.6. Comparison of DHA (P = 0.013; left) and EPA (P > 0.05; right) content of egg samples taken from broodstock fed Sardines and Squid (natural) versus broodstock fed pelletised feeds. Data are grouped over all cycles.

3.2.6.2. Manuscript - Impact of changing the diet of Yellowtail Kingfish broodstock (Seriola lalandi) on growth, fecundity and gut microbiome.

Mark Booth[a], Sarah Catalano[b], Andrew Oxley[b] and D. Stewart Fielder[a],

[a] New South Wales Department of Primary Industries, Locked Bag 1, Nelson Bay, NSW 2315
[b] South Australian Research and Development Institute Aquatic Sciences Centre, West Beach, SA 5024

This manuscript may be referenced as: Booth, M., Catalano, S., Oxley, A. and Fielder, D.S. (2019). 3.2.6.2. Manuscript - Impact of changing the diet of Yellowtail Kingfish broodstock (*Seriola lalandi*) on growth, fecundity and gut microbiome. In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.473-505.

Abstract

This is the second broodstock experiment undertaken at the Port Stephens Fisheries Institute (PSFI) Marine Fish Hatchery to investigate how different feeding strategies affect the growth and fecundity of Yellowtail Kingfish (*Seriola lalandi*; YTK). The 122 day qualitative experiment examined differences in the growth rate, feed conversion ratio (FCR), fecundity and gut (rectal swab) microbiome of broodstock brought about by the use of different feed types (i.e. a commercial pellet diet versus a PSFI "best-practice" regime of Australian Sardines [*Sardinops sagax*; Sardine] and Atlantic Squid [(*Doryteuthis pealeii*; Squid]). Seawater samples were also collected at the start and end of the experiment from each tank in order to evaluate and compare the microbiome of the surrounding environment to that observed in the gut of broodstock fish. Two tanks of broodstock (wild origin) were maintained on the best-practice regime feed (Sardines and Squid) and two tanks of broodstock (F1 of wild domesticated stock repatriated from a farm environment) were fed a commercial pellet formulated for grow-out of YTK. These F1 fish had been reared on commercial pellets since weaning at the PSFI Hatchery. All tanks were physically independent, but shared the same influent estuarine water source. No tanks of broodstock spawned following thermal-photoperiod manipulation at the end of the experiment. Reasons broodstock did not spawn are unclear, but they could relate to the sexual naivety of the wild and F1 origin animals or the additional stress placed on the broodstock when taking initial weightings and microbiome samples at the inception of the trial. Although not compared statistically, the dry basis FCR of fish fed commercial feed was approximately 1.3 units better than fish fed natural food sources equating to an improvement in FCR of about 37%. Results indicated there was a highly significant difference between the weight (SGR_{Wt}) and fork length based (SGR_{FL}) growth rate of different broodstock tanks. The SGR_{Wt} of broodstock was higher in tanks fed Sardines and Squid and lower in tanks fed pelleted diet. A similar pattern was observed in the SGR_{FL} of broodstock. There were no significant differences between the global bacterial community structure of the environmental microbiome samples taken from the four tanks; either at the inception (before samples) or conclusion (after samples) of the experiment. However, significant differences were recorded between the YTK gut and seawater samples, suggesting YTK are able to select, regulate and maintain their own environmentally-independent microbiome communities. Significant differences were also observed in the gut global bacterial community structure of wild and F1 broodstock through time for fish in the same tank and on the same diet (e.g. tank 7 before vs tank 7 after – wild broodstock fed Sardines and Squid; and tank 3 before vs tank 3 after – F1 broodstock fed the commercial pellet), as well as between the two tanks of wild origin broodstock (Tanks 7 and 9) and two tanks of F1 broodstock (Tanks 3 and 4) when sampled at the same time point (either before or after samples). This suggests on a global bacterial level, other factors apart for diet (e.g. host genetics, natural maturation process and

environment) are influencing the gut community structure. Nonetheless, as changes in the gut global bacterial community structure through time were more pronounced in the wild origin compared to F1 fish, the former of which were fed the Squid and Sardines diet may still have an underlying role in shaping the gut microbiome. Overall, good gut health was recorded from the wild origin and F1 broodstock irrespective of diet, with high species richness and evenness along with diversity at the phyla and taxa levels. Characteristics of a dysbiotic state, whereby the microbiome is imbalanced due to increased abundance and/or dominance of a single taxon resulting in reduced diversity and functionality, were not observed at any time point for any of the broodstock fish. This experiment is the first record of the microbiome in YTK broodstock and how it may be impacted by diet selection. As such it will provided an important benchmark for further research into the health and fecundity of this important commercial species.

Introduction

Research on the nutritional requirements of most broodstock is lacking. For this reason the NRC (2011) has recently advocated for more research on broodstock nutrition, with an emphasis on specialised species-specific diets (Migaud et al., 2013). Marine broodstock has traditionally been fed natural foods that are thought to approximate the type of prey the species would normally consume in the wild. It is commonplace to use frozen squid and oily fish such as pilchards to feed Yellowtail Kingfish (*Seriola* lalandi; YTK) broodstock (Kolkovski, 2005). However, there are good reasons for wanting to use manufactured feeds in hatcheries such as reducing biosecurity risks to broodstock and offspring posed by using natural food stuffs that may act as vectors for the introduction of diseases (Watanabe and Vassallo-Agius, 2003). Use of natural feeds in modern recirculating aquaculture systems can also lead to deterioration in water quality resulting in sub-standard environments and additional labour costs (Morais et al., 2014). The use of manufactured feeds also allows broader manipulation of nutrient quality and density via formulation as well as effective inclusion of feed supplements (e.g. vitamins, minerals, astaxanthin and attractants). Operating fish hatcheries is expensive and the cost of feeding can account for a large proportion of the operating budget. For this reason alone there may be good economic reasons to use commercial feeds rather than rely on natural foods.

A previous long term broodstock experiment found that fecundity in wild and mature progeny of wild YTK was higher using natural food sources (i.e. Australian Sardines [*Sardinops sagax*; Sardine] and Atlantic Squid [*Doryteuthis pealeii*; Squid]) rather than proprietary commercial fish pellets or specialised broodstock preparations (see Manuscript 3.2.6.1). As a result of that experiment NSW DPI broodstock have been sustained on natural food sources using the 'best-practice' feeding regime espoused by Fielder and Heasman (2011). Nonetheless, it was recognised at the time of that experiment that future broodstock trials should consider ways to improve the consumption of commercial feeds in YTK, as their use would improve hatchery operations as well as reduce the biosecurity risks associated with using natural feeds in the hatchery. The recent collection of 32 broodstock new from the New Sout Wales Department of Primary Industries (NSW DPI) / Huon Aquaculture marine aquaculture research lease (MARL) has provided a unique opportunity to re-examine the potential of a commercial feed to support acceptable reproductive output from broodstock YTK. Fish repatriated from the MARL to the Port Stephens Fisheries Institute (PSFI) have been reared on pellets their entire lives and, therefore, have no aversion to consuming pelletised aquafeed.

The current broodstock experiment at PSFI is qualitative in design and aims to determine how the reproductive output, health and performance of broodstock YTK is affected by feeding a commercial diet (9 mm Huon; Skretting Select) compared to a natural diet of squid and Australian Sardines. Apart from traditional measures of fecundity and performance this experiment will also evaluate potential changes in the gut (rectal swab) microbiome of broodstock. This area of research is in its infancy and provides great opportunity to link the intestinal health of broodstock to hatchery wide outcomes. Importantly, this experiment aims to see if there is a discernible difference between the gut microbiome of broodstock fed natural foods or a commercial pellet, and if these differences are in any way related to fecundity responses. Understanding the impact of feed type on the structure and diversity of the gut microbiome will provide valuable insights into fish health and an enable, where possible, reproductive

output to be correlated with changes in the microbiome. NSW DPI has collaborated with the South Australian Research and Development Institute (SARDI) on this experiment utilising the expertise of microbiologists studying the microbiome of wild YTK from South Australian (SA) waters and farmed YTK in sea-cages operated by Clean Seas Seafood.

Methods

This study is being performed under the NSW DPI Fisheries Animal Care and Ethics (ACEC) Research Authority known as 'Aquaculture Nutrition ACEC 93/5 – Port Stephens'. Care, husbandry and termination of fish were carried out according to methods outlined in 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2015). Broodstock were always sedated using an appropriate amount of AQUI-S before any handling procedures to prevent damage to the animals or harm to personnel.

Broodstock housing and feeding regimes

Two groups of broodstock were used in this experiment. A group of wild fish captured from the Port Stephens local area in April 2017 and 32 × F1 broodstock collected from the NSW DPI / Huon Aquaculture MARL and transported back to PSFI with the assistance of Huon Aquaculture staff and vessels. The F1 broodstock were the progeny of an older group of wild-captured, domesticated broodstock held at PSFI. The repatriated broodstock were stocked into sea pens on the MARL in late October and December 2016 as 30 g animals. The weight of repatriated YTK ranged between 3.2-6.3 kg. New broodstock arrivals were given a freshwater bath, weighed, measured, electronically tagged, sexed and fin clipped before being split equally among two 20 kL broodstock holding systems.

Although the wild and F1 groups of fish in this experiment have different origins, from a practical viewpoint they are all likely to be related because the F1 fish are all progeny of locally caught, wild YTK from Port Stephens being held at the PSFI Hatchery. The FI group may be more similar genetically, but nonetheless they come from the same geographic lineage.

There were 29 female and 18 male broodstock identified at the start of the experiment, however the sex of three fish could not be ascertained using non-destructive methods. The number of fish and the sex ratio within tanks was different. Tank 7 (wild) contained 8 female and 2 male fish; Tank 9 (wild) contained 7 female, 1 male and one unidentified animal; Tank 3 (F1) contained 7 female, 8 male and one unidentified animal; Tank 4 (F1) contained 7 female, 7 male and one unidentified animal.

Groups of broodstock were reared in four independent 25 kL recirculating systems (RAS). Each RAS consisted of a 20 kL rearing tank, a 200 L sump for collecting eggs, a rotating biological contactor (biofilter) and a micro-bead particle filter. Water temperature is controlled using a reverse cycle refrigeration unit. Fish were held at approximately 17-18 °C to prevent spawning. Tanks were fitted with lids that contained a fluorescent light to control photoperiod. Water was constantly circulated through the RAS via a 2.1 kW centrifugal pump (300-400 L min^{-1}). Each RAS was fed with high quality, filtered estuarine water (< 15 um) drawn from the intersection of Fenningham's Island and Tilligerry Creeks (adjacent PSFI). Rearing tanks were siphoned weekly to remove build-up of organic material. Broodstock at PSFI were generally managed under the best-practice regimes advocated by Fielder and Heasman (2011). Two of the research tanks contained wild broodstock and two tanks contained F1 YTK repatriated from the MARL sea-cages. All fish used in the experiment were fitted with individual electronic tags, sexed, weighed and measured (fork length) prior to being distributed to rearing tanks.

Prior to commencing the experiment, the wild origin fish were being reared on a ration of Australian Sardines (*Sardinops sagax*; Sardine) and Atlantic Squid (*Doryteuthis pealeii*; Squid) supplied by Tweed Bait Pty Ltd (www.tweedbait.com.au). These fish were maintained on natural feeds for the duration of the experiment. Fish repatriated from the MARL were maintained on Huon 9 mm diameter diet pellets (Skretting Select) for the duration of the experiment; i.e. the same diet they were being fed at sea. The justifications for maintaining 'wild' fish on the PSFI best-practice regime (i.e. Sardines and Squid 3

days week^{-1}) and repatriated fish on the Huon commercial pellet were varied, but in the first instance it was to ensure that broodstock continued to eat a satisfactory amount of food at each meal. Evidence from the last broodstock experiment indicated fish took an inordinate amount of time to accept changes to their diet, if at all. Secondly, the outcomes of this research should not be greatly affected by the fact that experimental animals are of wild or hatchery origin because it is highly probable all the fish used in this experiment are related (i.e. the hatchery offspring are derived from local, wild-caught broodstock from the Port Stephens area). In short, although the hatchery fish repatriated from the MARL may be more genetically similar, they nonetheless come from the same geographic area.

All broodstock were fed to apparent satiation once per day at approximately 13:00 h (Monday, Wednesday and Friday). This feeding frequency is in keeping with current hatchery practices at PSFI and the same as that used in the previous broodstock experiment. All uneaten feed was counted and recorded on a daily basis.

Fish spawning and egg collection

The experiment was stocked on the 23 March 2018 and concluded on the 23 August 2018 (122 days). Broodstock were only conditioned to spawn once and were induced to spawn naturally using temperature (i.e. increasing the water temperature in each RAS from 17 °C to 22 °C within 24-48 h) and photoperiod cues in the last week of the experiment. Spawning procedures and the collection, preparation and enumeration of eggs and measures of egg quality followed protocols described by Fielder and Heasman (2011).

Major physical response variables

The following performance variables were used to assess the response of YTK to different feed treatments;

- Initial weight of fish (g) = individual weight of fish at stocking
- Final weight of fish (g) = individual weight of fish at conclusion
- Specific growth rate (SGR_{Wt}) (% d^{-1}) = [Ln(final weight) – Ln(initial weight)] / 122d × 100
- Specific growth rate (SGR_{FL}) (% d^{-1}) = [Ln(final length) – Ln(initial length)] / 122d × 100
- Condition factor K = [individual weight of fish (g) / fork length of fish (mm)^3] × 10^5
- Food conversion ratio (FCR) = feed intake per tank (g) / wet weight gain per tank (g)

Chemical analysis of samples

The natural and commercial food sources were analysed for dry matter, crude protein, gross energy (bomb calorimetry), lipid (FAME) and ash content, respectively. Fertilized eggs were analysed to determine FAME composition. Chemical analysis was done by CSIRO (Agriculture and Food, St Lucia, QLD 4067, Australia) or the NSW DPI Feed Quality Service Laboratory (Wagga Wagga, NSW, 2650, Australia).

General statistical procedures

Biometric treatment data was compared using one way ANOVA. Alpha for ANOVA and the post-hoc multiple comparison procedure (Tukey-Kramer Test) was set at 0.05. Data subjected to ANOVA was statistically analysed using NCSS-8.0.23 after assumptions related to normality and sample variance were satisfied (Hintze, 2012). Individually tagged fish were used as replicates to assess differences in growth rates among different tanks.

Microbiome sampling

Gut (rectal swab) microbiome samples from all fish were collected at the start and end of the experiment to coincide with the weighing of fish. A 1 L sample of water (filtered estuarine) was also taken from each rearing tank at the start and end of the experiment and processed in parallel to control for the influence the environment may have on the structure and composition of the gut bacterial community (note: these water samples were taken before the addition of the anaesthetic). The procedure commenced by lowering the volume of water in each rearing tank to a level that allowed two personnel to safely enter the tank. All fish in the tank were then group sedated using AQUI-S. Individual fish were then guided into a plastic lined 'surf bag', the bag was zipped closed and they were carefully lifted from the tank. Fish and bag were immediately weighed using a clock-face hanging scale after which the bag was gently placed on the floor. The bag was opened and a rectal swab was collected from the fish. Rectal swabs were taken using sterile FLOQSwabs (Copan Flock Technologies) and immediately placed in a 15 mL falcon tube containing stabilising buffer (RNAlater™, Ambion), labelled and stored at 4 °C for 1-2 days and then for a month at -20 °C prior to RNA extraction. Each fish was scanned to identify the individual and their fork length was recorded. Fish were then moved to a recovery tank and the procedure was repeated. In addition, after removal of fish, the surf bags were hosed clean with high pressure fresh water to prevent inadvertent cross-contamination of mucus samples. All broodstock were moved back into their original tank after it was flushed and cleaned. Samples were refrigerated and then shipped to the Molecular Sciences Laboratory (SARDI, West Beach, SA) for preparation and extraction of genetic material. The gut microbiota of broodstock YTK was elucidated following similar procedures and sequencing methods outlined in Legrand et al. (2018).

In addition, for comparative analyses, the NSW broodstock dataset was merged and analysed together with the seven wild charter fish collected in SA waters via line fishing (Four Hummocks, approximately 40 km south-west off the coast of Coffin Bay, SA). Refer to Manuscript 3.1.1.1 for collection and sampling methods of these wild charter fish.

RNA extraction of rectal microbiome samples

RNA was extracted on ice from stabilised samples according to the methods detailed in Szafranska et al. (2014). In brief, the tip was taken out of the stabilizing buffer and placed in a lysing matrix B tube (MP Biomedicals) containing 1 mL of cold (4 °C) RLT buffer supplemented with 1% β-mercaptoethanol. Samples were disrupted via bead-beating using the FastPrep-24™ 5G instrument (MP Biomedicals) at an intensity of 5.5 for 45 s, placed on ice for 3 min then disrupted a second time as described above prior to centrifugation at 14,000 × g for 10 min at 4 °C. The supernatant was transferred to 1.5 mL RNase-free Biopur centrifuge tubes (Eppendorf) and the RNeasy minikit (Qiagen) was used to extract the RNA according to manufacturer's instructions. RNA was eluted in 30 µL of RNase free water, passed through the spin column twice to concentrate each sample and quantified using a NanoDrop 2000 spectrophotometer. To remove any source of potential contaminating gDNA, a routine DNase treatment was performed for all samples using the Turbo DNA-free™ kit (Life Technologies) following the manufacturer's instructions. All samples were precipitated with ethanol using standard procedures, reconstituted in 30 µL of RNase free water and the RNA re-quantified using NanoDrop. Samples were stored at -80 °C prior to use in down-stream procedures.

DNA extraction of environmental samples

One litre of estuarine water was collected in a sterile Schott bottle from each broodstock tank. Samples were labelled and stored at 4 °C prior to filtration and DNA extraction. Each water sample was filtered onto separate sterile 0.22 µM filters (Nalgene®) prior to DNA extraction using the FastDNA™ Spin Kit for Soil (MP Biomedicals) following the manufacturer's instructions. In brief, the filter paper was placed in a lysing matrix E tube with sodium phosphate and MT buffer and cells were lysed via bead-beating using the FastPrep-24™ 5G instrument (MP Biomedicals) at an intensity of 5.5 for 45 s. Samples were subsequently centrifuged for 10 min at 14,000 × g and the supernatant transferred to 1.5

mL DNA LoBind tubes (Eppendorf). Following the addition of a protein precipitation solution, the samples were mixed and centrifuged to pellet the precipitate before the supernatant was transferred to a clean 15 mL centrifuge tube supplemented with Binding Matrix solution. The DNA was captured on SPIN filter tubes and washed, re-eluted in 100 µL of DES and quantified using a NanoDrop 2000 spectrophotometer followed by precipitation with ethanol using standard procedures. The pelleted DNA was reconstituted in 30 µL of RNase free water, re-quantified using the NanoDrop and stored at 4 °C prior to use in down-stream procedures.

cDNA synthesis, PCR amplification, AMPure bead-purification and Picogreen quantification

For the rectal swab samples only, the RNA extracts were converted to cDNA to assess for the active (and likely resident) bacterial community members using the Superscript™ III First Strand Synthesis System (Life Technologies) following the manufacturer's instructions and stored at -20 °C prior to PCR amplification. The V1-V2 hypervariable region of the 16S rRNA gene was amplified for all samples (DNA and cDNA samples) as described by Camarinha-Silva et al. (2014); though included an initial pre-enrichment of the V1-V2 target region by conducting a 20 cycle PCR reaction with primers 27F and 338R as described by Chaves-Moreno et al. (2015). Specifically, 2 µL of cDNA and 5 µL of each environmental DNA extract was used as template in the first round of PCR, with 1 µL aliquots from this reaction used as template in a second 15 cycle PCR reaction to append sample specific barcodes and reverse adapter sequences complementary to the Illumina platform specific adaptors. One µL aliquots of the second PCR reaction were subsequently used as a template in a third 10 cycle PCR to append the Illumina multiplexing sequencing and index primers. PCR amplicons were visualised via gel electrophoresis and products of the expected size (~438 bp) were purified using Agencourt AMPure XP beads (Beckman Coulter). Samples were quantified in duplicate using the Quant-iT™ Picogreen® dsDNA kit (Life Technologies) following the manufacturer's instructions. Approximately 100 samples were pooled for each library in equimolar ratios and sequenced on the MiSeq platform (Illumina, San Diego, CA) using 250 nt paired-end sequencing chemistry through the Australian Genome Research Facility (AGRF). As a sequencing control, amplicons generated from a single bacterial species (*Lactobacillus reuteri*) were included within each Illumina index within each of the libraries. The final list of samples that generated high-quality microbiomic libraries are presented in Table 3.2.6.2.1.

Bioinformatics analysis

In total, 11,549,675 million sequence reads were derived from 97 samples (of the 97 that were collected). No samples failed to amplify enough material to produce good-quality NGS libraries. Sequence reads were paired using PEAR (version 0.9.5) (Zhang et al., 2014), where primers were identified and removed. Paired-end reads were quality filtered, with removal of low-quality reads, full-length duplicate sequences (after being counted) and singleton sequences using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010), USEARCH (version 8.0.1623) (Edgar, 2010; Edgar et al., 2011) and UPARSE software (Edgar, 2013). Reads were mapped to Operational Taxonomic Units (OTUs) using a minimum identity of 97%, and putative chimeras removed using the RDP-gold database as a reference (Cole et al., 2014). These OTUs were further filtered as conducted previously (Zhang et al., 2016) where only those that contributed to > 0.01% of the host-associated dataset (gut samples only) or > 0.01% of the environmental tank water samples were used (see Table 3.2.6.2.2 for a summary of OTUs remaining post-filtering). Rarefaction curves were used to inspect (retrospectively) sampling depth for each gut sample (Figure 3.2.6.2.9). Due to low sequence reads and outlier placement in the non-metric multidimensional scaling (nMDS) plot, two samples (530RS and 755RS) were removed from the dataset (see Table 3.2.6.2.2, Figure 3.2.6.2.9). Further interrogation of the resultant OTUs was conducted using the Seqmatch function of the RDP database (Wang et al., 2007) as well as SILVA (Quast et al., 2013), whereby lineages based on the SILVA taxonomy and best hits from RDP were assigned for each OTU alongside the corresponding RDP sequence similarity value (SeqMatch, S_ab score). The S_ab score represents the number of unique 7-base oligomers shared between an OTU and a known sequence contained in the RDP database divided by the lowest number

of unique oligos in either of the two sequences. A S_ab score of 1.000 represents an identical match to the nearest database sequence, with values closer to 1.000 providing greater confidence in the identification OTU sequence.

Statistical procedures related to microbiome analysis and interpretation

In order to explore for patterns across the global bacterial communities, a data matrix comprising the percent standardised abundances of OTUs was used to construct a sample-similarity matrix using the Bray-Curtis algorithm (Bray and Curtis, 1957), where samples were then ordinated using non-metric multidimensional scaling (nMDS) with 50 random restarts (Clarke et al., 2001). Significant differences between a priori pre-defined groups of samples (e.g. wild charter vs wild-caught vs progeny vs tank water) were evaluated using permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations, allowing for type III (partial) sums of squares, fixed effects sum to zero for mixed terms, and exact p-values generated using unrestricted permutation of raw data (Anderson, 2001). Groups of samples were considered significantly different if the *P*-value is < 0.05. Pairwise tests in PERMANOVA were used to determine which a priori pre-defined categories (e.g. wild-caught before vs wild-caught after) were significantly different. The multivariate analyses, relative percent abundance of bacterial phyla and rarefaction curves were performed and calculated using PRIMER (v.7.0.11), PRIMER-E, Plymouth Marine Laboratory, UK (Clarke et al., 2001).

Conventional measures of species diversity, richness and evenness were calculated using algorithms for total OTUs (S), Pielou's evenness (J'), Shannon diversity (H') and Simpson (1-λ), while taxonomic diversity was calculated using algorithms for taxonomic distinctness: average taxonomic distinctness (avTD - delta+) and variation in taxonomic distinctness (varTD - lambda+) using PRIMER (v.7.0.11) (Clarke et al., 2001). These univariate indicators of diversity (S, J', H', 1-λ, avTD, varTD) were compared between a priori pre-defined groups of samples for the wild-caught cohort (e.g. before tank 7, after tank 7, before tank 9 and after tank 9) separate to the progeny cohort (e.g. before tank 3, after tank 3, before tank 4 and after tank 4) using one-way ANOVA and plotted in Prism v. 7.01 (Graphpad Software Inc.). Variables within each of the two cohorts (wild-caught or progeny) were considered to be significantly different if $P < 0.05$, for which a Tukey's post-hoc multiple comparisons test was then performed comparing the means of each group (Prism v. 7.01). For further presentation of data, relative abundance plots of the top 15 most abundant gut OTUs were constructed in Excel. To identify the closest cultured bacterial species for each of the most abundant OTUs, the corresponding sequence was blasted against the RDP isolate database only. A similarity (S_ab) score in parenthesis is presented for each OTU in the top 15 OTUs plot.

Results

General observations

The chemical analysis of different feed sources is presented in Table 3.2.6.2.3. Fish remained healthy throughout the experiment, readily consuming feeds and gaining weight. Fish repatriated from the MARL were smaller at the beginning of the feeding experiment (4.75-5.36 kg; 95% CL) than wild fish (6.83-8.39 kg; 95% CL). The status of individual broodstock at the beginning and end of the experiment are presented in Table 3.2.6.2.4 and Table 3.2.6.2.5, respectively. Each individual can be identified via their unique electronic alphanumeric identification code. Three unexplained mortalities occurred during the feeding trial; two fish from tank 4 and one fish each from tank 7 and tank 9. These fish were far smaller than other fish from the same tanks, which may indicate these fish were not eating.

Growth and feed conversion ratio

The dry basis feed intake and biomass gain of each broodstock tank is presented in Table 3.2.6.2.6. The initial biomass of tanks 3, 4, 7 and 9 was 83.2, 73.6, 81.9 and 62.8 kg, respectively. Total dry matter

intake among tanks ranged from 40.0-55.8 kg feed and tank based FCR ranged from 2.0:1-3.6:1 based on dry matter feed intake (Table 3.2.6.2.6). Although not compared statistically, the dry basis FCR of fish fed pelletised feed was approximately 1.3 units better than fish fed natural food sources. This equates to an improvement in FCR of about 37%.

Differences in the specific growth rate of tanks (weight and fork length based) were examined using one-way ANOVA setting tank as the fixed factor and individually tagged fish as the replicate values. Results indicated there was a highly significant difference between the SGR_{Wt} ($F_{3,42} = 21.3$; $P < 0.0001$) and SGR_{FL} ($F_{3,42} = 27.5$; $P < 0.0001$) of different tanks. The SGR_{Wt} of broodstock was higher in tanks fed Sardines and Squid and lower in tanks fed pelleted diet. A similar pattern was repeated for the SGR_{FL} of broodstock (Table 3.2.6.2.7).

The average Fulton's condition factor *K* for each tank was significantly different at the beginning of the experiment ($F_{3,46} = 2.9$; $P = 0.045$), but the multiple comparisons procedure could not separate the treatment means at the 95% CI. Generally, the condition factor of F1 broodstock was higher than that of wild broodstock at the start of the experiment. The average condition factor of all broodstock was also significantly different at the end of the experiment ($F_{3,42} = 3.37$; $P = 0.027$), but the multiple comparisons procedure could not separate the treatment means at the 95% CI. The condition factor of all fish improved during the feeding trial, but remained highest in F1 broodstock (Table 3.2.6.2.7). On an individual basis the condition factor of several fish decreased during the experiment as indicated by Figure 3.2.6.2.1, which depicts these fish as falling below the line representing a gradient of 1.

Spawning

Both wild origin and F1 origin broodstock failed to spawn in this experiment. Possible reasons for this are canvassed in the discussion.

Gut (rectal swab) microbiome changes in broodstock - global community structure

In the nMDS plot of all the samples, both the wild charter SA fish and NSW tank water samples clustered as separate groups, with some overlap between the NSW broodstock YTK sample groups (wild-caught and progeny) (Figure 3.2.6.2.2A). From PERMANOVA pairwise comparisons, a significant difference was recorded between each group (Table 3.2.6.2.8A). When the NSW broodstock trial samples (wild-caught and progeny) were split into before and after attempted spawning groups, they clustered separately with a significant difference between the wild-caught before vs wild-caught after ($P = 0.0001$, Table 3.2.6.2.8B) and progeny before vs progeny after ($P = 0.0001$, Table 3.2.6.2.8B) (Figure 3.2.6.2.2B). A significant difference was also recorded between wild-caught vs progeny samples both before and after attempted spawning (Table 3.2.6.2.8B). No significant difference was recorded between the four tank water before and four tank water after samples ($P = 0.1978$, Table 3.2.6.2.8B).

As the global community structure of wild-caught and progeny YTK in the broodstock trial were significantly different, they were split into two datasets to evaluate tank effects. As observed in the nMDS plots and confirmed be PERMANOVA pairwise comparisons, there was a significant difference between the wild-caught before fish (tank 7 vs tank 9, $P = 0.0001$, Table 3.2.6.2.9A), along with a significant difference between wild-caught before and after in both tank 7 ($P = 0.0001$, Table 3.2.6.2.9A) and tank 9 ($P = 0.0002$, Table 3.2.6.2.9A) (Figure 3.2.6.2.3A). The same was observed for the progeny samples, with a significant difference between the before fish (tank 3 vs tank 4, $P = 0.0004$, Table 3.2.6.2.9B), along with a significant difference between before and after in both tank 3 ($P = 0.0001$, Table 3.2.6.2.9B) and tank 4 ($P = 0.0001$, Table 3.2.6.2.9B) (Figure 3.2.6.2.3B).

Gut (rectal swab) microbiome changes in broodstock - bacterial phyla

For the wild-caught YTK in tanks 7 and 9, bacterial phyla *Proteobacteria* and *Bacteroidetes* dominated both the before and after attempted spawning samples. However, for tank 7 before attempted spawning samples, there was also representation from additional phyla, including *Firmucutes*, *Actinobacteria* and *Spirochaetes* (Figure 3.2.6.2.4).

Greater phyla diversity was observed for the progeny YTK compared to wild-caught YTK (Figures 3.2.6.2.4 and 3.2.6.2.5). For the progeny samples, the before attempted spawning fish in tanks 3 and 4 shared similarities in phyla composition and abundance, with representation from bacterial phyla *Proteobacteria*, *Bacteroidetes*, *Tenericutes*, *Spirochaetes* and *Cyanobacteria* (Figure 3.2.6.2.5). The same was true for the after attempted spawning fish in both these tanks, with similar representation by phyla observed in the before attempted spawning samples (e.g. *Proteobacteria*, *Bacteroidetes* and *Tenericutes*) but with additional phyla, including *Latescibacteria* and *Chloroflexi*, observed in these after attempted spawning samples (Figure 3.2.6.2.5).

Gut (rectal swab) microbiome changes in broodstock - top 15 OTUs

For the wild-caught YTK in tanks 7 and 9, the taxa composition and relative abundance of the top 15 OTUs differed between tanks and between before and after attempted spawning samples, even though all fish were fed the same natural diet of Sardines and Squid throughout the trial (Figure 3.2.6.2.6). Within each of the four groups (e.g. before tank 7, after tank 7, before tank 9 and after tank 9) the taxa composition and relative abundance remained relatively consistent, with little heterogeneity observed (Figure 3.2.6.2.6). OTU 2, with closest sequence similarity to *Photobacterium damselae* subsp. *damselae/P. leiognathi* (similarity [S_ab] score 1.000) and OTU 4, with closest sequence similarity to *Pseudoalteromonas* sp. (S_ab score 1.000), were two taxa that were not recorded in the before attempted spawning samples in tanks 7 and 9, yet were observed in fish from both tanks after attempted spawning (Figure 3.2.6.2.6). No single OTU was observed as a dominant constituent in any of the samples, with additional taxa beyond the top 15 contributing to the total relative abundance of all these samples (Figure 3.2.6.2.6).

For the progeny YTK in tanks 3 and 4, similarities in phyla composition and relative abundance were observed both between tanks and between sampling points before and after attempted spawning (Figure 3.2.6.2.7). Some taxa, such as OTU 3 (*Mycoplasma insons*, S_ab score 0.420) and OTU 7 (*Amphritea atlantica*, S_ab score 0.622) were recorded in all of the four sample groups (e.g. before tank 3, after tank 3, before tank 4 and after tank 4), whereas others taxa were only recorded in the before attempted spawning groups in both tanks (e.g. OTU 13, *Fulvivirga kasyanovii*, S_ab score 0.511), or after attempted spawning groups in both tanks (e.g. OTU 1, *Photobacterium phosphoreum*/*P. iliopiscarium*, S_ab score 1.000) (Figure 3.2.6.2.7). Between the wild-caught and progeny samples, shared taxa from the top 15 OTUs were observed, including OTU 4 (*Pseudoalteromonas* sp., S_ab score 1.000), OTU 2253 (*Photobacterium phosphoreum/P. leiognathi/P. kishitanii*, S_ab score 0.965) and OTU 12 (*Brevinema andersonii*, S_ab score 0.741) (Figures 3.2.6.2.6 and 3.2.6.2.7). Similar to the wild-caught samples, no single OTU was observed as a dominant constituent in any of the samples, with additional taxa beyond the top 15 contributing to the total relative abundance of all these samples (Figure 3.2.6.2.7).

Gut (rectal swab) microbiome changes in broodstock - diversity indices

For the wild-caught cohort, species richness and evenness (lambda+) was significantly greater in tank 7 after attempted spawning samples (Table 3.2.6.2.10A, Figure 3.2.6.2.8). Species diversity (Shannon, Simpson and delta+) was not significantly different between the four groups (e.g. before tank 7, after tank 7, before tank 9 and after tank 9), although greater individual variation in terms of evenness (Pielou's) and diversity (Shannon and Simpson) was recorded for the after attempted spawning samples in both tanks compared to the before samples (Table 3.2.6.2.10A, Figure 3.2.6.2.8).

For the progeny cohort, while there was no significant difference in species richness, a significant difference was recorded between groups for all other diversity indices (Table 3.2.6.2.10B, Figure 3.2.6.2.8). Most consistently was higher species evenness (Pielou's) and diversity (Shannon, Simpson and delta+) for the before attempted spawning samples in both tanks compared to the after attempted spawning samples in tank 3 (Figure 3.2.6.2.8). Between the wild-caught and progeny cohorts, similarities in the values for the six diversity indices were observed, with a pattern of one group having higher or lower richness, evenness or diversity compared to the other not recorded (Figure 3.2.6.2.8). Interestingly species evenness (lambda+) was significantly greater in the after attempted spawning tanks (7, 9, 3 and 4) for both groups (wild-caught and progeny) compared to the before attempted spawning samples (in tanks 7, 9, 3 and 4) (Figure 3.2.6.2.8).

Discussion

Impact of dietary changes on the growth of wild and F1 broodstock

There was a highly significant difference between the community based SGR_{Wt} and SGR_{FL} of different holding tanks at the conclusion of the experiment. The SGR_{Wt} of broodstock was higher in tanks fed natural food sources (i.e. Sardines and Squid) and lower in tanks fed the pelleted commercial diet. A similar pattern was repeated for the SGR_{FL} of broodstock. These differences may reflect the origin of each set of animals (i.e. wild vs F1) or differences in the age of fish. The average condition factor of F1 animals was higher than the condition factor of wild broodstock at the start of the experiment. Nonetheless, the condition factor of all broodstock improved during the trial, but it remained highest in F1 fish. On an individual basis the condition factor of several fish decreased during the experiment as indicated by Figure 3.2.6.2.1, which depicts these fish as falling below the line representing a gradient of 1. Although not compared statistically, the dry basis FCR of fish fed pelletised feed was approximately 1.3 units better than fish fed natural food sources. This equates to an improvement in FCR of about 37%.

Understanding or monitoring the growth and development of individual broodstock and being able to match growth metrics with fecundity data will ultimately assist in the selection and retention of the best broodstock.

Impact of dietary changes on the fecundity of wild and F1 broodstock

Both wild caught and F1 broodstock failed to spawn in this experiment. Spawning typically occurs 3-4 days after thermal-photoperiod manipulation (Fielder and Heasman, 2011). Reasons broodstock did not spawn after thermal-photoperiod manipulation are unclear, but they could relate to the sexual naivety of the wild caught and F1 stock, or the additional stress placed on stock at the beginning of the experiment as a result of weighing and microbiome sampling. The latter is unlikely given more than 115 days had elapsed since the fish were handled. As the historical feeding regimes and feed sources for each group of fish was unchanged it also seems unlikely these factors were responsible for the spawning outcome. Additionally, most fish gained a reasonable amount of weight and showed no reluctance to consume their allocated food source. Therefore, it seems unlikely the nutrient and energy intake of fish was inadequate.

Impact of dietary changes on the gut (rectal) microbiome of wild and F1 broodstock

Significant differences in the global community structure of the tank water and NSW broodstock rectal swab samples highlights that YTK are able to select, regulate and maintain their own environmentally-independent communities, as has been presented in the baseline dataset in manuscript 3.1.1.1 of this report.

Significant differences were also observed in the global community structure between SA wild fish vs NSW broodstock trial samples, which were originally wild-caught or represent progeny of wild-caught fish. This could be due to different fish stocks (SA vs NSW), but more likely due to cultivation strategy, as the NSW 'wild-caught' fish had been maintained in the PSFI Hatchery system since April 2017 before these samples were collected. Differences in gut microbiome structure and dynamics has also been observed between wild-caught SA YTK and farmed SA YTK (onshore and offshore) (see manuscript 3.1.1.1.).

Wild-caught before and after fish sampled four months apart were all fed a natural diet of Squid and sardines, yet differences were observed in the global community structure and relative percent abundance of the top 15 OTUs for these groups, suggesting other factors aside from diet have an influence on the gut community structure and dynamics.

Progeny before and after fish sampled four months apart were all fed a formulated feed (Huon 9 mm pellets) and significant differences were observed in the global community structure of these groups. At the bacterial phyla level, similarities were observed between the before fish (tanks 3 and 4) and after fish (tanks 3 and 4), with these similarities also observed at the taxa level. However, clear differences were recorded at the bacterial phyla and taxa level between the before and after samples from the same tank (either tank 3 or tank 4), even though fish were fed the same formulated feed, again suggesting other factors aside from diet have an influence on the gut community structure and dynamics.

The environmental tank water sample remained the same through time - no significant difference in the global community structure between the four before tank samples compared to four after tank samples. Therefore again, differences observed for the gut samples are not a result of the surrounding environment as this did not change yet gut community structure did.

Tank effect was observed, more so for the wild-caught YTK compared to the progeny YTK. Gut community structure, phyla composition and taxa abundance were different in the before tank 7 samples compared to before tank 9 samples for the wild-caught fish. Although the wild-caught after tank 7 and after tank 9 samples were similar in terms of bacterial phyla composition and relative abundance, differences between these groups were observed at the taxa level. For the progeny samples, while significant difference in global community structure was observed between before tank 3 and before tank 4, as well as between after tank 3 and after tank 4, similarities in composition and relative abundance were recorded at the bacterial phyla and taxa level for these groups.

Diversity indices were consistent between the wild-caught and progeny cohorts. For the wild-caught groups, highest species richness and evenness (lambda+) was recorded for the after tank 7 samples. For the progeny samples, both the before tanks (3 and 4) had greater evenness (Pielou's) and diversity (Shannon, Simpson and delta+) compared to the after tanks (3 and 4).

For these samples, observing high taxa diversity (additional taxa beyond the top 15 OTUs contributing to the relative abundance), no dominance by a single organism and consistent global community structure between sample groups (see Figure 3.2.6.2.2), supports the notion of good gut health in both the wild-caught and progeny cohorts, irrespective of diet type. Although differences were observed between tanks and through time (before and after attempted spawning), a dysbiotic state characterised by complete dominance by single/few taxa with closest sequence similarity to known opportunistic pathogens and reduction in diversity indices was not observed. OTU 2, with closest sequence similarity to *Photobacterium damselae* subsp. *damselae*/*P. leiognathi* (similarity [S_ab] score 1.000), does warrant further investigation as it may be an opportunistic pathogen (was observed enriched in the after wild-caught fish from both tanks and *P. damselae* subsp. *damselae* is known to be an opportunistic pathogen, although no reports of pathology or disease have been associated with *P. leiognathi* in the literature so could also be commensal as this OTU is unresolved).

OTU 3, with low sequence similarity to *Mycoplasma insons* (S_ab 0.420), which was recorded in all the progeny YTK samples, also warrants further investigation. This taxon has previously been recorded enriched in gut enteritis disease samples collected as part of the health vs disease component – see Manuscript 3.3.1.3. However, abundances of this taxon recorded here in the broodstock trial are not as high or dominantly so across all samples as has been observed in the gut enteritis disease samples.

Comparing to SA tank trials, the samples from the broodstock trial had much greater species richness, evenness and diversity. SA tank trial samples have been consistently recorded with dominance by single taxon (e.g. *Brevinema andersonii* for the lipid/emulsifier trial and formulated feed vs natural diet trial, and *Mycoplasma insons* for the fatty acid and fish meal replacement trials - see Manuscript 3.2.1.1). Note the fish used in the SARDI trials are farmed fish, whereas, the broodstock trial had fish that were wild-caught as the starting point. We have already shown in our baseline dataset that there is a difference in microbiome structure and dynamics between wild and farmed fish, so this could be a reason why we are seeing much more diversity in the broodstock trial samples compared to SA trial samples.

Conclusions and Recommendations

Unfortunately, none of the wild origin or F1 broodstock spawned in this experiment. Reasons broodstock did not spawn are unclear, but they could relate to the sexual naivety of the wild and F1 origin animals or the additional stress placed on the broodstock when taking initial weightings and microbiome samples at the inception of the trial. Although not compared statistically, the dry basis FCR of fish fed commercial feed was approximately 1.3 units better than fish fed natural food sources, equating to an improvement in FCR of about 37%. Results also indicated there was a highly significant difference between the SGR of different broodstock tanks, with SGR being higher in tanks fed Sardines and Squid and lower in tanks fed pelleted diet. There were no differences found between the global community structure of water-borne microbiome samples taken from the four tanks; either at the inception or conclusion of the experiment. However, there were significant differences recorded between the gut samples from YTK compared to the water-borne tank samples, suggesting YTK are able to select, regulate and maintain their own environmentally-independent microbiome communities. Differences were observed in the global community structure among all tanks of broodstock at the inception and conclusion of the experiment and through time, suggesting other factors apart from diet selection are influencing gut community structure and dynamics. Nonetheless, both wild origin and F1 broodstock appear to have good gut health irrespective of the diet they were consuming, with no evidence of a dysbiotic state observed in any tank of broodstock.

Findings

- This experiment is the first record of the microbiome in YTK broodstock and how it may be impacted by diet selection. As such it will provide an important benchmark for further research into the health and fecundity of this important commercial species
- Differences were observed in the global community structure among all tanks of broodstock at the inception and conclusion of the experiment and through time, suggesting other factors apart from diet selection are influencing gut community structure and dynamics.
- Changes in the global community structure of broodstock over time were somewhat greater in wild origin fish than in F1 fish. This may be indicative of greater changes in the composition of Sardines and Squid compared to the commercial pellets or indeed differences in the maturation state on the wild and F1 broodstock.
- Our understanding of broodstock nutrition remains in its infancy. However the implications of farming progeny of poorly maintained and malnourished broodstock are profound, having negative ramifications across the whole nursery and production cycle. In addition, the implications of quickly shifting from ‘best-practice’ broodstock and hatchery regimes to newer regimes without proper evidence is also profound, as the consequences of getting it wrong can be long lasting. Manipulative nutrition trials with large broodstock animals are challenging due to the scale of systems, the size of animals, the duration of experiments (especially those involving long terms spawning cycles) and often low replication. The basic nutrition research conducted on broodstock in this and other experiments (Manuscript 3.2.6.1) has mostly been qualitative, but it has indicated that manipulating feeds and spawning cycles can impact the

fecundity of wild origin and F1 animals. Difference in feed type (natural vs manufactured) also appears to impact the microbiome of broodstock in definable ways. These results demonstrate we need to pay close attention to these issues in YTK hatcheries and develop better and more rapid methods to assess the impacts of diet or abiotic shifts on the fecundity and quality of output from broodstock animals.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, New South Wales Department of Primary Industries (NSW DPI) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

We would also like to acknowledge the technical assistance of Luke Vandenberg, Ian Russell, Brendan Findlay, Georgie Whelan, Daniel Yee, Nicholas Gibson and Emily Collier (NSW DPI). We thank Cedric Simon, Barney Hines and staff at CSIRO for conducting the chemical analyses on samples generated from this experiment. Dr Michael Salini and Simon Tabrett were actively involved in supplying many of the raw materials used to manufacture the experimental feeds (Ridley). The authors would also like to acknowledge the support of SARDI and provision of the Molecular Sciences Laboratory facilities at West Beach, South Australia.

References

ACEC, 2015. A Guide to Acceptable Procedures and Practices for Aquaculture and FisheriesResearch, 4th Edition. In: NSW DPI Animal Care and Ethics Committee, pp. 56.

Agius, R.V., Watanabe, T., Satoh, S., Kiron, V., Imaizumi, H., Yamazaki, T., Kawano, K. 2001. Supplementation of paprika as a carotenoid source in soft-dry pellets for broodstock yellowtall Seriola quinqueradiata (Temminck & Schlegel). Aquacult. Res. 32, 263-272.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology 26, 32-46.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7, 335-336.

Clarke, K.R., Warwick, R.M.R.M., Laboratory, P.M., 2001. Change in marine communities: an approach to statistical analysis and interpretation: Plymouth, U.K.: PRIMER-E Ltd.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research* 42 (Database issue), D633-D642.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19), 2460-2461.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nature Methods 10, 996-998.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194-2200.

Fielder, D.S., Heasman, M., 2011. Hatchery manual for the production of Australian Bass, Mulloway and Yellowtail Kingfish, Industry & Investment NSW & Aquafin CRC.

Hintze, J., 2012. NCSS 8. NCSS, LLC. Kaysville, Utah, USA. NCSS, Kaysville, Utah, USA., www.ncss.com.

Kolkovski, S., 2005. Evaluation of Yellowtail Kingfish (Seriola lalandi) grow out in land-based systems and broodstock management. In: A Final Report to the Aquaculture Development Council and the Department of Fisheries Western Australia. Department of Fisheries – Research Division, Western Australian Fisheries and Marine Research Laboratories, North Beach, Western Australia, pp. 64.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herraez, M.P., Carrillo, M., 2013. Gamete quality and broodstock management in temperate fish. Reviews in Aquaculture 5, S194-S223.

Morais, S., Mendes, A.C., Castanheira, M.F., Coutinho, J., Bandarra, N., Dias, J., Conceicao, L.E.C., Pousao-Ferreira, P., 2014. New formulated diets for Solea senegalensis broodstock: Effects of parental nutrition on biosynthesis of long-chain polyunsaturated fatty acids and performance of early larval stages and juvenile fish. Aquaculture 432, 374-382.

NRC, 2011. Nutrient Requirements of Fish and Shrimp, The National Academic Press, Washington, D.C.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41, D590-D596.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology 73, 5261-5267.

Watanabe, T., Vassallo-Agius, R., 2003. Broodstock nutrition research on marine finfish in Japan. Aquaculture 227, 35-61.

Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30, 614-20.

Zhang, M., Sun, Y., Liu, Y., Qiao, F., Chen, L., Liu, W-T, Du, Z., Li, E., 2016. Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference. Aquaculture 454, 72.

Table 3.2.6.2.1. Sample information pertaining to broodstock microbiome trial (rectal swab samples) collected from wild-caught and F1 Yellowtail Kingfish before (b) and after (a) attempted spawning.

Sample type	Fork length (cm)	Weight (kg)	Sex	Location	Site	Tank ID	Date sample collected	Library size	# bacterial OTUs
water sample-b	-	-		NSW	PSFI	Tank 7	23/04/18	82090	353
water sample-b	-	-		NSW	PSFI	Tank 9	23/04/18	90335	336
water sample-b	-	-		NSW	PSFI	Tank 3	24/04/18	80382	342
water sample-b	-	-		NSW	PSFI	Tank 4	24/04/18	82284	299
water sample-a	-	-		NSW	PSFI	Tank 7	22/08/18	187357	436
water sample-a	-	-		NSW	PSFI	Tank 9	23/08/18	147837	387
water sample-a	-	-		NSW	PSFI	Tank 3	22/08/18	242573	400
water sample-a	-	-		NSW	PSFI	Tank 4	22/08/18	199472	384
wild charter SA	78	5.8		SA	Four Hummocks	-	05/02/18	69976	59
wild charter SA	92	8.9		SA	Four Hummocks	-	05/02/18	192958	51
wild charter SA	61	2.85		SA	Four Hummocks	-	05/02/18	151009	42
wild charter SA	67	5.4		SA	Four Hummocks	-	05/02/18	74727	29
wild charter SA	106	12.4		SA	Four Hummocks	-	06/02/18	117980	47
wild charter SA	78.5	5.5		SA	Four Hummocks	-	06/02/18	108639	52
wild charter SA	76	5.19		SA	Four Hummocks	-	06/02/18	119163	30
wild caught-b	76.0	6.6	F	NSW	PSFI	Tank 7	23/04/18	45759	375
wild caught-b	80.0	9.0	F	NSW	PSFI	Tank 7	23/04/18	56355	327
wild caught-b[1]	85.0	9.1	M	NSW	PSFI	Tank 7	23/04/18	42271	219
wild caught-b	78.0	6.6	F	NSW	PSFI	Tank 7	23/04/18	79711	317
wild caught-b	80.0	7.5	F	NSW	PSFI	Tank 7	23/04/18	72610	358
wild caught-b	85.0	10.4	M	NSW	PSFI	Tank 7	23/04/18	52959	442
wild caught-b	89.0	10.2	F	NSW	PSFI	Tank 7	23/04/18	47891	355
wild caught-b	82.0	8.1	F	NSW	PSFI	Tank 7	23/04/18	77328	359
wild caught-b	83.0	8.5	F	NSW	PSFI	Tank 7	23/04/18	109111	404
wild caught-b	77.0	7.2	F	NSW	PSFI	Tank 9	23/04/18	54362	412
wild caught-b	83.5	8.0	F	NSW	PSFI	Tank 9	23/04/18	71161	362
wild caught-b	76.0	6.5	F	NSW	PSFI	Tank 9	23/04/18	42052	404
wild caught-b	79.0	7.5	F	NSW	PSFI	Tank 9	23/04/18	32875	422
wild caught-b	74.0	6.25	F	NSW	PSFI	Tank 9	23/04/18	67940	442
wild caught-b	77.0	7.5	F	NSW	PSFI	Tank 9	23/04/18	64998	413
wild caught-b	78.0	7.3	F	NSW	PSFI	Tank 9	23/04/18	85849	376
wild caught-b	84.0	9.0	M	NSW	PSFI	Tank 9	23/04/18	63136	431
progeny-b	66.0	4.8	M	NSW	PSFI	Tank 3	24/04/18	40589	398
progeny-b	70.0	5.5	F	NSW	PSFI	Tank 3	24/04/18	95361	332
progeny-b	63.0	3.2	M	NSW	PSFI	Tank 3	24/04/18	38302	346
progeny-b	69.0	4.25	M	NSW	PSFI	Tank 3	24/04/18	101304	439
progeny-b	66.0	5.4	M	NSW	PSFI	Tank 3	24/04/18	83991	325
progeny-b	67.0	5.2	F	NSW	PSFI	Tank 3	24/04/18	53428	382
progeny-b	69.0	5.0	M	NSW	PSFI	Tank 3	24/04/18	71555	378
progeny-b	66.0	5.1	F	NSW	PSFI	Tank 3	24/04/18	62630	439
progeny-b	70.0	5.5	U	NSW	PSFI	Tank 3	24/04/18	36893	310
progeny-b	64.0	5.8	F	NSW	PSFI	Tank 3	24/04/18	46249	380
progeny-b	69.5	6.9	F	NSW	PSFI	Tank 3	24/04/18	73388	432
progeny-b	70.0	6.1	F	NSW	PSFI	Tank 3	24/04/18	47933	340
progeny-b	70.0	5.3	M	NSW	PSFI	Tank 3	24/04/18	36643	413
progeny-b	63.0	5.0	M	NSW	PSFI	Tank 3	24/04/18	35750	436
progeny-b	66.0	4.6	M	NSW	PSFI	Tank 3	24/04/18	44810	426
progeny-b	70.0	5.5	F	NSW	PSFI	Tank 3	24/04/18	42700	345
progeny-b	64.0	5.5	M	NSW	PSFI	Tank 4	24/04/18	60206	359
progeny-b	57.0	3.5	M	NSW	PSFI	Tank 4	24/04/18	49469	361

progeny-b	65.0	6.0	F	NSW	PSFI	Tank 4	24/04/18	55002	399
progeny-b	64.0	4.0	F	NSW	PSFI	Tank 4	24/04/18	30004	300
progeny-b	66.0	5.5	F	NSW	PSFI	Tank 4	24/04/18	56519	271
progeny-b	65.0	5.2	F	NSW	PSFI	Tank 4	24/04/18	125525	388
progeny-b	70.0	4.0	F	NSW	PSFI	Tank 4	24/04/18	71922	415
progeny-b	67.0	4.0	M	NSW	PSFI	Tank 4	24/04/18	73480	442
progeny-b	67.0	4.6	F	NSW	PSFI	Tank 4	24/04/18	67643	349
progeny-b	71.0	5.1	U	NSW	PSFI	Tank 4	24/04/18	113121	445
progeny-b	66.0	5.4	F	NSW	PSFI	Tank 4	24/04/18	67362	394
progeny-b	64.0	5.1	M	NSW	PSFI	Tank 4	24/04/18	70859	338
wild caught-a	95.0	13.5	F	NSW	PSFI	Tank 7	22/08/18	125157	499
wild caught-a	84.0	8.8	F	NSW	PSFI	Tank 7	22/08/18	156212	527
wild caught-a	84.5	10.0	F	NSW	PSFI	Tank 7	22/08/18	175458	532
wild caught-a	92.0	11.6	F	NSW	PSFI	Tank 7	22/08/18	179827	568
wild caught-a	87.5	11.1	F	NSW	PSFI	Tank 7	22/08/18	118545	454
wild caught-a	91.0	12.0	M	NSW	PSFI	Tank 7	22/08/18	184522	547
wild caught-a	85.0	8.6	F	NSW	PSFI	Tank 7	22/08/18	203750	502
wild caught-a	86.5	9.6	F	NSW	PSFI	Tank 7	22/08/18	138909	501
wild caught-a	90.5	11.7	M	NSW	PSFI	Tank 7	22/08/18	241224	495
wild caught-a	83.0	9.5	F	NSW	PSFI	Tank 9	23/08/18	170344	425
wild caught-a	86.5	10.5	F	NSW	PSFI	Tank 9	23/08/18	186260	420
wild caught-a	90.0	10.5	F	NSW	PSFI	Tank 9	23/08/18	127868	364
wild caught-a	84.0	9.9	F	NSW	PSFI	Tank 9	23/08/18	171059	377
wild caught-a	89.0	10.5	F	NSW	PSFI	Tank 9	23/08/18	175845	417
wild caught-a	84.0	9.5	F	NSW	PSFI	Tank 9	23/08/18	132117	408
wild caught-a	83.0	9.5	F	NSW	PSFI	Tank 9	23/08/18	184432	463
wild caught-a	89.0	12.4	M	NSW	PSFI	Tank 9	23/08/18	151289	420
progeny-a	69.5	4.5	M	NSW	PSFI	Tank 3	22/08/18	171218	370
progeny-a	65.5	3.5	M	NSW	PSFI	Tank 3	22/08/18	122551	292
progeny-a	72.5	6.75	F	NSW	PSFI	Tank 3	22/08/18	133422	367
progeny-a	68.5	6.15	M	NSW	PSFI	Tank 3	22/08/18	152537	481
progeny-a	65.5	6.0	M	NSW	PSFI	Tank 3	22/08/18	143325	460
progeny-a	72.5	6.1	M	NSW	PSFI	Tank 3	22/08/18	206099	434
progeny-a	72.5	6.15	M	NSW	PSFI	Tank 3	22/08/18	143718	484
progeny-a	68.5	6.0	M	NSW	PSFI	Tank 3	22/08/18	116282	451
progeny-a	70.0	7.0	F	NSW	PSFI	Tank 3	22/08/18	91367	437
progeny-a	72.0	5.5	F	NSW	PSFI	Tank 3	22/08/18	78162	435
progeny-a[1]	68.0	7.2	F	NSW	PSFI	Tank 3	22/08/18	73	28
progeny-a	70.0	6.4	U	NSW	PSFI	Tank 3	22/08/18	56808	345
progeny-a	71.0	7.8	F	NSW	PSFI	Tank 3	22/08/18	84541	451
progeny-a	70.0	5.8	M	NSW	PSFI	Tank 3	22/08/18	204508	417
progeny-a	69.5	6.4	F	NSW	PSFI	Tank 3	22/08/18	103189	407
progeny-a	68.0	6.4	F	NSW	PSFI	Tank 3	22/08/18	164543	346
progeny-a	68.0	6.6	F	NSW	PSFI	Tank 4	22/08/18	128891	487
progeny-a	72.0	4.7	F	NSW	PSFI	Tank 4	22/08/18	148124	478
progeny-a	66.0	4.25	F	NSW	PSFI	Tank 4	22/08/18	138685	467
progeny-a	68.5	6.4	F	NSW	PSFI	Tank 4	22/08/18	104349	484
progeny-a	69.5	4.5	M	NSW	PSFI	Tank 4	22/08/18	118545	532
progeny-a	68.0	6.4	F	NSW	PSFI	Tank 4	22/08/18	108327	446
progeny-a	68.0	7.0	F	NSW	PSFI	Tank 4	22/08/18	214652	284
progeny-a	60.5	4.0	M	NSW	PSFI	Tank 4	22/08/18	182854	460
progeny-a	66.0	6.5	M	NSW	PSFI	Tank 4	22/08/18	132755	497
progeny-a	73.0	6.5	U	NSW	PSFI	Tank 4	22/08/18	234164	516
progeny-a	69.5	5.6	F	NSW	PSFI	Tank 4	22/08/18	232402	503

progeny-a	66.0	6.4	M	NSW	PSFI	Tank 4	22/08/18	244978	297

Abbreviations: a, after; b, before; F, female; M, male; NSW, New South Wales; PSFI, Port Stephens Fisheries Institute; SA, South Australia; U, unknown.
[1]Samples removed from dataset due to low sequence reads/outlier placement in nMDS plot.

Table 3.2.6.2.2. Summary of sequenced sample parameters.

Data-set	**No. of samples**	**Total reads**	**Median library size**	**Range**	**Bacterial OTUs post-filtering**
Broodstock NSW	105	11,549,675	101,304	73 - 244,978	1,037

Table 3.2.6.2.3. Measured nutrient content of feed sources used in the broodstock experiment (dry matter basis; g kg^{-1} or MJ kg^{-1}; FAME as mg g^{-1} lipid)

	Diet type (as-fed basis)		
Parameter	Huon 9 mm**	Sardines (*Sardinops sagax*)**	Squid (*Doryteuthis pealeii*)**
Dry Matter (%)	1000.0	1000	1000
Moisture content (%)	0.0	0.0	0.0
Ash (%)	114.8	153.5	74.7
Total nitrogen (%)	77.2	95.3	126.2
Crude protein (%)	482.3	598.5	788.5
Total Lipid (%)	189.7	241.6	101.9
Carbohydrate (%)	212.7	9.1	34.9
Gross energy (MJ/Kg)	23.3	23.9	23.1
CP:GE ratio	20.7	25.0	34.1
FAME (mg g^{-1} lipid)			
∑SAT	350.1	348.1	159.5
∑MONO	104.4	113.1	44.1
∑n-3 PUFA	126.3	307.2	239.2
∑n-6 PUFA	148.3	34.01	16.5
Total FAME identified	729.1	802.5	459.4
n-3:n-6 ratio	0.85	9.0	14.5

**Analysed by CSIRO Laboratories.

Table 3.2.6.2.4. Description and status of broodstock tanks at beginning of the experiment.

Tank	Origin	Feed type	Sex	Chip#	Start weight (kg)	Start fork length (CM)	K start
7	Wild	S+SQ	F	007839A45	10.2	89.0	1.45
7	Wild	S+SQ	F	00783B9A2	6.6	78.0	1.39
7	Wild	S+SQ	F	006C99E83	6.6	76.0	1.50
7	Wild	S+SQ	F	007AC3278	8.5	83.0	1.49
7	Wild	S+SQ	F	007AC885B	8.1	82.0	1.47
7	Wild	S+SQ	M	007B01A0A	10.4	85.0	1.69
7	Wild	S+SQ	F	007B02097	7.5	80.0	1.47
7	Wild	S+SQ	F	007AC8AE1	9.0	80.0	1.76
7	Wild	S+SQ	M	007ACOEA7	9.1	85.0	1.48
7	Wild	S+SQ	F	007AC4562	5.9	79.0	1.20
9	Wild	S+SQ	F	006C99ED0	6.3	74.0	1.54
9	Wild	S+SQ	F	007AFD7EC	7.5	79.0	1.52
9	Wild	S+SQ	F	00783A2F9	8.0	83.5	1.37
9	Wild	S+SQ	F	007AFDASC	7.3	78.0	1.54
9	Wild	S+SQ	F	007AC9C4C	7.5	77.0	1.64
9	Wild	S+SQ	F	007AC4687	6.5	76.0	1.48
9	Wild	S+SQ	F	007ABFD4C	7.2	77.0	1.58
9	Wild	S+SQ	M	007AFD59C	9.0	84.0	1.52
9	Wild	S+SQ	U	007B00A20	3.5	66.0	1.22
3	F1 SC	P	M	06B965A0	4.3	69.0	1.29
3	F1 SC	P	M	06C983D1	3.2	63.0	1.28
3	F1 SC	P	F	06B94832	5.5	70.0	1.60
3	F1 SC	P	M	06A2B435	5.4	66.0	1.88
3	F1 SC	P	M	07B0197F	5.0	63.0	2.00
3	F1 SC	P	M	06C97D13	5.0	69.0	1.52
3	F1 SC	P	M	06A2AFFF	5.3	70.0	1.55
3	F1 SC	P	M	06A27D02	4.8	66.0	1.67
3	F1 SC	P	F	06B98353	6.1	70.0	1.78
3	F1 SC	P	F	06A28AEA	5.5	70.0	1.60
3	F1 SC	P	F	06C98CE3	5.8	64.0	2.21
3	F1 SC	P	U	06C98829	5.5	70.0	1.60
3	F1 SC	P	F	07B0DCBD	6.9	69.5	2.06
3	F1 SC	P	M	06A287C7	4.6	66.0	1.60
3	F1 SC	P	F	06A2A90F	5.2	67.0	1.73
3	F1 SC	P	F	06B9ADAB	5.1	66.0	1.77
4	F1 SC	P	F	06C98B70	5.5	66.0	1.91
4	F1 SC	P	F	068933EE	4.0	70.0	1.17
4	F1 SC	P	F	06C99E74	4.0	64.0	1.53
4	F1 SC	P	F	07B00D45	5.4	66.0	1.88

4	F1 SC	P	M	008CEE3	4.0	67.0	1.33
4	F1 SC	P	F	06A29F2E	5.2	65.0	1.89
4	F1 SC	P	F	06A2B4FB	6.0	65.0	2.19
4	F1 SC	P	M	06B93BD7	3.5	57.0	1.89
4	F1 SC	P	M	06B94F39	6.2	70.0	1.81
4	F1 SC	P	M	06A2955E	5.1	64.0	1.95
4	F1 SC	P	U	06A29113	5.1	71.0	1.43
4	F1 SC	P	F	06A288D0	4.6	67.0	1.53
4	F1 SC	P	M	07B0DF9A	5.5	64.0	2.10
4	F1 SC	P	M	06B93A33	4.0	69.5	1.19
4	F1 SC	P	M	06C99808D	5.5	68.0	1.75

Table 3.2.6.2.5. Description and status of broodstock tanks at the completion of the experiment.

Tank	Origin	Feed type	Sex	Chip#	End weight (kg)	End fork length (CM)	K end
7	Wild	S+SQ	F	007839A45	13.5	95.0	1.58
7	Wild	S+SQ	F	00783B9A2	8.8	84.0	1.49
7	Wild	S+SQ	F	006C99E83	10.0	84.5	1.66
7	Wild	S+SQ	F	007AC3278	11.6	92.0	1.49
7	Wild	S+SQ	F	007AC885B	11.1	87.5	1.66
7	Wild	S+SQ	M	007B01A0A	12.0	91.0	1.59
7	Wild	S+SQ	F	007B02097	8.6	85.0	1.40
7	Wild	S+SQ	F	007AC8AE1	9.6	86.5	1.48
7	Wild	S+SQ	M	007ACOEA7	11.7	90.5	1.58
7	Wild	S+SQ	F	007AC4562	mort	mort	mort
9	Wild	S+SQ	F	006C99ED0	9.5	83.0	1.66
9	Wild	S+SQ	F	007AFD7EC	10.5	86.5	1.62
9	Wild	S+SQ	F	00783A2F9	10.5	90.0	1.44
9	Wild	S+SQ	F	007AFDASC	9.9	84.0	1.67
9	Wild	S+SQ	F	007AC9C4C	10.5	89.0	1.49
9	Wild	S+SQ	F	007AC4687	9.5	84.0	1.60
9	Wild	S+SQ	F	007ABFD4C	9.5	83.0	1.66
9	Wild	S+SQ	M	007AFD59C	12.4	89.0	1.76
9	Wild	S+SQ	U	007B00A20	mort	mort	mort
3	F1 SC	P	M	06B965A0	4.5	69.5	1.34
3	F1 SC	P	M	06C983D1	3.5	65.5	1.25
3	F1 SC	P	F	06B94832	6.8	72.5	1.77
3	F1 SC	P	M	06A2B435	6.2	68.5	1.91
3	F1 SC	P	M	07B0197F	6.0	65.5	2.14
3	F1 SC	P	M	06C97D13	6.1	72.5	1.60
3	F1 SC	P	M	06A2AFFF	6.2	72.5	1.61
3	F1 SC	P	M	06A27D02	6.0	68.5	1.87
3	F1 SC	P	F	06B98353	7.0	70.0	2.04
3	F1 SC	P	F	06A28AEA	5.5	72.0	1.47
3	F1 SC	P	F	06C98CE3	7.2	68.0	2.29
3	F1 SC	P	U	06C98829	6.4	70.0	1.87
3	F1 SC	P	F	07B0DCBD	7.8	71.0	2.18
3	F1 SC	P	M	06A287C7	5.8	70.0	1.69
3	F1 SC	P	F	06A2A90F	6.4	69.5	1.91
3	F1 SC	P	F	06B9ADAB	6.4	68.0	2.04
4	F1 SC	P	F	06C98B70	6.6	68.0	2.10
4	F1 SC	P	F	068933EE	4.7	72.0	1.26
4	F1 SC	P	F	06C99E74	4.3	66.0	1.48

4	F1 SC	P	F	07B00D45	6.4	68.5	1.99
4	F1 SC	P	M	008CEE3	4.5	69.5	1.34
4	F1 SC	P	F	06A29F2E	6.4	68.0	2.04
4	F1 SC	P	F	06A2B4FB	7.0	68.0	2.23
4	F1 SC	P	M	06B93BD7	4.0	60.5	1.81
4	F1 SC	P	M	06B94F39	7.0	71.0	1.96
4	F1 SC	P	M	06A2955E	6.5	66.0	2.26
4	F1 SC	P	U	06A29113	6.5	73.0	1.67
4	F1 SC	P	F	06A288D0	5.6	69.5	1.67
4	F1 SC	P	M	07B0DF9A	6.4	66.0	2.23
4	F1 SC	P	M	06B93A33	mort	mort	mort
4	F1 SC	P	M	06C99808D	mort	mort	mort

Table 3.2.6.2.6. Feed intake, biomass gain and biological food conversion ratio of broodstock (tank basis).

		As-fed basis (kg)				Dry basis (kg)	Tank basis (kg)	Tank basis
Tank	Origin	Pellet	Squid	Sardines	Feed sum	Feed sum	Biomass gain	FCR
7	Wild	-	95.5	126.6	222.1	55.8	23.2	3.6
9	Wild	-	103.1	112.6	215.7	53.6	26.5	3.3
3	F1 SC	54.6	-	-	54.6	51.8	14.5	2.4
4	F1 SC	42.1	-	-	42.1	40.0	12.1	2.0

Dry matter feed input based on dry matter content of Huon 9 mm feed, Sardines and Squid being 95%, 28% and 22%, respectively. Biological FCR accounts for mortality in tanks 4, 7 and 9.

Table 3.2.6.2.7. Weight based and fork-length based specific growth rate (SGR_{Wt}; SGR_{FL}) and Fulton's condition factor *K* of YTK broodstock (mean + SEM; tank basis).

Tank	Origin	Feed type	SGR_{Wt}	SGR_{FL}	Condition factor *K* @ stocking*	Condition factor *K* @ harvest*
7	Wild	Natural	0.2009^{b}	0.0621^{b}	1.489	1.546
9	Wild	Natural	0.2711^{c}	0.0750^{b}	1.490	1.613
3	F1 SC	Pellet	0.1295^{a}	0.0264^{a}	1.697	1.810
4	F1 SC	Pellet	0.1349^{a}	0.0285^{a}	1.702	1.847
F-value			*21.29*	*27.52*	*2.91*	*3.37*
P-value			*<0.0001*	*<0.0001*	*0.0446*	*0.027*

ANOVA results based on using individually tagged fish in each tank as replicate.
*Although ANOVA was significant the multiple comparisons procedure could not separate means at the 95% CI.

Table 3.2.6.2.8. One-way PERMANOVA: Pairwise test between A) SA wild charter YTK and NSW broodstock trial samples (wild-caught, progeny and tank water) and B) NSW broodstock trial samples (wild-caught, progeny and tank water) before and after attempted spawning.

Sample type	***P***	**Significant?**
A) Wild charter, wild-caught, progeny and tank water		
wild charter, wild-caught	**0.0001**	Yes
wild charter, progeny	**0.0001**	Yes
wild charter, tank water	**0.0003**	Yes
wild-caught, progeny	**0.0001**	Yes
wild-caught, tank water	**0.0001**	Yes
progeny, tank water	**0.0001**	Yes
B) Before and after attempted spawning		
wild-caught before, wild-caught after	**0.0001**	Yes
progeny before, progeny after	**0.0001**	Yes
wild-caught before, progeny before	**0.0001**	Yes
wild-caught before, progeny after	**0.0001**	Yes
wild-caught after, progeny before	**0.0001**	Yes
wild-caught after, progeny after	**0.0001**	Yes
TW before, TW after	0.1978	No

Abbreviations: TW, tank water.
[1]Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.2.6.2.9. One-way PERMANOVA: Pairwise test between NSW broodstock trial samples before and after attempted spawning for A) wild-caught YTK in tanks 7 and 9 and B) progeny YTK in tanks 3 and 4.[1]

Sample type	***P***	**Significant?**
A) wild-caught		
before tank 7, before tank 9	**0.0001**	Yes
before tank 7, after tank 7	**0.0001**	Yes
before tank 9, after tank 9	**0.0002**	Yes
after tank 7, after tank 9	**0.0002**	Yes
B) progeny		
before tank 3, before tank 4	**0.0004**	Yes
before tank 3, after tank 3	**0.0001**	Yes
before tank 4, after tank 4	**0.0001**	Yes
after tank 3, after tank 4	**0.0463**	Yes

[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.2.6.2.10. ANOVA results for diversity indices comparing NSW broodstock trial samples before and after attempted spawning for A) wild-caught YTK in tanks 7 and 9 and B) progeny YTK in tanks 3 and 4.[1]

Diversity measure	ANOVA summary[2]	Tukey's posthoc test[3]	Adjusted P-value
A) Wild-caught			
Species richness (S)	F = 17.99 ***P* < 0.0001**	W before T7 vs W after T7 W after T7 vs W before T9 W after T7 vs W after T9	**<0.0001** **0.0012** **<0.0001**
Pielou's evenness (J')	F = 4.104 ***P* = 0.0152**	W before T7 vs W after T9	**0.0373**
Shannon's diversity (H')	F = 3.586 *P* = 0.0522		
Simpson's diversity (1-λ)	F = 2.825 *P* = 0.0561		
Delta+ (Δ+)	F = 4.378 *P* = 0.0500		
Lambda+ (λ+)	F = 32.18 ***P* < 0.0001**	W before T7 vs W after T7 W before T7 vs W after T9 W after T7 vs W before T9 W before T9 vs W after T9	**<0.0001** **<0.0001** **<0.0001** **0.0004**
B) Progeny			
Species richness (S)	F = 1.757 *P* = 0.1672		
Pielou's evenness (J')	F = 6.546 ***P* = 0.0008**	P before T3 vs P after T3 P after T3 vs P before T4	**0.0016** **0.0057**
Shannon's diversity (H')	F = 5.895 ***P* = 0.0016**	P before T3 vs P after T3 P after T3 vs P before T4	**0.0024** **0.0094**
Simpson's diversity (1-λ)	F = 4.341 ***P* = 0.0085**	P before T3 vs P after T3 P after T3 vs P before T4	**0.0142** **0.0197**
Delta+ (Δ+)	F = 22.25 ***P* < 0.0001**	P before T3 vs P after T3 P before T3 vs P after T4 P after T3 vs P before T4 P before T4 vs P after T4	**<0.0001** **0.0003** **<0.0001** **0.0025**
Lambda+ (λ+)	F = 23.65 ***P* < 0.0001**	P before T3 vs P after T3 P before T3 vs P after T4 P after T3 vs P before T4 P before T4 vs P after T4	**<0.0001** **<0.0001** **<0.0001** **<0.0001**

Abbreviations: P, progeny; T, tank; W, wild-caught.

[1] Significant difference denoted by $P < 0.05$, bolded if significant.

[2] Where a significant difference ($P < 0.05$) is observed then the Tukey's pairwise test was performed.

[3] Only significant pairwise comparisons are shown.

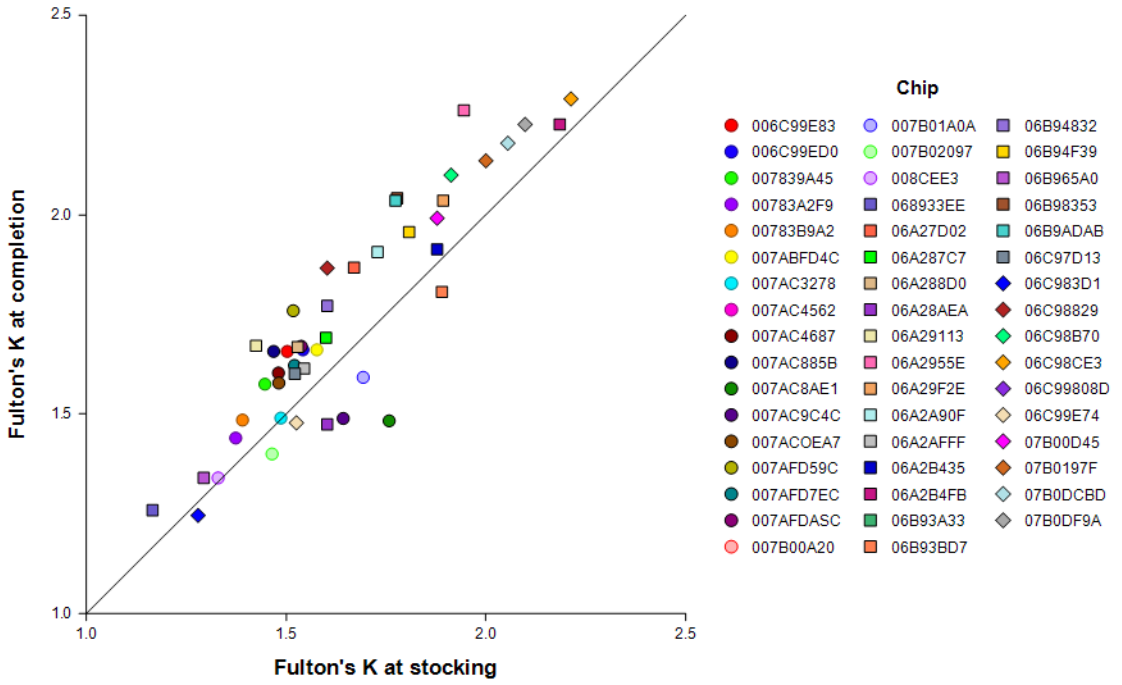


Figure 3.2.6.2.1. Change in Fulton's condition factor *K* of individual Yellowtail Kingfish broodstock.

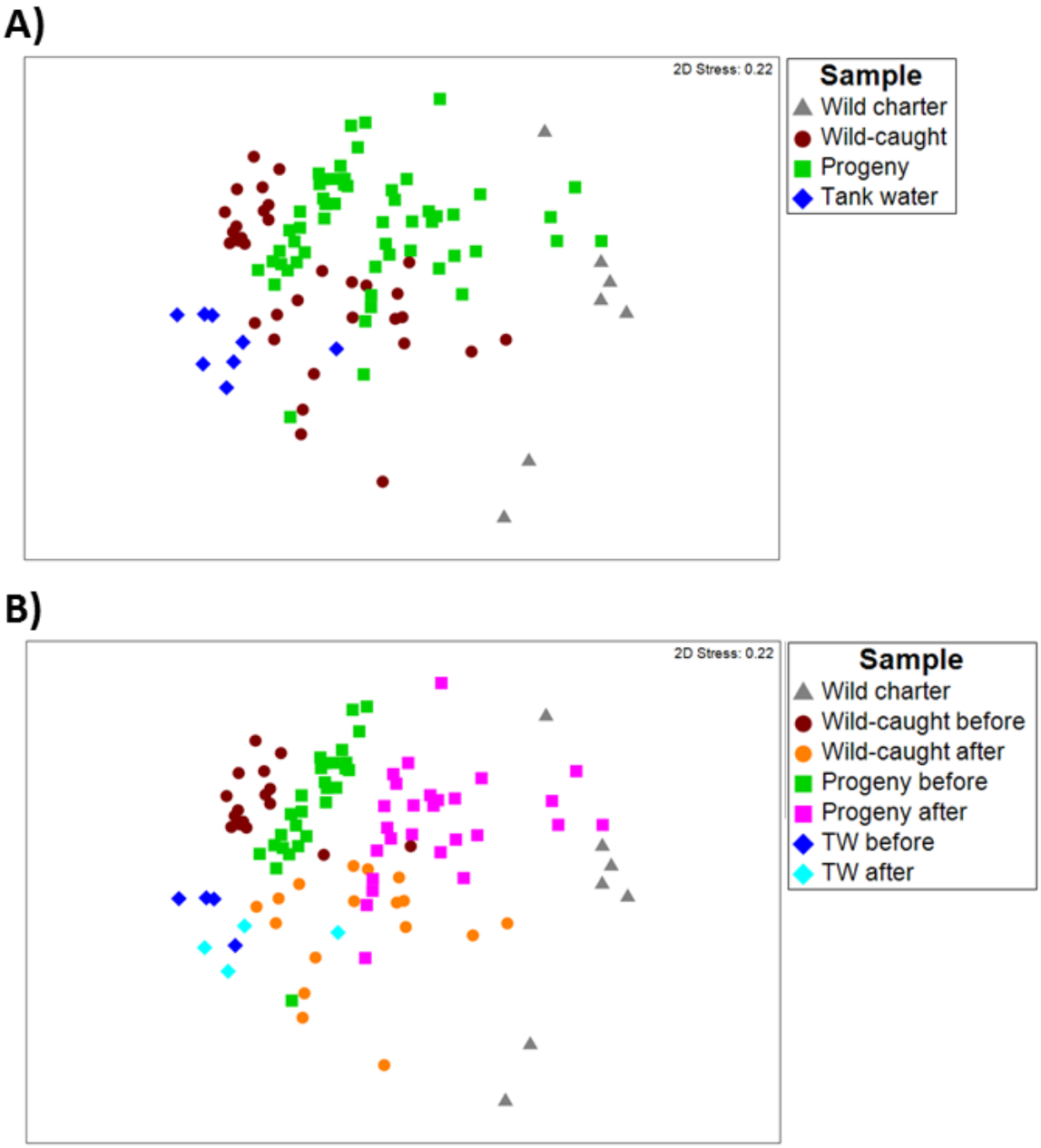


Figure 3.2.6.2.2. Difference between the global community structure of all 103 samples comparing A) wild charter SA YTK with NSW broodstock trial samples (wild-caught YTK, progeny YTK and tank water) and B) as above with the NSW broodstock trial samples split into before and after attempted spawning groups as analysed by non-metric multidimensional scaling (nMDS).[1,2]

Abbreviations: NSW, New South Wales; SA, South Australia; TW, tank water.
[1] Hindgut scrapings from 7 wild charter fish collected in SA waters and rectal swab samples from 88 NSW broodstock YTK (33 wild caught and 55 progeny) collected at two time points before and after attempted spawning. Eight environmental tank water samples collected from each of the tanks containing the wild-caught and progeny YTK at both time points before and after attempted spawning.
[2] Before samples collected in April 2018, after samples collected four months later in August 2018.

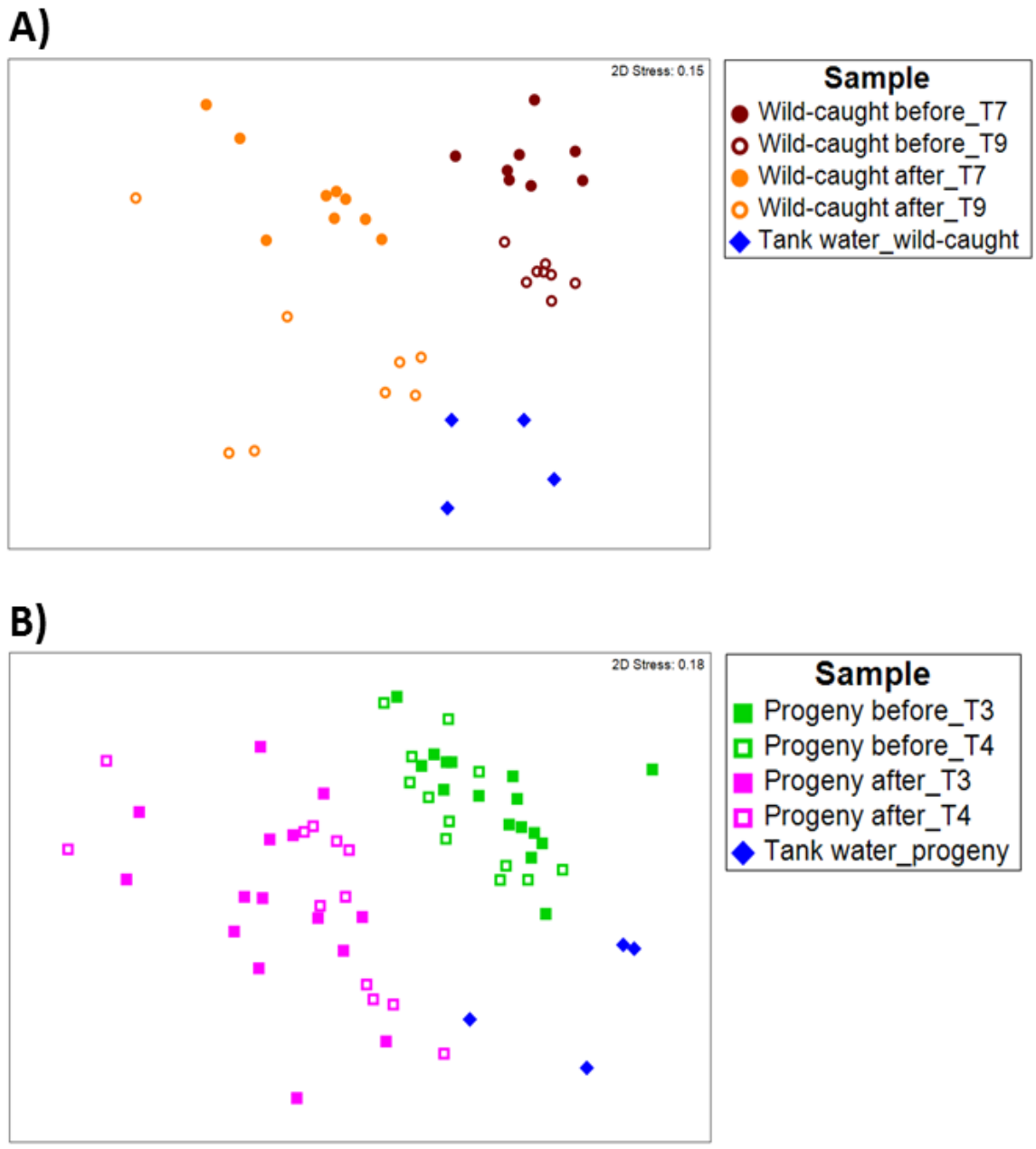


Figure 3.2.6.2.3. Difference between the global community structure of NSW broodstock trial samples before and after attempted spawning for A) wild-caught YTK and B) progeny YTK as analysed by non-metric multidimensional scaling (nMDS).[1]

Abbreviations: T, tank.
[1] Before samples collected in April 2018, after samples collected four months later in August 2018.

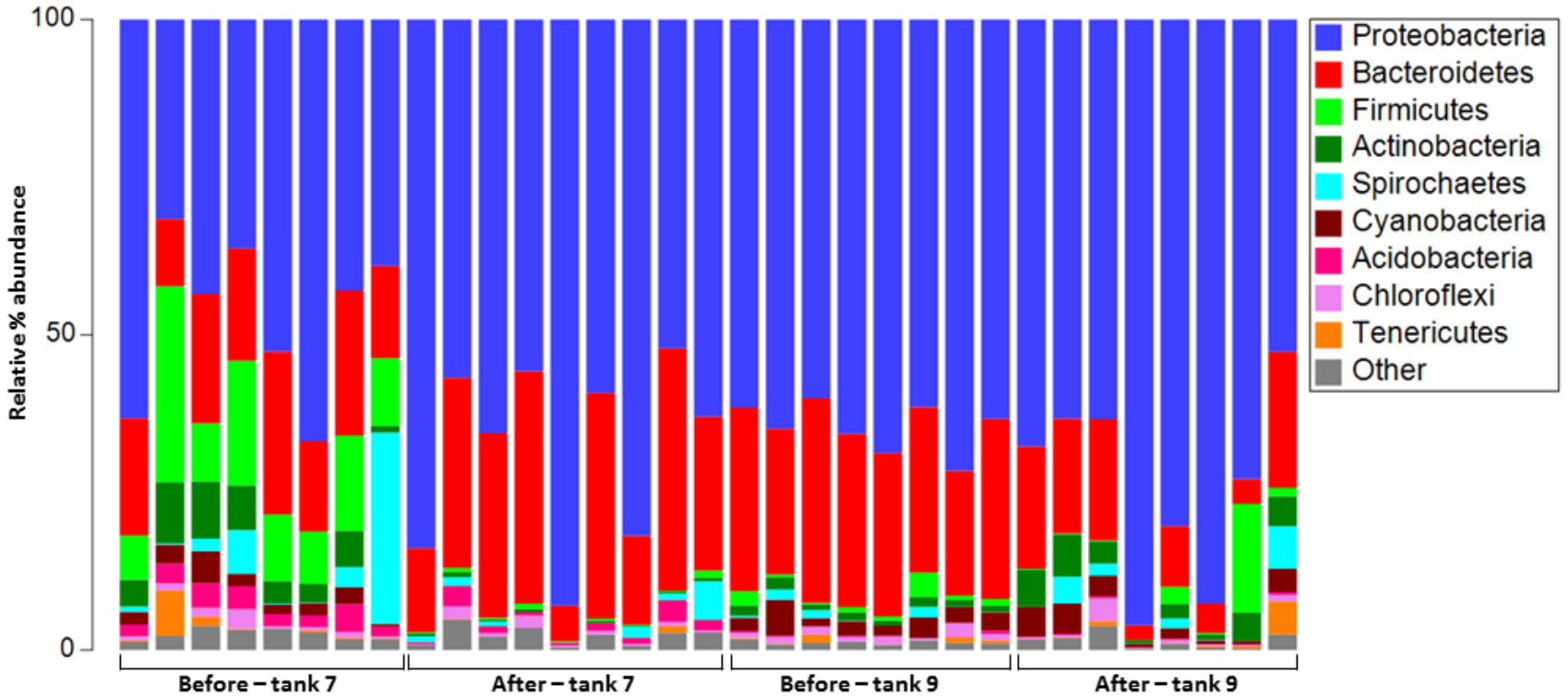


Figure 3.2.6.2.4. Relative percent abundance of bacterial phyla associated with the NSW broodstock trial wild-caught YTK in tanks 7 and 9 sampled before and after attempted spawning.[1,2]

[1] Before samples collected in April 2018, after samples collected four months later in August 2018.
[2] All fish fed a natural diet of Squid and Sardines for the duration of the trial.

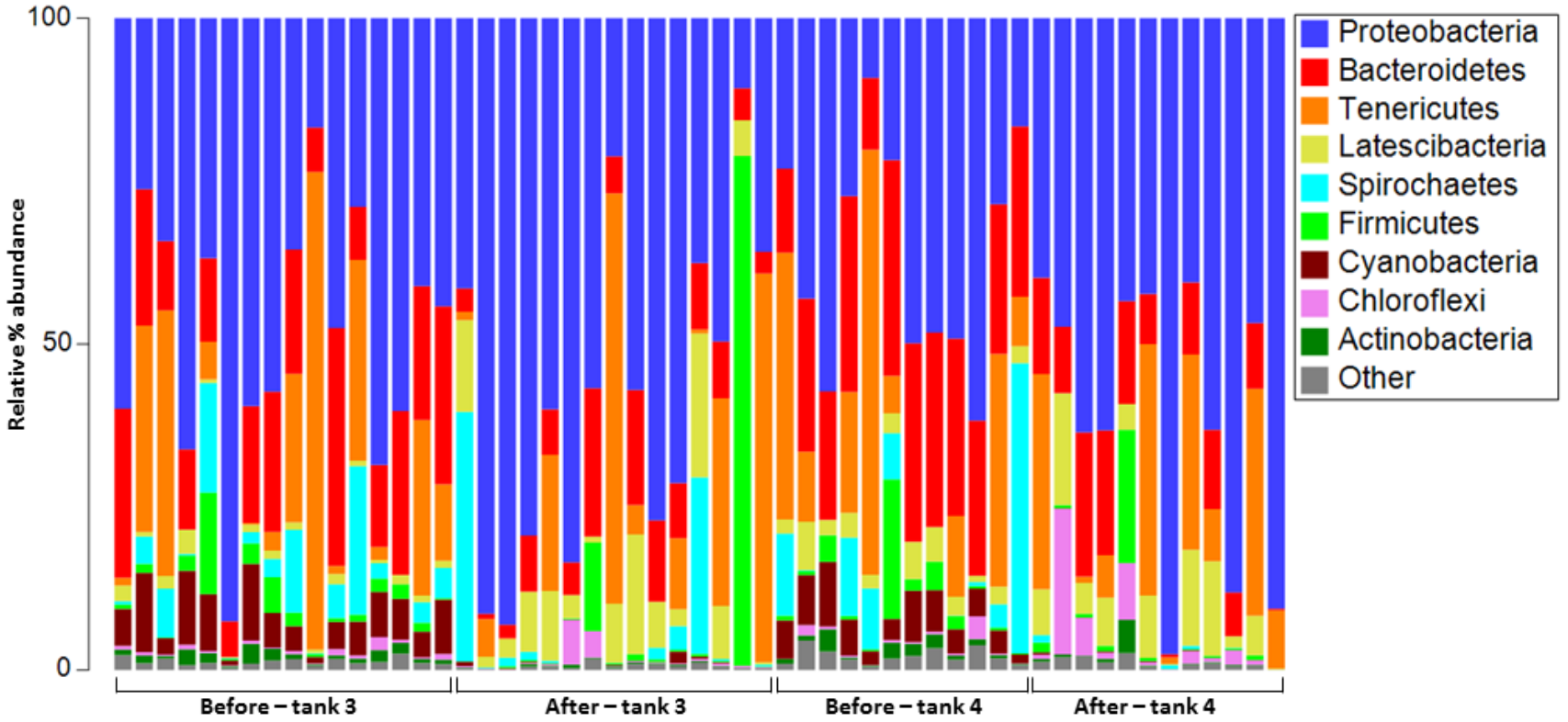


Figure 3.2.6.2.5. Relative percent abundance of bacterial phyla associated with the NSW broodstock trial progeny YTK in tanks 3 and 4 sampled before and after attempted spawning.[1,2]

[1] Before samples collected in April 2018, after samples collected four months later in August 2018.
[2] All fish fed a formulated fed (Huon pellet) for the duration of the trial.

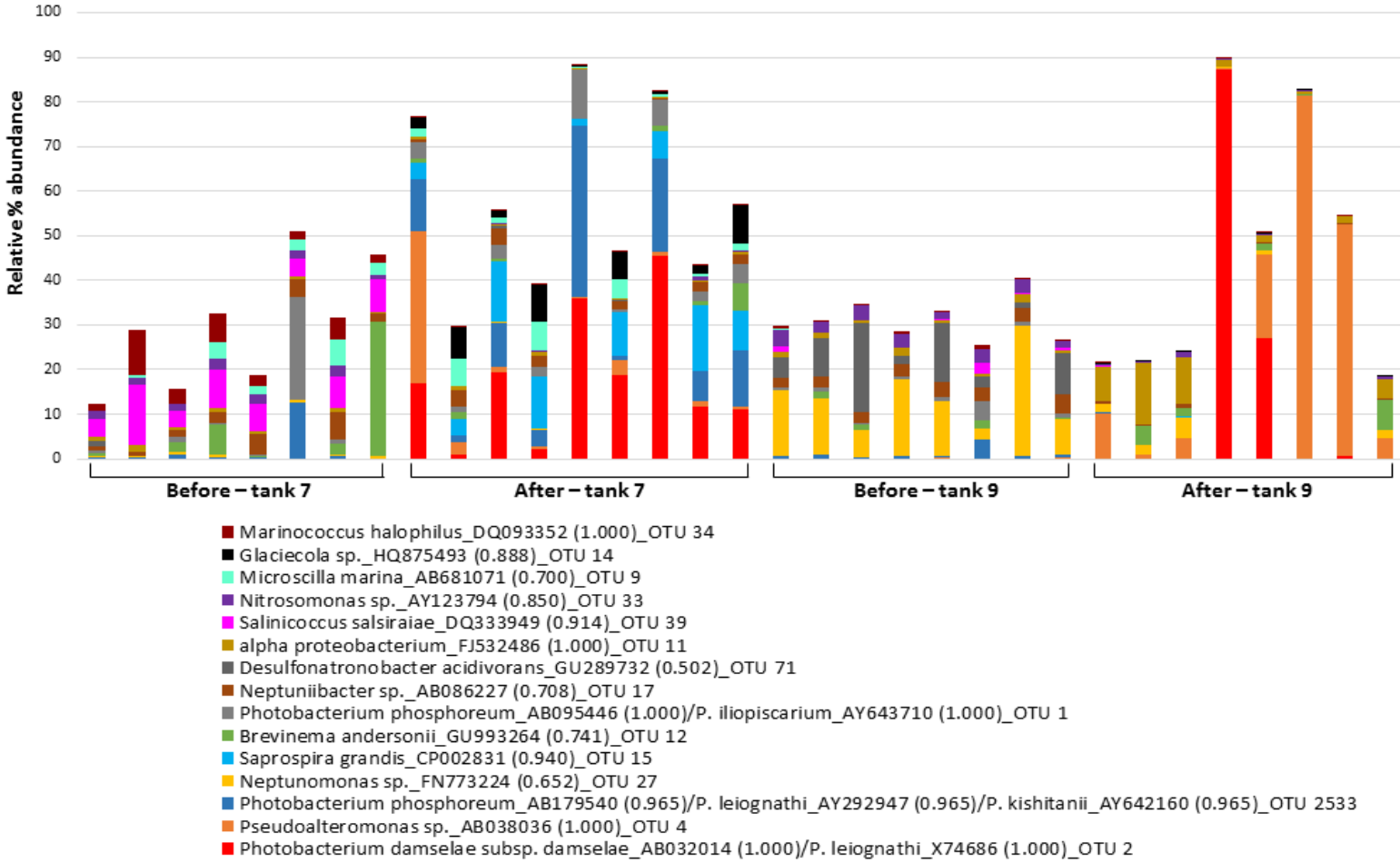


Figure 3.2.6.2.6. Relative percent abundance of the 15 most abundant bacterial OTUs from the NSW broodstock trial wild-caught YTK in tanks 7 and 9 sampled before and after attempted spawning.[1,2]

[1] Before samples collected in April 2018, after samples collected four months later in August 2018.

[2] All fish fed a natural diet of Squid and Sardines for the duration of the trial.

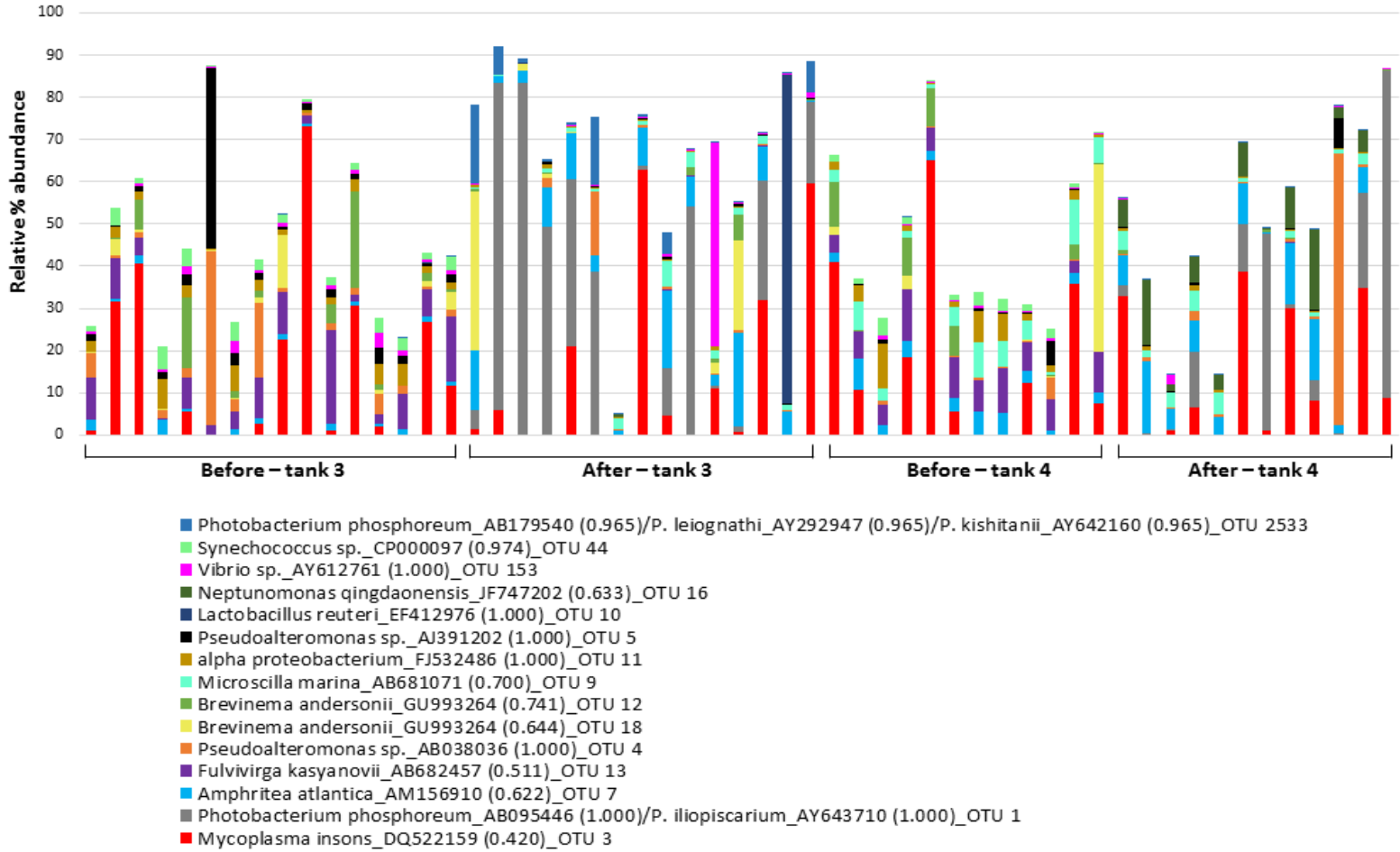


Figure 3.2.6.2.7. Relative percent abundance of the 15 most abundant bacterial OTUs from the NSW broodstock trial progeny YTK in tanks 3 and 4 sampled before and after attempted spawning.[1,2]

[1] Before samples collected in April 2018, after samples collected four months later in August 2018.

[2] All fish fed a formulated fed (Huon pellet) for the duration of the trial.

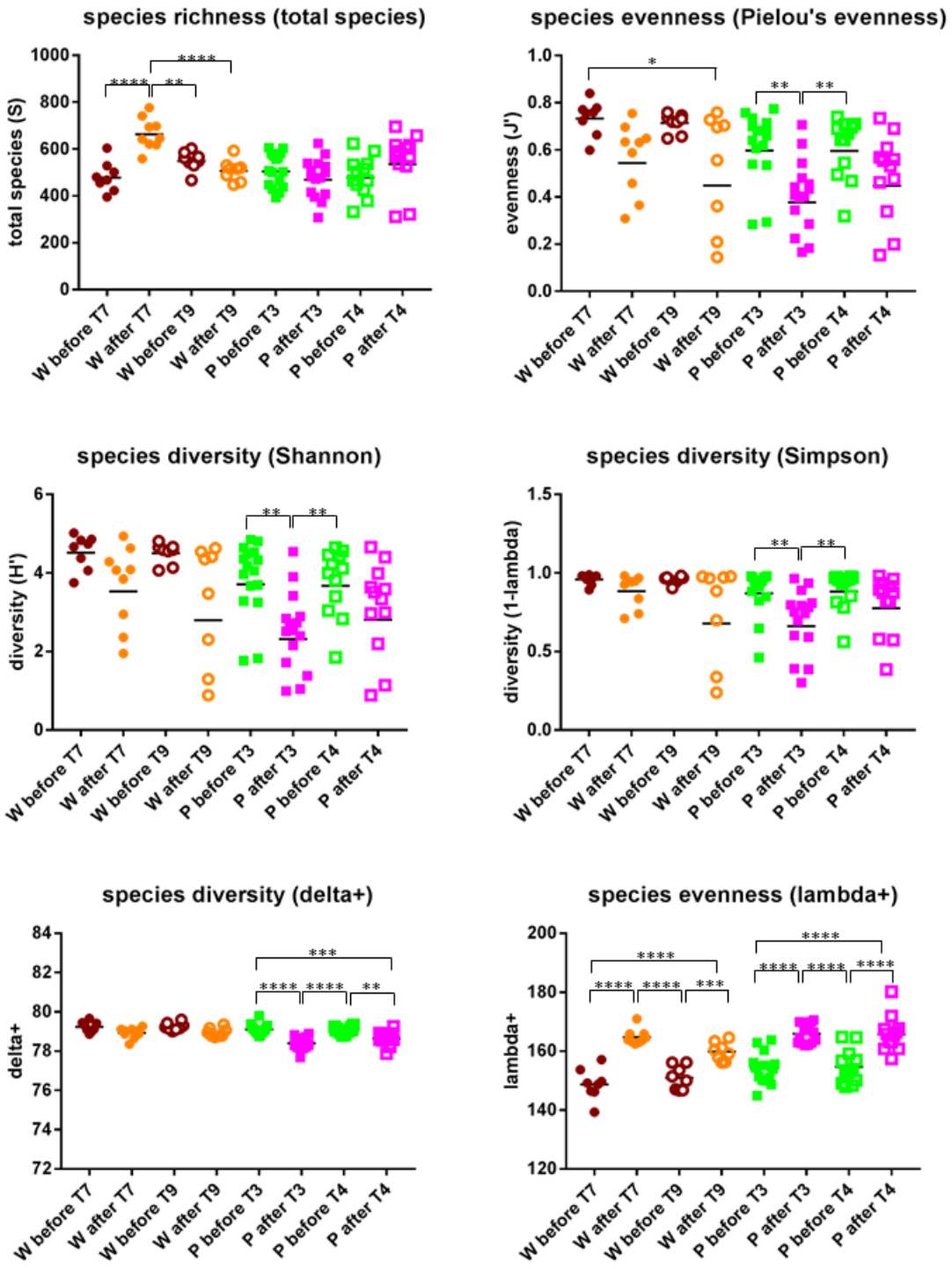


Figure 3.2.6.2.8. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for NSW broodstock trial sample groups.[1,2]

Abbreviations: P, progeny; T, tank; W, wild-caught.
[1] Mean values are plotted for each of the groups of interest – wild-caught YTK sampled before and after attempted spawning in tanks 7 and 9 and progeny YTK sampled before and after attempted spawning in tanks 3 and 4.
[2] Before samples collected in April 2018, after samples collected four months later in August 2018.

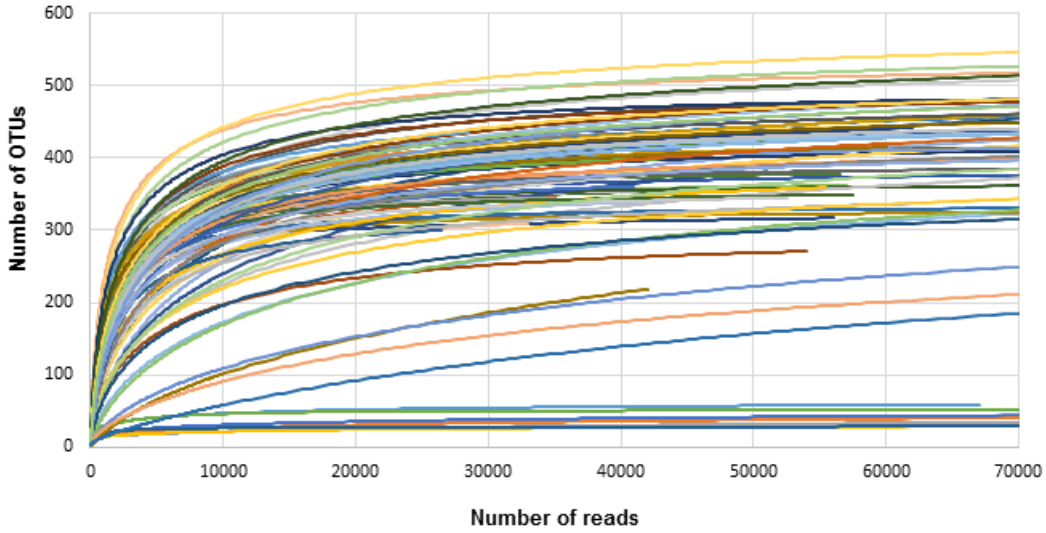


Figure 3.2.6.2.9. Rarefaction curves portraying the number of resolved OTUs against sequencing depth of each sample from the NSW broodstock experimental component.

3.2.6.3. Manuscript - Impact of high quality booster feed on performance and fecundity in Yellowtail Kingfish broodstock (Seriola lalandi).

Mark Booth [a], Luke Cheviot [a], Brendan Findlay [a] and D. Stewart Fielder [a],

[a] New South Wales Department of Primary Industries, Port Stephens Fisheries Institute, Taylors Beach, NSW, 2316, Australia

This manuscript may be referenced as: Booth, M., Cheviot, L., Findlay, B., and Fielder, S. (2019). 3.2.6.3. Manuscript - Impact of high quality booster feed on performance and fecundity in Yellowtail Kingfish broodstock (*Seriola lalandi*) (Output 3b). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.506-517.

Abstract

This is the third qualitative broodstock experiment done at the New South Wales Department of Primary Industries (NSW DPI) Port Stephens Fisheries Institute (PSFI) Marine Fish Hatchery to investigate how different feeding strategies affect the growth and fecundity of Yellowtail Kingfish (*Seriola lalandi*; YTK). The 111 day trial examined differences in the growth rate, feed conversion ratio (FCR) and fecundity of broodstock brought about by the use of different feed types (i.e. a combination of dry commercial diet (Huon 9 mm pellet diameter) + booster feed (Breed-M) versus a PSFI "best-practice" regime of Australian Sardines (*Sardinops sagax*; Sardines) and Atlantic Squid (*Doryteuthis pealeii*; Squid). Two tanks of broodstock (local caught wild origin) were maintained on the PSFI best-practice regime and two tanks of broodstock (F1 of wild domesticated stock), were fed the commercial preparations. The fish allocated to the commercial feed regime were fed the Huon feed for all but the last four weeks of a three month breeding cycle and then fed Breed-M for a month leading up to induction. All tanks were physically independent, but shared the same influent estuarine water source. Fish were fed three times weekly to apparent satiation at approximately 13:00 h on Mondays, Wednesdays and Fridays. Fish were not fed during spawning. None of the wild or F1 broodstock spawned in this experiment following a normal thermal-photoperiod manipulation (rising from 16 °C to 22 °C in 24-48 h). The reasons broodstock did not spawn after thermal-photoperiod manipulation are unclear, but they could relate to the sexual naivety of the wild caught and F1 stock, or the additional stress placed on stock at the beginning of the experiment as a result of weighing and handling procedures. The latter is unlikely given more than 111 days had elapsed since the fish were last handled. As the historical feeding regimes and feed sources for the groups of YTK fed Sardines and Squid was unchanged it also seems unlikely these factors were responsible for the spawning outcomes in that group. All groups of broodstock in this experiment showed no reluctance to consume their allocated food sources, at least until F1 broodstock were switched to Breed-M. Therefore it seems unlikely the nutrient and energy intake of fish was inadequate; especially that of the broodstock fed Sardine and Squid. This outcome means the impact of the pelletised feeding regime could not be assessed against the best-practice regime with respect to investigations of fecundity and egg quality. There were some minor impacts of feeding regime on growth rate. Based on statistical analysis using individual fish as replicates, the SGR_{Wt} of broodstock trended towards being significantly higher in broodstock fed under the best practice regime as opposed to the dry pelletised feed regime. Further long term trials will be necessary to evaluate the efficacy of feeding dry pelletised rations and booster feeds to broodstock at PSFI.

Introduction

Research on the nutritional requirements of most broodstock is lacking. For this reason the National Research Council (NRC) (2011) has recently advocated for more research on broodstock nutrition, with an emphasis on specialised species-specific diets (Migaud et al., 2013). Marine broodstock has traditionally been fed natural foods which are thought to approximate the type of prey the species would normally consume in the wild. It is commonplace to use frozen squid and oily fish such as Australian Sardines (*Sardinops sagax*; Sardines) to feed Yellowtail Kingfish (*Seriola lalandi*; YTK) broodstock (Kolkovski, 2005). However, there are good reasons for wanting to use manufactured feeds in hatcheries such as reducing biosecurity risks to broodstock and offspring posed by using natural food stuffs which may act as vectors for the introduction of diseases (Watanabe and Vassallo-Agius, 2003). Use of natural feeds in modern recirculating aquaculture systems can also lead to deterioration in water quality resulting in sub-standard environments and additional labour costs (Morais et al., 2014). The use of manufactured feeds also allows broader manipulation of nutrient quality and density via formulation as well as effective inclusion of feed supplements (e.g. vitamins, minerals, astaxanthin and attractants). Operating fish hatcheries is expensive and the cost of feeding can account for a large proportion of the operating budget. For this reason alone there may be good economic reasons to use commercial feeds rather than rely on natural foods.

A previous long term broodstock experiment at New South Wales Department of Primary Industries (NSW DPI) Port Stephens Fisheries Institute (PSFI) found that fecundity in wild and mature progeny of wild YTK was higher using natural food sources (i.e. Sardines and Squid) rather than proprietary commercial fish pellets or specialised broodstock preparations (see Manuscript 3.2.6.1). As a result of that experiment, NSW DPI broodstock have been sustained on natural food sources using the 'best-practice' feeding regime espoused by Fielder and Heasman (2011). Nonetheless, it was recognised at the time of that experiment that future broodstock trials should consider ways to improve the consumption of commercial feeds in YTK as their use would improve hatchery operations as well as reduce the biosecurity risks associated with using natural feeds in the hatchery.

The current broodstock experiment at PSFI is qualitative in design and aims to determine how the performance and reproductive output of broodstock YTK is affected by feeding a commercial diet (9 mm pellet diameter Huon Skretting Select) followed by longer exposure to feeding on a booster diet (Breed-M; INVE Aquaculture) versus the PSFI best practice feeding regime. The trial was conducted immediately after a former broodstock experiment at PSFI that investigated the impact of diet selection on fecundity and microbiome of broodstock (see Manuscript 3.2.6.2). The same groups of broodstock and tank systems were employed in this study to aid in the continuity of the research.

Methods

This study is being performed under the NSW DPI Fisheries Animal Care and Ethics (ACEC) Research Authority known as 'Aquaculture Nutrition ACEC 93/5–Port Stephens'. Care, husbandry and termination of fish were carried out according to methods outlined in 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' (ACEC, 2015). Broodstock were always sedated using an appropriate amount of AQUI-S before any handling procedures to prevent damage to the animals or harm to personnel.

Experimental feeds

The commercial feeds chosen for this trial were based on earlier research (Manuscripts 3.2.6.1 and 3.2.6.2). A high quality proprietary broodstock preparation (Fish Breed-M) was obtained from INVE Aquaculture (www.inveaquaculture.com). The commercial manufactured pelletised YTK feed was obtained from Huon Aquaculture. Fish Breed-M is claimed to incorporate highly refined and digestible raw materials. The powder is usually mixed with water in order to make a stable

moist-paste or moist-sausage with around 50% moisture (this preparation was trialed in Manuscripts 3.2.6.1).

Australian Sardines (*Sardinops sagax*; Sardines) and Atlantic Squid (*Doryteuthis pealeii*; Squid) were purchased frozen (Tweed Bait Pty Ltd; www.tweedbait.com.au) and thawed prior to each daily feeding event. A roughly 50:50 mix of Sardines and Squid was prepared each day for each tank. The commercial manufactured pelletised diet (Huon 9 mm pellet diameter) was used as is. The Breed-M powder preparation was mixed with an appropriate amount of tap-water, formed into a moist mash / dough and then extruded through a meat mincer fitted with a 15 mm aperture. The sausage was cut into discs approximately 10-15 mm wide and dried in a convection drier until moisture content was < 10%. Both Huon and Breed-M feeds were stored frozen until used. The chemical analysis of different feed sources is presented in Table 3.2.6.3.1 and Table 3.2.6.3.2.

Broodstock housing and feeding regimes

Two groups of broodstock were used in this experiment. A group of wild fish captured from the Port Stephens local area in April 2017 and F1 broodstock collected from the NSW DPI / Huon Marine Aquaculture Research (MARL) and transported back to PSFI with the assistance of Huon staff and vessels. The F1 broodstock were the progeny of an older group of wild-captured, domesticated broodstock held at PSFI (see Manuscript 3.2.6.2 for background on origin of broodstock).

Although the wild and F1 groups of fish in this experiment have different origins, from a practical viewpoint, they are all likely to be related because the F1 fish are all progeny of locally caught wild YTK from Port Stephens being held at the PSFI Hatchery. The F1 group may be more similar genetically, but nonetheless they come from the same geographic lineage.

There were 28 female and 16 male broodstock identified at the start of the experiment, however the sex of two fish could not be ascertained using non-destructive methods. The number of fish and the sex ratio within tanks was different. Tank 7 (wild origin) contained 7 female and 2 male fish; Tank 9 (wild origin) contained 7 female, 1 male fish; Tank 3 (F1) contained 7 female, 8 male and one unidentified animal; Tank 4 (F1) contained 7 female, 5 male and one unidentified animal.

Groups of broodstock were reared in four independent 25 kL recirculating systems (RAS). Each RAS consisted of a 20 kL rearing tank, a 200 L sump for collecting eggs, a rotating biological contactor (biofilter) and a micro-bead particle filter. Water temperature is controlled using a reverse cycle refrigeration unit. Fish were held at approximately 16-17 °C to prevent spawning. Tanks were fitted with lids that contained a fluorescent light to control photoperiod. Water was constantly circulated through the RAS via a 2.1 kW centrifugal pump (300-400 L min^{-1}). Each RAS was fed with high quality, filtered estuarine water (< 15 µm) drawn from the intersection of Fenningham's Island and Tilligerry Creeks (adjacent PSFI). Rearing tanks were siphoned weekly to remove build-up of organic material. Broodstock at PSFI were generally managed under the best-practice regimes advocated by Fielder and Heasman (2011). All fish used in the experiment were fitted with individual electronic tags, sexed, weighed and measured (fork length) prior to being distributed to rearing tanks.

Prior to commencing the experiment, the wild origin fish were reared exclusively on a ration of Sardines and Squid and F1 animals had been reared exclusively on a commercial manufactured pelletised feed (Huon 9 mm pellet diameter; Skretting Select) in the PSFI Hatchery for several months (see Manuscript 3.2.6.2). In this experiment wild origin fish were maintained on their regular ration of Sardines and Squid. The FI broodstock were continued on the dry pelletised Huon 9 mm pellet diameter diet for all but the last four weeks of a three month breeding cycle and then fed the "Breed-M" in the month leading up to photo-thermal induction. The justifications for maintaining 'wild' fish on the PSFI best-practice regime and F1 fish on the Huon commercial pellet + Breed-M were varied, but in the first instance it was to ensure that broodstock continued to eat a satisfactory amount of food at each meal. Evidence from the last broodstock experiment (see

Manuscript 3.2.6.1) indicated fish took an inordinate amount of time to accept changes to their diet, if at all. Secondly, the outcomes of this research should not be greatly affected by the fact that experimental animals are of wild or hatchery origin because it is highly probable all the fish used in this experiment are related.

All broodstock were fed to apparent satiation once per day at approximately 13:00 h (Monday, Wednesday and Friday). This feeding frequency is in keeping with current hatchery practices at PSFI and the same as that used in the previous broodstock experiments (Manuscripts 3.2.6.1 and 3.2.6.2). Feed input was recorded on a daily basis.

Fish spawning and egg collection

The experiment was stocked on the 22-23 August 2018 and concluded on the 10-12 December 2018 (≈111 days). Broodstock were conditioned and induced to spawn in the last week of the experiment using photoperiod-temperature cues (i.e. increasing the water temperature in each RAS from 16 °C to 22 °C within 24-48 h). Spawning procedures and the collection, preparation and enumeration of eggs and measures of egg quality followed protocols described by Fielder and Heasman (2011).

Major physical response variables

The following performance variables were used to assess the response of YTK to different feed treatments;

- Initial weight of fish (g) = individual weight of fish at stocking
- Final weight of fish (g) = individual weight of fish at conclusion
- Specific growth rate (SGR_{Wt}) (% d^{-1}) = [Ln(final weight) – Ln(initial weight)]/days × 100
- Specific growth rate (SGR_{FL}) (% d^{-1}) = [Ln(final length) – Ln(initial length)]/days × 100
- Condition factor K = [individual weight of fish (g) / fork length of fish $(mm)^3$] × 10^5
- Food conversion ratio (FCR) = dry basis feed intake per tank (g) / wet weight gain per tank (g)

Chemical analysis of samples

The natural and commercial food sources were analysed for dry matter, crude protein, gross energy (bomb calorimetry), lipid (FAME) and ash content, respectively. Fertilized eggs were analysed to determine FAME composition. Chemical analysis was done by CSIRO (Agriculture and Food, St Lucia, QLD 4067, Australia) or the NSW DPI Feed Quality Service Laboratory (Wagga Wagga, NSW, 2650, Australia) (as per Manuscripts 2.3.6.1 and 2.3.6.2).

General statistical procedures

Biometric treatment data was compared using one way ANOVA. Individually tagged fish were used as replicates to assess differences in growth rates among different tanks. Alpha for ANOVA and the post-hoc multiple comparison procedure (Tukey-Kramer Test) was set at 0.05. Data subjected to ANOVA was statistically analysed using NCSS-8.0.23 after assumptions related to normality and sample variance were satisfied (Hintze, 2012). Where data was heterogeneous a Welch's test of means allowing for unequal variances was used.

Results

Growth and condition factor

Fish remained healthy throughout the experiment. Feed consumption was regular in tanks offered fresh feeds. Feed consumption was steady in fish reared on the Huon feed, but declined significantly when these fish were changed to the dry Breed-M pellet. Fish gained an average of 421 g, 288 g, 1178 g and 1175 g, respectively, in Tank 3, Tank 4, Tank 7 and Tank 9. The status of individual broodstock at the beginning and end of the experiment is presented in Table 3.2.6.3.3. Individuals can be identified via their unique electronic alphanumeric identification code.

There was a significant difference in the average condition factor of fish in different tanks at the start of the experiment ($P < 0.05$), but the Tukey's multiple comparison test could not separate the treatment means at the 95% CI; Nonetheless, F1 broodstock tanks had a higher condition score than wild origin broodstock tanks. At the end of the experiment there were no statistical difference in the SGR_{FL} or SGR_{Wt} of broodstock in different tanks (both $P > 0.05$; Welch's test). There was also no difference in the average condition factor of fish in different tanks ($P > 0.05$; ANOVA) (Table 3.2.6.3.4). The condition factor of broodstock in Tanks 3, 7 and 9 remained similar or increased slightly during the experiment, whereas the condition factor of fish in Tank 4 declined.

Pooling data by diet type (i.e. raw feeds vs pelletised feeds) indicated there was no effect of diet regime on SGR_{FL} ($n = 17$, raw = 0.022 d^{-1}; $n = 29$, pellet = 0.020% d^{-1}; $P > 0.05$) or SGR_{Wt} ($n = 17$, raw = 0.089 d^{-1}; $n = 29$, pellet = 0.052% d^{-1}; $P > 0.05$); however, the test on SGR_{Wt} approached the level of significance ($P = 0.052$; Welch's test). Pooling data by diet type also indicated there was a significant difference in the average condition factor of fish at harvest (both ANOVA and Welsch's test $P < 0.05$). The average condition factor of broodstock reared on the pelletised regime was higher (1.80; $n = 29$) than the condition factor of fish reared on the raw feed regime (1.63; $n = 17$). This may simply reflect the different morphology of F1 and wild origin animals as F1 animals from this hatchery exhibit a compressed fusiform shape (heavier per unit length) whereas wild origin fish tend to be relatively longer, but lighter per unit length.

The dry basis feed intake and biomass gain of each broodstock tank is presented in Table 3.2.6.3.5. Biomass gain was higher and FCR was lower (based on dry matter feed intake) in broodstock tanks fed on the raw feed regime (i.e. Tank 7 and Tank 9) compared to broodstock fed the pelletised feed regime (Tank 3 and Tank 4). FCR recorded in Tank 7 and 9 in this experiment (i.e. 2.19 and 23.0, respectively), was slightly better than the FCR recorded on the same broodstock tanks held on the same feeding regime in Manuscript 2.3.6.2 (i.e. 3.6 and 3.3, respectively). FCR of fish held exclusively on Huon 9 mm diameter pellets in Manuscript 2.3.6.2 was much lower (i.e. 2.0-2.4) than the FCR of the same tanks in this experiment (i.e. 4.63 to 6.01), indicating broodstock in these tanks did not agree with the sudden change in their diet 4 weeks from induction. This response is evident by the large decrease in feed intake of Breed-M in Table 3.2.6.3.4 and reflects the response of broodstock to sudden changes in diet discussed in Manuscript 2.3.6.1. In terms of the relative contribution of each feed source to each tank; Breed-M contributed 30.6% and 20.9% of total intake in Tank 3 and Tank 4, respectively. Squid contributed 43.0% and 45.2% of total intake, respectively (Table 3.2.6.3.5).

Spawning

Both wild origin and F1 origin broodstock failed to spawn in this experiment. Possible reasons for this are being investigated.

Discussion

Impact of dietary changes on the fecundity of wild and F1 broodstock

Both wild caught and F1 broodstock failed to spawn in this experiment. Spawning typically occurs 3-4 days after thermal-photoperiod manipulation (Fielder and Heasman, 2011). Reasons broodstock did not spawn after thermal-photoperiod manipulation are unclear, but they could relate to the sexual naivety of the wild caught and F1 stock, or the additional stress placed on stock at the beginning of the experiment as a result of weighing and handling procedures. The latter is unlikely given more than 111 days had elapsed since the fish were last handled. As the historical feeding regimes and feed sources for the groups of YTK fed Sardines and Squid was unchanged it also seems unlikely these factors were responsible for the spawning outcomes in that group. Broodstock in this experiment showed no reluctance to consume their allocated food sources, at least until F1 broodstock were switched to Breed-M. Therefore it seems unlikely the nutrient and energy intake of fish was inadequate; especially that of the broodstock fed Sardines and Squid. Similar outcomes were recorded in the previous broodstock experiment at PSFI (Manuscript 3.2.6.2).

Impact of dietary changes on broodstock

Based on statistical analyses using individual fish as replicates, the SGR_{Wt} of broodstock trended towards being significantly higher in broodstock fed under the best practice regime as opposed to the dry pelletised feed regime. The absolute weight gain of broodstock was also far higher in groups of fish fed Sardines and Squid. The choice of the pelletised feed regime used in this experiment was based on the results of research undertaken in Manuscripts 3.2.6.1 and 3.2.6.2. In Manuscript 3.2.6.1 soft-pelletised sausages were made from either a commercial pellet (Pelagica; Ridley) or from the Breed-M powder and these preparations were trialled on broodstock YTK in comparison to the best-practice regime. However, broodstock were slow to acclimate from Sardines and Squid to the soft pellets. In addition, the fecundity of YTK broodstock was significantly lower in groups reared on the soft commercial pellets after 9 months of feeding, irrespective of the quality of the commercial or Breed-M booster feed. The composition of viable eggs also seemed to be adversely altered in fish fed pelletised rations. Similar conclusions cannot be drawn from the current study as none of the broodstock spawned after being induced. However there are similarities in the response to feed type and feed intake between the experiments.

Conclusions and Recommendations

None of the wild origin or F1 broodstock in this experiment could be induced to spawn following normal practice at PSFI. The reasons broodstock did not spawn are unclear, but they could relate to the sexual naivety of the wild and F1 origin animals or the additional stress placed on the broodstock when taking initial weightings and possible the microbiome samples at the inception of the trial (see Manuscript 3.2.6.2). Therefore, the impact of the pelletised feeding regime could not be assessed against the best-practice regime with respect to investigations of fecundity and egg quality and hatch rate. There were some minor impacts of feeding regime on growth and FCR, but these indices are not of great concern in terms of hatchery output, so long as broodstock remain healthy. This may be inferred from condition indices among other metrics. Further long term trials will be necessary to evaluate the efficacy of feeding pelletised rations to broodstock at PSFI.

Findings

- The feeding regimes used in this experiment had minor impacts on the performance of YTK broodstock.

- YTK broodstock could not be induced to spawn in this experiment, irrespective of whether they were reared on Sardines and Squid or pelletised feeds; including a proprietary booster feed.
- These results are still being interpreted at the time or writing.
- Manipulative nutrition trials with large broodstock animals are challenging due to the scale of systems, the size of animals, the duration of experiments (especially those involving long terms spawning cycles) and often low replication. The basic nutrition research conducted on broodstock in this and other experiments (Manuscript 3.2.6.1, Manuscript 3.2.6.2) has been qualitative, but it has indicated that manipulating feeds and spawning cycles can impact the fecundity of wild origin and F1 animals.
- Difference in feed type (natural vs manufactured) also appears to impact the microbiome of broodstock in definable ways. These results demonstrate we need to pay close attention to these issues in YTK hatcheries and develop better and more rapid methods to assess the impacts of diet or abiotic shifts on the fecundity and quality of output from broodstock animals.
- Despite being problematic, further research should be conducted on YTK broodstock nutrition to ensure the YTK industry is working with the highest quality fingerlings.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, New South Wales Department of Primary Industries (NSW DPI) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

We would also like to acknowledge the technical assistance of Luke Vandenberg, Ian Russell, Georgie Whelan, Daniel Yee, Nicholas Gibson and Emily Collier (NSW DPI). We thank Cedric Simon, Barney Hines and staff at CSIRO and staff at NSW DPI Wagga Wagga Feed Laboratory for conducting the chemical analyses on samples generated from this experiment.

References

ACEC, 2015. A Guide to Acceptable Procedures and Practices for Aquaculture and FisheriesResearch, 4th Edition. In: NSW DPI Animal Care and Ethics Committee, pp. 56.

Fielder, D.S., Heasman, M., 2011. Hatchery manual for the production of Australian Bass, Mulloway and Yellowtail Kingfish, Industry & Investment NSW & Aquafin CRC.

Hintze, J., 2012. NCSS 8. NCSS, LLC. Kaysville, Utah, USA. NCSS, Kaysville, Utah, USA., www.ncss.com.

Kolkovski, S., 2005. Evaluation of Yellowtail Kingfish (Seriola lalandi) grow out in land-based systems and broodstock management. In: A Final Report to the Aquaculture Development Council and the Department of Fisheries Western Australia. Department of Fisheries – Research Division,

Western Australian Fisheries and Marine Research Laboratories, North Beach, Western Australia, pp. 64.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herraez, M.P., Carrillo, M., 2013. Gamete quality and broodstock management in temperate fish. Reviews in Aquaculture 5, S194-S223.

Morais, S., Mendes, A.C., Castanheira, M.F., Coutinho, J., Bandarra, N., Dias, J., Conceicao, L.E.C., Pousao-Ferreira, P., 2014. New formulated diets for *Solea senegalensis* broodstock: Effects of parental nutrition on biosynthesis of long-chain polyunsaturated fatty acids and performance of early larval stages and juvenile fish. Aquaculture 432, 374-382.

NRC, 2011. Nutrient Requirements of Fish and Shrimp. The National Academic Press, Washington, D.C.

Watanabe, T., Vassallo-Agius, R., 2003. Broodstock nutrition research on marine finfish in Japan. Aquaculture 227, 35-61.

Table 3.2.6.3.1. Measured nutrient content of feed sources used in the broodstock experiment (dry matter basis; g kg^{-1} or MJ kg^{-1}; FAME as mg g^{-1} lipid; same sources as used in Manuscript 3.2.6.2).

	Diet type (as-fed basis)		
Parameter	**Huon 9 mm****	**Sardines (*Sardinops sagax*)****	**Squid (*Doryteuthis pealeii*)****
Dry Matter (%)	1000.0	1000	1000
Moisture content (%)	0.0	0.0	0.0
Ash (%)	114.8	153.5	74.7
Total nitrogen (%)	77.2	95.3	126.2
Crude protein (%)	482.3	598.5	788.5
Total Lipid (%)	189.7	241.6	101.9
Carbohydrate (%)	212.7	9.1	34.9
Gross energy (MJ Kg^{-1})	23.3	23.9	23.1
CP:GE ratio	20.7	25.0	34.1
FAME (mg g^{-1} lipid)			
∑SAT	350.1	348.1	159.5
∑MONO	104.4	113.1	44.1
∑n-3 PUFA	126.3	307.2	239.2
∑n-6 PUFA	148.3	34.01	16.5
Total FAME identified	729.1	802.5	459.4
n-3:n-6 ratio	0.85	9.0	14.5

**CSIRO Laboratory.

Table 3.2.6.3.2. Typical nutrient and amino acid composition of feed types used in experiment (dry matter basis; g kg^{-1} or MJ kg^{-1}).

Parameter	**Breed-M***	**Sardines (*Sardinops sagax*)****	**Squid (*Doryteuthis pealeii*)****
Dry Matter (%)	1000.0	1000.0	1000.0
Moisture content (%)	0.0	0.0	0.0
Ash (%)	141.1	142.1	100.0
Total nitrogen (%)	108.5	104.4	120.2
Crude protein (%)	678.3	652.2	751.4
Total Lipid (%)	145.3	194.0	93.9
Carbohydrate (%)	35.4	11.7	54.8
Gross energy (MJ Kg^{-1})	23.5	23.2	22.4
CP:GE ratio	28.9	28.1	33.6
Alanine	30.7	38.3	28.4
Arginine	34.5	38.0	42.8
Aspartic acid	46.0	52.3	104.7
Cysteine	-	8.2	7.9
Glutamic acid	102.7	90.0	91.2
Glycine	33.2	38.6	14.4
Histidine	13.7	33.5	29.3
Hydroxyproline	4.2	1.8	2.3
Isoleucine	25.7	29.6	27.9
Leucine	42.9	51.7	43.7
Lysine	38.2	55.6	42.8
Methionine	16.8	15.4	14.0
Phenylalanine	25.1	27.2	22.8
Proline	34.5	26.3	27.4
Serine	24.9	24.8	25.1
Taurine	17.2	8.2	41.4
Threonine	23.4	29.2	27.0
Tryptophan	-	2.2	5.1
Tyrosine	19.6	21.5	17.2
Valine	30.2	33.2	25.6
Sum of AAs (excl. tryp.)	563.6	625.7	640.9

*NSW DPI Wagga Wagga Laboratory **CSIRO Laboratory.

Table 3.2.6.3.3. Stock (*ca.* 22-8-18) and harvest data (*ca.* 12-12-18) condition factor and specific growth rate recorded in Yellowtail Kingfish broodstock experiment.

			Stocking data			Harvest data			Growth performance		
	Sex	**Pit Tag**	**Weight (kg)**	**Fork length (cm)**	**K factor**	**Weight (kg)**	**Fork length (cm)**	**K factor**	**Daily gain (g)**	**SGR_{FL} (% d^{-1})**	**SGR_{Wt} (% d^{-1})**
F1 origin											
BST 3	Female	06A28AEA	5.5	72.0	1.47	6.8	73.0	1.75	11.82	0.01	0.19
BST 3	Female	06A2A90F	6.4	69.5	1.91	6.3	70.0	1.84	-0.91	0.01	-0.01
BST 3	Female	06B94832	6.8	72.5	1.77	7.3	74.0	1.80	5.00	0.02	0.07
BST 3	Female	06B98353	7.0	70.0	2.04	7.5	71.0	2.10	4.55	0.01	0.06
BST 3	Female	06B9ADAB	6.4	68.0	2.04	6.6	70.0	1.92	1.82	0.03	0.03
BST 3	Female	06C98CE3	7.2	68.0	2.29	7.6	69.0	2.31	3.64	0.01	0.05
BST 3	Female	07B0DCBD	7.8	71.0	2.18	8.5	73.0	2.18	6.36	0.03	0.08
BST 3	Male	06A27D02	6.0	68.5	1.87	6.2	70.0	1.81	1.82	0.02	0.03
BST 3	Male	06A287C7	5.8	70.0	1.69	6.4	72.0	1.71	5.45	0.03	0.09
BST 3	Male	06A2AFFF	6.2	72.5	1.61	6.5	74.0	1.60	3.18	0.02	0.05
BST 3	Male	06A2B435	6.2	68.5	1.91	6.3	69.0	1.92	1.36	0.01	0.02
BST 3	Male	06B965A0	4.5	69.5	1.34	4.6	71.0	1.29	0.91	0.02	0.02
BST 3	Male	06C97D13	6.1	72.5	1.60	6.7	74.0	1.65	5.45	0.02	0.09
BST 3	Male	06C983D1	3.5	65.5	1.25	4.0	67.0	1.33	4.55	0.02	0.12
BST 3	Male	07B0197F	6.0	65.5	2.14	6.5	67.0	2.16	4.55	0.02	0.07
BST 3	Unknown	06C98829	6.4	70.0	1.87	6.6	71.0	1.84	1.82	0.01	0.03
F1 origin											
BST 4	Female	068933EE	4.7	72.0	1.26	5.0	74.0	1.23	2.73	0.02	0.06
BST 4	Female	06A288D0	5.6	69.5	1.67	6.0	72.0	1.61	3.64	0.03	0.06
BST 4	Female	06A29F2E	6.4	68.0	2.04	6.5	70.0	1.90	0.91	0.03	0.01
BST 4	Female	06A2B4FB	7.0	68.0	2.23	7.3	70.0	2.13	2.73	0.03	0.04
BST 4	Female	06C98B70	6.6	68.0	2.10	7.3	69.0	2.22	6.36	0.01	0.09
BST 4	Female	06C99E74	4.3	66.0	1.48	4.5	68.0	1.43	2.27	0.03	0.05
BST 4	Female	07B00D45	6.4	68.5	1.99	7.0	73.0	1.80	5.45	0.06	0.08
BST 4	Male	0008CEE3	4.5	69.5	1.34	4.5	71.0	1.26	0.00	0.02	0.00
BST 4	Male	06A2955E	6.5	66.0	2.26	6.6	67.0	2.19	0.91	0.01	0.01
BST 4	Male	06B93BD7	4.0	60.5	1.81	4.0	63.0	1.60	0.00	0.04	0.00
BST 4	Male	06B94F39	7.0	71.0	1.96	7.6	74.0	1.88	5.45	0.04	0.07
BST 4	Male	07B0DF9A	6.4	66.0	2.23	6.8	68.0	2.16	3.64	0.03	0.06
BST 4	Unknown	06A29113	6.5	73.0	1.67	6.5	74.0	1.60	0.00	0.01	0.00
Wild origin											
BST 7	Female	0007AC3278	11.6	92.0	1.49	14.4	95.0	1.68	25.00	0.03	0.19
BST 7	Female	0006C99E83	10.0	84.5	1.66	11.6	87.0	1.76	14.29	0.03	0.13
BST 7	Female	0007AC8AE1	9.6	86.5	1.48	10.2	87.0	1.55	5.36	0.01	0.05
BST 7	Female	000783B9A2	8.8	84.0	1.48	9.5	85.0	1.55	6.25	0.01	0.07
BST 7	Female	0007AC885B	11.1	87.5	1.66	11.8	89.0	1.67	6.25	0.02	0.05
BST 7	Female	0007839A45	13.5	95.0	1.57	14.3	97.0	1.57	7.14	0.02	0.05
BST 7	Female	0007B02097	8.6	85.0	1.40	8.0	86.0	1.26	-5.36	0.01	-0.06
BST 7	Male	0007AC0EA7	11.7	90.5	1.58	12.8	92.0	1.64	9.82	0.01	0.08
BST 7	Male	0007B01A0A	12.0	91.0	1.59	14.9	92.0	1.91	25.89	0.01	0.19
Wild origin											
BST 9	Female	0006C99ED0	9.5	83.0	1.66	11.0	90.0	1.51	13.51	0.07	0.13
BST 9	Female	0007AC4687	9.5	84.0	1.60	11.0	89.0	1.56	13.51	0.05	0.13
BST 9	Female	0007AC9C4C	10.5	89.0	1.49	12.5	89.0	1.77	18.02	0.00	0.16
BST 9	Female	000783A2F9	10.5	90.0	1.44	12.0	93.0	1.49	13.51	0.03	0.12
BST 9	Female	0007ABFD4C	9.5	83.0	1.66	9.5	83.0	1.66	0.00	0.00	0.00
BST 9	Female	0007AFD7EC	10.5	86.5	1.62	11.2	88.0	1.64	6.31	0.02	0.06
BST 9	Female	0007AFDA5C	9.9	84.0	1.67	10.5	86.0	1.65	5.41	0.02	0.05
BST 9	Male	0007AFD59C	12.4	89.0	1.76	14.0	90.0	1.92	14.41	0.01	0.11

Table 3.2.6.3.4. Weight based and fork-length based specific growth rate (SGR_{Wt}; SGR_{FL}) and Fulton's condition factor *K* of broodstock (mean + SEM; tank basis).

Tank	Origin	Feed type	SGR_{Wt}	SGR_{FL}	Condition factor *K* @ stocking*	Condition factor *K* @ harvest
3	F1	Pelletised	0.0619	0.0181	1.811	1.826
4	F1	Pelletised	0.0407	0.0277	1.849	1.770
7	Wild	Natural	0.0833	0.0167	1.546	1.621
9	Wild	Natural	0.0950	0.0250	1.613	1.650
F-value			2.62	1.92	5.74	1.48
P-value			$P > 0.05$	$P > 0.05$	$P < 0.05$	$P > 0.05$

ANOVA results based on using individually tagged fish in each tank as replicate.
*Although ANOVA was significant the multiple comparisons procedure could not separate means at the 95% CI.

Table 3.2.6.3.5. Biomass gain and feed data recorded in Yellowtail Kingfish broodstock experiment.

		As-fed basis feed intake (kg tank^{-1})		Dry matter basis feed intake (kg tank^{-1})				
Origin	Tank	Huon	Breed-M	Huon	Breed-M	Total feed intake (kg tank^{-1})	Biomass Gain (kg tank^{-1})	Dry basis FCR
F1	3*	22.83	10.06	21.69	9.56	31.24	6.75	4.63
F1	4*	18.77	4.97	17.83	4.72	22.55	3.75	6.01
		Squid	Sardines	Squid	Sardines			
Wild origin	7**	46.47	61.51	9.99	13.23	23.22	10.60	2.19
Wild origin	9**	45.53	55.21	9.79	11.87	21.66	9.40	2.30

*Tank 3, Breed-M = 30.6% of total intake; Tank 4, Breed-M = 20.9% of total intake.
**Tank 7, Squid = 43.0% of total intake; Tank 9, Squid = 45.2% of total intake.

3.3. Theme - Nutrional Health

3.3.1. Chapter - Investigating the microbiome of Yellowtail Kingfish (*Seriola lalandi*) from wild and farmed stocks.

3.3.1.1. Manuscript - Differentiating the natural effects of environment, cultivation practice and growth on the microbiome of Yellowtail Kingfish (Seriola lalandi).

Sarah R. Catalano [a], Andrew P.A. Oxley [a]

[a] South Australian Research and Development Institute, South Australia Aquatic Sciences Centre, West Beach, SA 5024, Australia.

This manuscript may be referenced as: Catalano, S.R. and Oxley, A.P.A. (2019). 3.3.1.1. Manuscript - Differentiating the natural effects of environment, cultivation practice and growth on the microbiome of Yellowtail Kingfish (*Seriola lalandi*) (Output 4d). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.518-557.

Abstract

The active components of the gut microbiome from farmed Yellowtail Kingfish (YTK) from onshore (land) and offshore (sea-cage) systems and across the commercial production cycle were characterised and compared to the microbiome of the surrounding environment (sea/tank water samples) and the gut of wild YTK. Differences were observed in the microbiome (bacterial assemblages) between the gut and water samples, highlighting that YTK are able to select, regulate and maintain their own environmentally-independent communities. In comparing the gut microbiome of wild and farmed YTK, significantly distinct differences were observed in the global community structure, bacterial phyla and order compositions and relative abundances of the top 15 taxa (as designated as Operational Taxonomic Units, OTUs). Higher species diversity and evenness were also observed for the wild fish compared to farmed fish. Within the farmed samples, differences in the microbiome structure were observed based on cultivation strategy (i.e. between onshore and offshore systems), as well as across the commercial production cycle (i.e. between different size classes/ages). Onshore (land) samples were primarily dominated by taxa from *Proteobacteria*, while offshore (sea-cage) samples were generally more diverse and comprised taxa from a broad range of phyla including *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Tenericutes*, *Bacteroidetes* and *Firmicutes*. Smaller, 'younger' fish were primarily dominated by *Proteobacteria* and *Cyanobacteria*, whereas larger, 'older' fish were characterised by *Proteobacteria* and *Firmicutes*, with lower levels of *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes*, *Spirochaetae* and *Fusobacteria*. Species richness significantly reduced with size class/age, along with increased inter-individual variation (in terms of diversity and evenness) in larger, 'older' fish. This study is the first to catalogue the active bacterial communities of the gut of wild and farmed YTK, elucidating the environmentally-independent selection processes that arise in these fish, along with characterising the 'natural' dynamics that occur between cultivation strategies (onshore and offshore systems) and across the commercial production cycle. In doing so, this work provides baseline information for future studies seeking to elucidate changes in the health and nutrition of farmed YTK.

Introduction

The indigenous microbiota (or microbiome) of teleosts and their influence on the host is of topical interest in fish health and nutrition research. In humans and other vertebrates, the microbiome has a tremendous influence on host immunity and governs many aspects of digestion and nutrient metabolism. The microbiota is composed of an ecological community of commensal, symbiotic and pathogenic microorganisms (Lederberg and McCray, 2001) and includes bacteria (which are the dominant microbes), archaea, viruses, protozoa and fungi (Eckburg et al., 2005; Gill et al., 2006; Ley et. al, 2008a, 2008b; Rajilić-Stojanović et al., 2007). Knowledge of the gut microbiome has been shown to be of relevance for the identification of both favourable and dysbiotic (or pathogenic) phenotypes and offers the prospect for downstream manipulation for optimising host health and nutrition and consequentially the productivity of farmed species (Parris et al., 2016). Next generation sequencing (NGS) technologies paired with the availability of 16S rRNA gene sequence databases (e.g. RDP, SILVA and Greengenes) provide a powerful tool to count, classify and describe the microbial communities, including uncultivable members, that occur in a system or under a given treatment or condition (Federici et al., 2015; Llewellyn et al., 2014). In studies of human and animal models, it is now well-known that a balanced microbiota is extremely important for maintaining host health; though this is yet to be elucidated for teleosts.

Yellowtail Kingfish (*Seriola lalandi*; YTK) is a valuable species in Australia that is in the early stages of aquaculture development with significant commercial potential. YTK has been successfully spawned and reared in sea-cages in southern Australian waters, however, mortality events have been recorded in past years in Western Australia with stock losses of $\geq$70% (Stephens and Savage, 2010). In aquaculture industries, it is important to control for such disease outbreaks and mortality events, as they can significantly constrain production, marketability and profitability, overall negatively influencing the economic development of this sector (Wang et al., 2010). As the fish gut microbiota contributes to digestion, nutrient acquisition and the immune response and can affect the growth, reproduction, development, overall population dynamics and vulnerability of the host to disease, understanding the natural dynamics of these communities and how they respond to particular stressors is pertinent, particularly for farmed species that are reared under artificial conditions and are prone to a range of stressors not normally encountered in the wild (Colston and Jackson, 2016; Ghanbari et al., 2015). Such research thus represents a valuable opportunity to identify novel microbial biomarkers of changing health and nutrition, as well as for informing improved management strategies and establishing (in the longer-term) novel therapeutics (e.g. probiotics).

While detailed knowledge of the gut microbiota of YTK is currently lacking, initial insights from a recent study of Ramirez and Romero (2017) from Chile, revealed that differences between wild and farmed (recirculating tank-reared) YTK may occur and may be associated with the use of artificial feed formulations. Despite such a finding, only a small number of individuals (i.e. 5 wild and 5 farmed YTK) were examined. Furthermore, only broad (phylum-level) compositional changes were reported from analysis of the total community DNA. Knowledge of the more pertinent active (and likely resident) bacterial constituents of the gut microbiota of YTK (as discerned using total community RNA rather than DNA), thus remains undescribed. Baseline data which highlights the natural or 'normal' community dynamics and structure of the microbiome across the commercial production cycle for offshore (sea-cage) farmed YTK is also lacking. Additionally, while the composition of the gut microbiome may be a reflection of the taxa found in the surrounding water as observed for other fish species (Colston and Jackson, 2016; Roeselers et al., 2011), the involvement of the external environment on shaping the gut microbiome of YTK is currently unknown. In this manuscript, baseline data on the gut microbiome of YTK is presented, with comparisons made between onshore (land) and offshore (sea-cage) cultivation systems, select size classes/ages across the commercial farm production cycle, the surrounding environment (sea/tank water), and between farmed and wild YTK. The baseline data generated in this manuscript thus serves as a critical reference for delineating the influence of changing nutrition and health status in farmed YTK across cultivation systems (as detailed in Manuscripts 3.3.1.2 and 3.3.1.3 respectively).

Aim

The aim of this experimental work is to provide baseline data of the 'normal' gut microbiome of YTK and to elucidate its dynamics by differentiating the natural effects of environment, cultivation strategy and growth; as observed from comparisons between wild and farmed YTK, farmed YTK under different rearing conditions (i.e. onshore, land-based systems vs offshore sea-cages treatments) and among major size classes across the commercial production cycle. Compositional comparisons were evaluated in relation to those of the surrounding sea/tank water to determine if environmentally-independent gut community assemblages are selected for in the gut.

A. Land vs sea

A. Methods

Experimental design

A total of 20 fish were sampled for this experiment and comprised 11 fish from the Clean Seas Seafood commercial hatchery at Arno Bay, South Australia (SA) (representing the land-based onshore rearing treatment group) and nine fish from a grow-out farm at Point Boston (cage AB16-1) off Port Lincoln, SA (representing the sea-cage offshore rearing treatment group). Fish were from the same year class (2016) but fed different diets, with hatchery fish fed Feed A and sea-cage fish fed Feed B. Three 'wild' fish caught off Kangaroo Island (KI) were also provided by Clean Seas Seafood and acted as controls for farming practice and as a reference for downstream comparisons (see Figure 3.3.1.1.1). Note that the true nature of these fish as representatives from wild populations is questionable, as these fish were captured and maintained within a holding well on a boat with other fish species (including potential prey species) for one week prior to sampling, which could have influenced the resultant gut microbiome community. Hence we refer to these fish as 'wild'. Additionally, to control for the influence the environment may have on the structure and composition of the gut bacterial community, a 1L sea/tank water sample was also taken and processed in parallel from both the sea-cage offshore and land-based onshore locations.

Fish sampling

Each fish was euthanised in AQUIS solution and measured (fork length, cm). Weights (g) were collected for the land (offshore) samples only. For microbiome and histological sampling, the body cavity was opened and the entire GI tract removed. For the farmed fish (offshore and onshore), the mid- and hindgut were separated from the foregut using a sterile scalpel blade and placed on a clean surface. Using a clean pair of forceps and sterile scalpel, an incision was made along the length of the mid- and hindgut to expose the inner surfaces, then a single scraping of the entire region was performed with a sterile glass slide to collect the gut contents/mucosa. For the 'wild' fish, the midgut was separated from the hindgut and a separate scraping of each region was then collected. All scrapings were immediately placed in individual 50 mL falcon tubes containing stabilising buffer (RNAlater™, Ambion), labelled and stored at 4°C for 1-2 days before being stored for up to a month at -20°C prior to RNA extraction. Gloves, aluminium foil and scalpel blades were discarded and forceps were cleaned with ethanol after sampling each fish to avoid cross contamination. Histology samples were collected from the three gut regions of the 3 'wild' fish, but are analysed with further wild samples collected as part of the wild charter operations for the health vs disease experimental components outlined in Manuscript 3.3.1.3.

RNA extraction for gut microbiome samples

RNA was extracted on ice from stabilised samples according to the methods detailed in Szafranska et al. (2014). In brief, the stabilising buffer was removed from each sample and 1 mL of cold (4 °C) RLT buffer supplemented with 1% β-mercaptoethanol was added and transferred to lysing matrix B tubes (MP Biomedicals). Samples were disrupted via bead-beating using the FastPrep-24™ 5G instrument

(MP Biomedicals) at an intensity of 5.5 for 45 s, placed on ice for 3 min then disrupted a second time as described above prior to centrifugation at 14,000 × *g* for 10 min at 4 °C. The supernatant was transferred to 1.5 mL RNase-free Biopur centrifuge tubes (Eppendorf) and the RNeasy minikit (Qiagen) was used to extract the RNA according to manufacturer's instructions. RNA was eluted in 30 µL of RNase free water, passed through the spin column twice to concentrate each sample and quantified using a NanoDrop 2000 spectrophotometer. To remove any source of potential contaminating gDNA, a routine DNase treatment was performed for all samples using the Turbo DNA-free™ kit (Life Technologies) following the manufacturer's instructions. All samples were precipitated with ethanol using standard procedures, reconstituted in 30 µL of RNase free water and the RNA re-quantified using the NanoDrop. Samples were stored at -80 °C prior to use in downstream procedures.

DNA extraction for environmental sample

One litre aliquots of tank/sea water was collected in sterile Schott bottles from each of the offshore and onshore locations (Arno Bay hatchery, pre-drum system [land]; and Point Boston sea-cage AB16-1 [sea], Port Lincoln). Each bottle was labelled with the site location and cage ID and stored at 4°C prior to filtration and DNA extraction. Each environmental water sample was filtered onto separate sterile 0.22 µM filters (Nalgene®), and the DNA extracted from the filter discs using the FastDNA™ Spin Kit for Soil (MP Biomedicals) following the manufacturer's instructions. In brief, the filter paper was placed in a lysing matrix E tube with sodium phosphate and MT buffer and cells were lysed via bead-beating using the FastPrep-24™ 5G instrument (MP Biomedicals) at an intensity of 5.5 for 45 s. Samples were subsequently centrifuged for 10 min at 14,000 × *g* and the supernatant transferred to 1.5 mL DNA LoBind tubes (Eppendorf). Following the addition of a protein precipitation solution, the samples were mixed and centrifuged to pellet the precipitate before the supernatant was transferred to a clean 15 mL centrifuge tube supplemented with Binding Matrix solution. The DNA was captured on SPIN filter tubes and washed, re-eluted in 100 µL of DES and quantified using a NanoDrop 2000 spectrophotometer followed by precipitation with ethanol using standard procedures. The pelleted DNA was reconstituted in 30 µL of RNase free water and re-quantified using the NanoDrop. Samples were stored at 4 °C prior to use in down-stream procedures.

cDNA synthesis, PCR amplification, AMPure bead-purification and Picogreen quantification

For the gut scraping samples only, the RNA extracts were converted to cDNA to assess for the active (and likely resident) bacterial constituents using the Superscript™ III First Strand Synthesis System (Life Technologies) following the manufacturer's instructions and stored at -20 °C prior to PCR amplification. The V1-V2 hypervariable region of the 16S rRNA gene was amplified for all samples (DNA and cDNA samples) as described by Camarinha-Silva et al. (2014); though included an initial pre-enrichment of the V1-V2 target region by conducting a 20 cycle PCR reaction with primers 27F and 338R as described by Chaves-Moreno et al. (2015). Specifically, 2 µL of cDNA and 5 µL of each environmental DNA extract was used as template in this first round of PCR, with 1 µL aliquots from this reaction used as template in a second 15 cycle PCR reaction to append sample specific barcodes and reverse adapter sequences complementary to the Illumina platform specific adaptors. One microlitre aliquots of the second PCR reaction were subsequently used as a template in a third 10 cycle PCR to append the Illumina multiplexing sequencing and index primers. PCR amplicons were visualised via agarose gel electrophoresis and products of the expected size (~438 bp) were purified using Agencourt AMPure XP beads (Beckman Coulter). Samples were quantified in duplicate using the Quant-iT™ Picogreen® dsDNA kit (Life Technologies) following the manufacturer's instructions. Approximately 100 samples were pooled for each library in equimolar ratios and sequenced on the MiSeq platform (Illumina, San Diego, CA) using 250 nucleotide (nt) paired-end sequencing chemistry through the Australian Genome Research Facility (AGRF). As a sequencing control, amplicons generated from a single bacterial species (*Lactobacillus reuteri*) were included within each Illumina index within each of the libraries. The final list of samples that generated good-quality microbiomic libraries for this component of work are presented in Table 3.3.1.1.1.

Bioinformatics analysis

In total, 1,088,094 million sequence reads were derived from 23 samples (of the 26 that were collected). Three samples failed to amplify enough material to produce good-quality NGS libraries. This was accounted for in the experimental approach, by allowing for ample replication of fish. Sequence reads were paired using PEAR (version 0.9.5) (Zhang et al., 2014), where primers were identified and removed. Paired-end reads were quality filtered, with removal of low-quality reads, full-length duplicate sequences (after being counted) and singleton sequences using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010), USEARCH (version 8.0.1623) (Edgar, 2010; Edgar et al., 2011) and UPARSE software (Edgar, 2013). Reads were mapped to Operational Taxonomic Units (OTUs) using a minimum identity of 97%, and putative chimeras removed using the RDP-gold database as a reference (Cole et al., 2014). These OTUs were further filtered as conducted previously (Zhang et al., 2016) where only those that contributed to >0.01% of the host-associated dataset (gut samples only) or >0.01% of the environmental seawater samples were used (see Table 3.3.1.1.2 for a summary of OTUs remaining post-filtering). Rarefaction curves were used to inspect (retrospectively) sampling depth for each gut sample (Appendix 1A). Further interrogation of the resultant OTUs was conducted using the Seqmatch function of the RDP database (Wang et al., 2007) as well as SILVA (Quast et al., 2013), whereby lineages based on the SILVA taxonomy and best hits from RDP were assigned for each OTU alongside the corresponding RDP sequence similarity value (SeqMatch, S_ab score). The S_ab score represents the number of unique 7-base oligomers shared between an OTU and a known sequence contained in the RDP database divided by the lowest number of unique oligos in either of the two sequences. A S_ab score of 1.000 represents an identical match to the nearest database sequence, with values closer to 1.000 providing greater confidence in the identification of the OTU sequence.

Statistical analysis

In order to explore for patterns across the global bacterial communities, a data matrix comprising the percent standardised abundances of OTUs was used to construct a sample-similarity matrix using the Bray-Curtis algorithm (Bray and Curtis, 1957), where samples were then ordinated using non-metric multidimensional scaling (nMDS) with 50 random restarts (Clarke et al., 2001). Significant differences between *a priori* pre-defined groups of samples (e.g. land vs sea) were evaluated using permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations, allowing for type III (partial) sums of squares, fixed effects sum to zero for mixed terms, and exact p-values generated using unrestricted permutation of raw data (Anderson, 2001). Groups of samples were considered significantly different if the p-value falls < 0.05. Pairwise tests in PERMANOVA were used to determine which *a priori* pre-defined categories (e.g. water samples vs gut scraping samples) were significantly different. The multivariate analyses, relative percent abundance of bacterial phyla and orders, and rarefaction curves were performed and calculated using PRIMER (v.7.0.11), PRIMER-E, Plymouth Marine Laboratory, UK (Clarke et al., 2001).

Conventional measures of species diversity, richness and evenness were calculated using algorithms for total OTUs (S), Pielou's evenness (J'), Shannon diversity (H') and Simpson (1-λ), while taxonomic diversity was calculated using algorithms for taxonomic distinctness: average taxonomic distinctness (avTD - delta+) and variation in taxonomic distinctness (varTD - lambda+) using PRIMER (v.7.0.11) (Clarke et al., 2001). These univariate indicators of diversity (S, J', H', 1-λ, avTD, varTD) were compared between *a priori* groups of samples (seawater, wild, sea-cage, land) using one-way ANOVA and plotted in Prism v. 7.01 (Graphpad Software Inc.). Variables were considered to be significantly different if the p-value falls < 0.05, for which a Tukey's post-hoc multiple comparisons test was then performed (Prism v. 7.01). For further presentation of data, relative abundance plots of the top 15 most abundant gut OTUs were constructed in Excel. To identify the closest cultured bacterial species for each of the most abundant OTUs, the corresponding sequence was blasted against the RDP isolate database only. A similarity (S_ab) score in parenthesis is presented for each OTU in the top 15 OTUs plot.

A. Results

Global community structure

In the nMDS plot there was a clear separation between the global community structure of the two environmental (seawater) samples and all of the gut samples collected from both the 'wild' and farmed fish, along with a separation based on cultivation strategy; offshore (sea-cages) compared to onshore (land-based tank) samples (Figure 3.3.1.1.2). This observation was confirmed by PERMANOVA, with a significant difference between environmental and gut samples (P = 0.0082, table not shown), and offshore (sea-cage) vs onshore (land-based tank) samples (P = 0.0015, table not shown).

Bacterial phyla

Dominance by bacterial taxa from the phylum *Proteobacteria* was observed across all samples (seawater, wild, offshore and onshore), however there was greater diversity of phyla in the seawater samples compared to gut scrapings (Figure 3.3.1.1.3). Onshore hatchery (land-based tank) samples were primarily dominated by taxa from *Proteobacteria*, while offshore (sea-cage) samples were generally more diverse with taxa representation from a range of phyla including *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Tenericutes*, *Bacteroidetes* and *Firmicutes* (Figure 3.3.1.1.3). The bacterial phyla composition in the 'wild' fish was distinctly different from the farmed samples, with a greater abundance of *Fusobacteria* and lower representation of taxa from *Tenericutes* and *Firmicutes* (Figure 3.3.1.1.3).

Top 15 OTUs

The fish from the onshore (land-based) rearing systems were consistently dominated by an unresolved *Photobacterium* species clade, with closest sequence similarity to the opportunist pathogen *P. damselae* subsp. *damselae* and *P. leiognathi* (OTU 2, similarity [S_ab] score 1.000), along with two *Vibrio* species, *V. ponticus* (OTU 21, S_ab score 1.000) and *Vibrio* sp. (OTU 16, S_ab score 1.000) (Figure 3.3.1.1.4). In comparison, offshore (sea-cage) reared fish had greater heterogeneity (or inter-individual variation) within the population, with variable dominance between individuals observed for various taxa including *Ehrlichia* sp. trout isolate (OTU 12, S_ab score 0.821), *Aliivibrio* sp. (OTU 4, S_ab score 0.948), *Mycoplasma insons* (OTU 1, S_ab score 0.420), *Pseudomonas veronii*/*P. azotoformans* (OTU 36, S_ab score 1.000) and *Chroococcidiopsis* sp. (OTU 62, S_ab score 0.774) (Figure 3.3.1.1.4). The *Photobacterium* clade was also observed in all 'wild' fish, however, with a lower level of dominance compared to the offshore, sea-cage fish. Additional distinct taxa (not observed in the farmed fish) were consistently present in the 'wild' fish samples, including those with closest sequence similarity to *Cetobacterium somerae* (OTU 39, S_ab score 0.774; and OTU 41, S_ab score 0.669), *Shewanella corallii* (OTU 34, S_ab score 0.727), *P. phosphoreum/P. iliopiscarium* (OTU 17, S_ab score 1.000) and *Clostridium neonatale* (OTU 84, S_ab score 0.579) (Figure 3.3.1.1.4).

Diversity indices

Although there were no significant differences in the six diversity indices between the gut scraping samples ('wild', offshore and onshore, table not shown), a greater spread of high and low evenness (Pielou's) and diversity (Shannon and Simpson) was observed for the offshore (sea-cage) fish compared to the onshore (land-based tank) fish (Figure 3.3.1.1.5). There was also a greater dominance of fewer species for the onshore fish compared to the offshore fish. While species richness (total species) was similar between 'wild' and farmed fish, a general pattern of greater species evenness (Pielou's) and diversity (Shannon and Simpson) was observed for the 'wild' fish compared to the farmed fish (Figure 3.3.1.1.5).

B. Commercial production cycle

B. Methods

Experimental design

A total of 40 fish were sampled for this experiment across the commercial production cycle, comprising 10 fish for time point 1 (T1, 100 g fish, sampled 21st January 2016), 10 fish from time point 2 (T2, 500 g fish, sampled 21st January 2016), 10 fish for time point 3 (T3, 1000 g fish, sampled 4th March 2016) and 10 fish for time point 4 (T4, 2000 g fish, sampled 18th July 2016). All fish were collected from Arno Bay, SA, AB3-AB Site 2, with T1 from cage AB 16-7 and T2, T3 and T4 from cage AB16-3. Fish were fed the same diet (Feed A) and were all from the same 2016 year class. Three 'wild' fish caught off KI, as detailed in section A. Methods, were also provided by Clean Seas Seafood and acted as controls for cultivation practice and as a reference for downstream comparisons (Figure 3.3.1.1.6). A 1L seawater sample was collected and processed in parallel from the Arno Bay sea-cage site to control for the influence the environment may have on the structure and composition of the gut bacterial community.

Fish sampling

Refer to section A. Methods. A combined total weight of all 10 fish from each time point was recorded (giving an average weight for each fish, see Table 3.3.1.1.3) instead of an individual weight for each fish.

RNA extraction for gut microbiome samples

Refer to section A. Methods.

DNA extraction for environmental sample

Refer to section A. Methods.

cDNA synthesis, PCR amplification, AMPure bead-purification and Picogreen quantification

Refer to section A. Methods. The final list of samples that generated good-quality microbiomic libraries for this component of the work are presented in Table 3.3.1.1.3.

Bioinformatics analysis

In total, 1,534,702 million sequence reads were derived from 28 samples (of the 47 that were collected). Nineteen samples failed to amplify enough material to produce good-quality NGS libraries. This was accounted for in the experimental design approach, by allowing for ample replication of fish. Table 3.3.1.1.4 provides a summary of OTUs remaining post-filtering. Rarefaction curves were used to inspect (retrospectively) sampling depth for each gut sample (Appendix 1B). For detailed methods on quality filtering and the mapping of the sequence reads, refer to section A. Methods.

Statistical analysis

Refer to section A. Methods. In addition, the Similarity Percentage (SIMPER) routine was applied in PRIMER (v.7.0.11) where there was a significant difference and moderate variation to seek for those OTUs that contribute mostly to the observed difference between *a priori* pre-defined categories (such as 100 g vs 500 g fish).

B. Results

Global community structure

In the nMDS plot there was a clear separation between the global community structure of the one environmental (seawater) sample and all gut samples collected from the 'wild' and farmed fish (Figure 3.3.1.1.7), which was confirmed by PERMANOVA (P = 0.0362, table not shown). Additionally, there was a significant difference between size classes (P = 0.0001, Table 3.3.1.1.5), although smaller 'younger' fish (100 g and 500 g) were similar with an overlap observed between the samples in the nMDS (Figure 3.3.1.1.7) and a non-significant difference established by PERMANOVA (P = 0.6274, Table 3.3.1.1.6). All other size classes clustered independently in the nMDS (Figure 3.3.1.1.7) and were significantly different to one another (Table 3.3.1.1.6).

Bacterial phyla

The smaller 'younger' fish in size classes 100 g and 500 g were dominated by bacterial taxa from the phyla *Proteobacteria* and *Cyanobacteria* with a shift to an almost complete dominance of *Proteobacteria* observed in the 1000 g fish (Figure 3.3.1.1.8). For the larger 'older' fish at 2000 g, dominance by *Proteobacteria* and *Firmicutes* were observed, with lower levels of taxa representative of the phyla *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes*, *Spirochaetae* and *Fusobacteria* (Figure 3.3.1.1.8). The bacterial phyla composition in the 'wild' fish was distinctly different from the farmed samples, with a greater abundance of *Fusobacteria* and lower representation of taxa from *Tenericutes* and *Firmicutes* (Figure 3.3.1.1.8).

Top 15 OTUs and greatest taxa contributors

The relative abundance and taxa composition of the top 15 OTUs in the smaller (100 g and 500 g) size class fish were similar, with representation from taxa with closest sequence similarity to *Photobacterium damselae* subsp. *damselae* (OTU 3, similarity [S_ab] score 1.000), *Synechococcus* sp. (OTU29, S_ab score 0.978; and OTU 23, S_ab score 1.000), *Ehrlichia* sp. trout isolate (OTU 16, S_ab score 0.853) and *Brevinema andersonii* (OTU 2, S_ab score 0.632) (Figure 3.3.1.1.9). Differences in taxa composition was then observed between these smaller 'younger' size classes of fish and the other two larger 'older' size classes of 1000 g and 2000 g fish (Figure 3.3.1.1.9). The observed difference between the 500 g and 1000 g size classes of fish were associated with an increased abundance of *Vibrio ichthyoenteri*/*V. scophthalmi* (OTU 12, S_ab score 1.000) and a reduced abundance of two *Synechococcus* species (OTU29, S_ab score 0.978; and OTU 23, S_ab score 1.000) and *Ehrlichia* sp. trout isolate (OTU 16, S_ab score 0.853) in the 1000 g fish (Table 3.3.1.1.7, Figure 3.3.1.1.9). However, between the 1000 g and 2000 g sized fish, the observed difference was associated with the replacement of *V. icthyoenteri*/*V. scophthalmi* (OTU 12, S_ab score 1.000) in the 1000 g fish by *Vibrio* sp. V774/*Aliivibrio finisterrensi* (OTU 4, S_ab score 1.000) in the 2000 g fish (Table 3.3.1.1.8, Figure 3.3.1.1.9). The larger 'older' fish (2000 g) were also characterised by enrichment of potentially beneficial taxa, including *Bacillus* sp./*Geobacillus stearothermophilus* (OTU 234, S_ab score 0.977), *Bacillus smithii* (OTU 83, S_ab score 1.000) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (OTU 279, S_ab score 1.000) (Table 3.3.1.1.8).

'Wild' fish were similar to the smaller (100 g and 500 g) size fish, though alongside *Photobacterium damselae* subsp. *damselae*/*P. leiognathi* (OTU 3, S_ab score 1.000) they also comprised several distinct taxa (not observed in the farmed fish), including those with closest sequence similarity to *Cetobacterium somerae* (OTU 57, S_ab score 0.779; and OTU 168, S_ab score 0.759), *Shewanella corallii* (OTU 60, S_ab score 0.781), *P. mandapamensis/P. leiognathi* (OTU 81, S_ab score 0.879) and *Clostridium perfringens* (OTU 113, S_ab score 0.642) (Figure 3.3.1.1.9).

Diversity indices

While species richness (total number of bacterial OTUs) was not significantly different between the 100 g, 500 g and 2000 g size classes, there was a significant reduction between these size classes and the 1000 g size class ($P < 0.05$, see Figure 3.3.1.1.10, Table 3.3.1.1.9). There were no further significant differences between size classes across the other diversity indices (Figure 3.3.1.1.10, Table 3.3.1.1.9).

C. Wild SA vs farmed YTK

C. Methods

Experimental design

A total of seven wild charter fish ranging in size from 2.85 to 12.4 kg (average = 6.58 kg) were caught via line fishing at Four Hummocks (approximately 40km south-west off the coast of Coffin Bay, SA) on the 5th and 6th February 2018 and were used to supplement the original three 'wild' YTK collected off of KI, SA (which were kept within a holding well on a boat for one week prior to sampling). Separate mid- and hindgut scrapings were collected from each fish for microbiomic evaluation and the resultant data merged and analysed together with the earlier baseline data derived from comparisons between the onshore (land) and offshore (sea-cage) systems, commercial production cycle and the three 'wild' fish (see sections A. Methods and B. Methods above). A seawater sample was also taken from the fishing site and processed in parallel to control for the influence the environment may have on the structure and composition of the gut bacterial community (Figure 3.3.1.1.11).

Fish sampling

Refer to section A. Methods. Histology samples were collected from the three gut regions of the seven wild charter fish, but are reported alongside samples taken from the initial three 'wild' YTK samples as part of the health vs disease experimental components outlined in Manuscript 3.3.1.3.

RNA extraction for gut microbiome samples

Refer to section A. Methods.

DNA extraction for environmental sample

Refer to section A. Methods.

cDNA synthesis, PCR amplification, AMPure bead-purification and Picogreen quantification

Refer to section A. Methods. The final list of samples that generated good-quality microbiomic libraries for this component of the work are presented in Table 3.3.1.1.10.

Bioinformatics analysis

In total, 3,459,141 million sequence reads were derived from 57 samples (of the 81 that were collected). Twenty-four samples failed to amplify enough material to produce good-quality NGS libraries. This was accounted for in the experimental design approach, by allowing for ample replication of fish. Table 3.3.1.1.11 provides a summary of OTUs remaining post-filtering. Rarefaction curves were used to inspect (retrospectively) sampling depth (Appendix 1C). For detailed methods on quality filtering and the mapping of the sequence reads, refer to section A. Methods.

Statistical analysis

Refer to section A. Methods.

C. Results

Global community structure

There was a clear separation in the global community structure between the seawater and gut samples, an observation confirmed to be significantly different by PERMANOVA ($P = 0.0179$, table not shown) (Figure 3.3.1.1.12). The samples from the wild YTK also clustered independently from those obtained from the farmed YTK, though the 'wild' and wild charter samples formed distinct clusters (with the exception of one wild charter sample which instead clustered with the original 'wild' YTK samples) (Figure 3.3.1.1.12). This was confirmed by PERMANOVA, with significant differences occurring between the wild charter and onshore vs offshore samples ($P = 0.0001$, table not shown), wild charter and commercial production cycle samples ($P = 0.0001$, table not shown) and wild charter and 'wild' samples ($P = 0.0006$, table not shown) (Figure 3.3.1.1.12).

Bacterial phyla

Despite some differences occurring in the bacterial phyla compositions between the two wild sample groups (i.e. 'wild' and wild charter), *Proteobacteria* were a predominant feature across all samples, including those from farmed YTK (Figure 3.3.1.1.13). Notable differences between the wild chartered and farmed YTK samples, however, were also observed and included the absence of certain phyla such as *Chloroflexi* (and to a lesser extent *Spirochaetes*) from the farmed samples (Figure 3.3.1.1.13). Instead, farmed samples appear to comprise an abundance of other select phyla, notably *Cyanobacteria* (Figure 3.3.1.1.13).

As factors such as size class may influence the microbiome composition (as revealed in section B. Results), comparisons between fish of a similar size range (i.e. $\geq$ 2 kg) revealed that farmed fish may also be dominated by *Firmicutes* and, to a lesser extent, *Actinobacteria* and *Bacteroidetes* (Figure 3.3.1.1.14). Additionally, while a greater diversity of bacterial taxa at the order level were observed for the wild charter fish (including members of the *Oceanospirillales*, *Vibrionales*, *Alteromonadales* and *Spirochaetales*), the farmed fish were primarily dominated by select groups, including *Vibrionales* and, to a lesser extent, *Bacillales* and *Synechococcales* (Figure 3.3.1.1.15).

Top 15 OTUs

Differences in the composition and contribution of the top 15 taxa were observed between the 'wild' and wild charter fish. Specifically, while the 'wild' fish primarily comprised a single taxon (namely *Photobacterium damselae* subsp. *damselae/P. leiognathi*; OTU 2, similarity [S_ab] score 1.000), the wild charter fish comprised a more diverse group of taxa including those with closest sequence similarity to *Aeromonas veronii/Vibrio agarivorans* (OTU 4, S_ab score 0.603), *P. phosphoreum/P. iliopiscarium* (OTU 5, S_ab score 1.000), *Ralstonia picketti* (OTU 16, S_ab score 1.000) and *Endozoicomonas elysicola* (OTU 19, S_ab score 1.000) (Figure 3.3.1.1.16). The observed diversity for the wild charter fish was also markedly different to those from farmed fish which instead, depending on the treatment group, comprised a range of other predominant bacterial constituents including *P. damselae* subsp. *damselae/P. leiognathi* (OTU 2, S_ab score 1.000), *Allivibrio finisterrensis/Vibrio* sp. (OTU 3, S_ab score 1.000), *Ehrlichia* sp. trout isolate (OTU 6, S_ab score 0.821), *Synechococcus* sp. (OTU 21, S_ab score 0.974) and *P. swingsii*/*Vibrio* sp. (OTU 3429, S_ab score 0.952) (Figure 3.3.1.1.16).

Diversity indices

Diversity indices were similar between the wild and farmed fish, with a significant difference only observed between the wild charter and onshore (land-based tank) farmed group for species evenness and diversity (Shannon's and Simpson's) (Figure 3.3.1.1.17, Table 3.3.1.1.12). For these two fish groups, species evenness and diversity was significantly higher in the wild charter fish compared to the land-based tank farmed fish (Figure 3.3.1.1.17).

Discussion

The aim of this experimental work was to provide baseline data of the 'normal' gut microbiome of YTK and to elucidate its dynamics by differentiating the natural effects of environment, cultivation practice and growth; as observed from comparisons between wild and farmed YTK, farmed YTK under different cultivation conditions (i.e. onshore, land-based tank systems vs offshore sea-cages) and among major size classes across the commercial production cycle. Compositional comparisons were also evaluated in relation to those of the surrounding seawater to determine if environmentally-independent gut community assemblages are selected for in the gut. Overall, the global bacterial community composition between the environmental samples (surrounding tank/sea water) and the gut samples was markedly different, highlighting that YTK are able to regulate and maintain their own environmentally-independent bacterial communities in the gut. This is feature also widely reported for other species, whereby host phylogeny and ecology are thought to be key drivers associated with regulation of the normal microbiota (Clements et al., 2014; Dehler et al., 2016; Ghanbari et al., 2015; Romero et al., 2014).

Broad variations in the global community composition were observed between wild and farmed YTK gut samples. Of note, *Proteobacteria* was a dominant feature of both the wild and farmed fish, though in fish of similar sizes (> 2kg), there was also greater representation of *Firmicutes* and *Actinobacteria* in the farmed compared to wild fish. *Proteobacteria* has been described as the most predominant phyla in both wild marine fish (Star et al., 2013) and wild freshwater fish (Liu et al., 2016), with our study further highlighting its dominance in farmed YTK. In the study of Ramirez and Romero (2017), the global microbiome (bacterial community) structure of five cultivated YTK from recirculating aquaculture systems in Chile, and five wild YTK (collected from Santiago, Chile) of similar size classes (~3-5 kg) were investigated. However, in contrast to our findings here, only the wild fish were dominated by *Proteobacteria* (83%), whereas the farmed fish were represented by *Firmicutes* (61%), *Proteobacteria* (20%) and *Actinobacteria* (14%). At the lower taxonomic (order) level, some similarities were also apparent, whereby Ramirez and Romero (2017) also reported *Alteromonadales* in wild and *Bacillales* in farmed YTK. Despite this, in our study, many differences were also apparent including the occurrence of bacterial taxa belonging to *Oceanospirillales*, *Vibrionales*, *Alteromonadales* and *Spirochaetales* in wild YTK, and the dominance of *Vibrionales* and, to a lesser extent, *Bacillales* and *Synechococcales* in farmed individuals. These differences are likely due to variations in the experimental design approach, as Ramirez and Romero (2017) investigated the global bacterial community structure from total DNA samples and targeted the V4 hypervariable region of the 16S rRNA gene, while we examined the active bacterial constituents of the microbiome from cDNA (as derived from total RNA), and targeted an alternative hypervariable region of the 16S rRNA gene (i.e. V1-V2).

Variation in the contribution and composition of the top 15 taxa also appear to vary between wild and farmed YTK. Specifically, samples collected from the wild charter YTK samples comprised markedly different species to those observed from the farmed fish, and included organisms known to occur as both commensal or symbiotic species in other fish or marine animals e.g. *Photobacterium phosphoreum, P. iliopiscarium, Endozoicomonas elysicola, Ralstonia picketti* (Beaz-Hidalgo et al., 2010; Labella et al., 2018; Larsen et al., 2014; Whitaker et al., 2006, Wu et al., 2010); though the latter species may also represent a potentially opportunistic pathogen in humans (Stelzmueller et al., 2006). Overall, the wild charter YTK samples comprised significantly higher species diversity and evenness (Shannon's and Simpson's) in comparison to the farmed (onshore tank-reared) YTK. Some of the samples from YTK farmed offshore in sea-cages also comprised a reduced diversity and evenness in

comparison to the wild charter YTK (though this was not statistically different and requires further examination of a larger number of individuals). Collectively, the results from this study and that of Ramirez and Romero (2017) indicate that the microbiome of wild and farmed YTK is different and may be a feature of broad differences in diet (or other stressors) whereby farmed fish are restricted to formulated feeds, while wild fish are able to forage and consume a wider array of prey species; which may require a more diverse community structure to be able to digest and metabolise these varied food items. Interestingly, differences in the microbiome compositions between the 'wild' and wild charter YTK were also observed here and may reflect differences among individual YTK populations. However, while it is tempting to postulate on the possible host genetic or dietary pressures that may drive differences in the gut microbiome of individual populations (as observed in other fish species, Webster et al., 2018), further work would be required to elucidate this due to the limited number of fish sampled and the potentially confounding effects from the original three 'wild' fish having been kept in a holding well on a boat for one week prior to sampling.

Within the farmed samples, differences were observed between onshore and offshore cultivation practices and across the commercial production cycle. Land (onshore, tank-reared YTK) samples were primarily dominated by taxa from *Proteobacteria*, while YTK farmed offshore in sea-cages were generally more diverse with taxa representation from a range of phyla including *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Tenericutes*, *Bacteroidetes* and *Firmicutes*. This suggests either a change in the gut microbiome of sea-cage fish after relocation from the onshore tanks for grow-out, or that the onshore tank-based conditions enrich for a unique bacterial profile during rearing. The onshore tank-reared fish were consistently dominated by an unresolved *Photobacterium* species clade, with closest sequence similarity to the opportunist pathogen *P. damselae* subsp. *damselae* and *P. leiognathi,* whereas the offshore sea-cage fish had greater heterogeneity (or inter-individual variation) within the population. *Photobacterium damselae* subsp. *damselae,* a marine bacterium from the family *Vibrionaceae* (Rivas et al., 2013), is known from a range of fish species, including Damselfish (*Chromis punctipinnis*), Eels (*Anguilla anguilla*), Brown Shark (*Carcharhinus plumbeus*), Seabream (*Sparus aurata*), Turbot (*Scophthalmus maximus*) and Yellowtail (*Seriola quinqueradiata*) (Fouz et al., 2000), and can cause disease symptoms in infected fish including skin ulcerative lesions and extensive hemorrhages, especially in the mouth, eyes and musculature (Fouz et al., 2000). Alternatively, *P. leiognathi* is a luminous marine bacterium found commonly in the light organs of leiognathid fish (Slipmouths) (Ast and Dunlap, 2004), and there are no reports of pathology or disease associated with this bacterial species in the current literature. As this *Photobacterium* species clade in the onshore fish is unresolved, it may either represent a commensal organism, or a potentially harmful opportunistic pathogen that is capable of causing disease in YTK. Additional sampling from the onshore system (and bacteriological testing) would be required to investigate this further.

Variations in the microbiome composition with size of the YTK during the commercial production cycle was also apparent. Specifically, smaller, 'younger' YTK (100-500 g) were observed to have a microbiome comprising similar bacterial taxa (at both a phyla and genus level) which, in turn, was significantly different to those of larger, 'older' fish (1000-2000 g). Interestingly, there was also a significantly higher number of bacterial species in smaller compared to larger fish, with a general trend of reduced taxonomic evenness and diversity observed with size/age (although this finding was not significantly different and requires further examination of a larger number of individuals). While this highlights the need for age-specific controls when comparing between different treatment groups (as compositional variations may be confounded by life-stage rather than an underlying condition), a trend of reduced bacterial species richness with growth has also been observed in other farmed fish and likely reflects the natural 'maturation' processes that occur between the microbiome and its host throughout growth and development. Indeed, within fish more broadly, it is well-known that the microbiome can shift significantly over an individual's lifespan (Parris et al., 2016), with the selection and enrichment of key groups occurring from hatching and continuing throughout development and maturity (Forberg et al., 2016; Llewellyn et al., 2016). In some species like Atlantic Salmon (*Salmo salar*) changes in species richness as adults was suggested to be attributed to fasting in the migratory phases as they return to freshwater environments to spawn (Llewellyn et al., 2016). However, for YTK which are reared and mature in a marine environment, it is likely that other factors are contributing to the variations observed between size classes and may include the adaptation of the fish to local environmental microbial constituents following transition of individuals from the hatchery to the sea-cages for grow-out, in

moving cages to alternative sites, and/or with changes in dietary formulations/pellet sizes during growth. Notably though, the larger (2000 g) fish comprised a number of potentially beneficial taxa as some of their major microbiome constituents, indicating the selective enrichment of species by the fish with growth/maturity. Some of these taxa included a number of *Bacillus* species (e.g. *B. smithii*, *Lactobacillus delbrueckii* subsp. *bulgaricus*) which are known to exert positive effects on the host through their ability to aid in digestion, enhance the immune response, compete with potential pathogens and produce inhibitory compounds (Merrifield et al., 2010; Romero et al., 2014) and in this regard are often used as probiotics to improve growth and survival in cultivated species (Schulze et al., 2006; Pérez et al., 2010). Further work is required, however, to elucidate the factors leading to the selection of these groups and their role within YTK.

Conclusions and Recommendations

In conclusion, we provide here a detailed analysis of the active bacterial components of the gut microbiome of wild and farmed YTK, establishing baseline data of the 'normal' gut microbiome and delineating the natural effects that environment, cultivation practice and growth have on its composition. The enrichment of environmentally-independent bacterial taxa was apparent in YTK, with marked differences also occurring in the compositions between wild and farmed individuals. Increased levels of diversity in wild fish indicate the possible influence of natural diets in their formation, while formulated feeds may contribute to the reduced diversity and/or enrichment of select taxa in farmed fish. Cultivation strategy also appears to influence the microbiome composition, with lower levels of diversity and the enrichment of potentially opportunistic bacterial species occurring in onshore (tank-based) compared to offshore (sea-cage) systems. Approaches or management strategies which aim to enhance gut microbiome diversity in onshore systems may be required for optimising the robustness of the fish and may improve the natural adaptive processes of the fish to local environmental microbial communities when transferred offshore to sea-cages for grow-out, and requires further elucidation. With changes in microbiome composition and diversity also observed among major size classes associated with the commercial production cycle, there is also a need to ensure that appropriate size/age-specific controls are taken when surveying the relevance of the microbiome in changing health and nutrition in future surveys.

Findings

This work found marked differences in the global bacterial community structure between environmental (tank/sea water) and YTK gut samples, along with differences between wild and farmed fish, including across cultivation strategies of onshore (land-based) and offshore (sea-cages) systems. Differences in the global community structure and diversity measures were also observed across size classes, with smaller fish having a significantly higher number of bacterial species than larger fish, with a general trend of reduced taxonomic evenness and diversity observed with size/age. This work demonstrates the capacity for YTK to selectively enrich environmentally independent bacterial taxa in the gut, with the community structure and dynamics of farmed fish appearing to be different to those from the wild. Within farmed fish, the gut microbiome also appears to be influenced by the different cultivation strategies associated with onshore and offshore systems, with compositional variations occurring with increasing size/age of the fish. While this likely reflects the natural 'maturation' processes that occur between the microbiome and its host throughout growth and development, other factors (e.g. diet) may also be contributing features.

Publications

No publications have resulted from this R&D to date.

Acknowledgements

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit program, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds. The authors would also like to acknowledge the support of SARDI and provision of the Molecular Sciences Laboratory facilities at West Beach, South Australia. Thanks to Dr Trent D'Antignana of Nutrisea, Dr Jay Dent and Dr Erin Bubner of Clean Seas Seafood, and Clean Seas Seafood health team staff for coordinating and assisting with sample collections on-farm. We would also like to thank Leigh Kuerschner, Ben Crowe, Samantha Chown and Thibault Legrand for their assistance in the wild charter fish collection, and Dr Melissa Wos-Oxley of Flinders University for supporting and guiding the statistical analyses.

References

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology 26, 32-46.

Ast, J.C., Dunlap, P.V. 2004. Phylogenetic analysis of the lux operon distinguishes two evolutionary distinct clades of *Photobacterium leiognathi*. Archives of Microbiology 181, 352-361.

Beaz-Hidalgo, R., Doce, A., Balboa, S., Barja, J.L., Romalde, J.L. 2010. *Aliivibrio finisterrensis* sp. nov., isolated from Manila clam, *Ruditapes philippinarum* and emended description of the genus *Aliivibrio*. International Journal of Systematic and Evolutionary Microbiology 60, 223-228.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. Ecological monographs 27(4), 325-349.

Camarinha-Silva, A., Jáuregui, R., Chaves-Moreno, D., Oxley, A.P.A., Schaumburg, F., Becker, K., Wos-Oxley, M.L., Pieper, D.H., 2014. Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. Environmental Microbiology 16(9), 2939-2952.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7(5), 335-336.

Chaves-Moreno, D., Plumeier, I., Kahl, S., Krismer, B., Peschel, A., Oxley, A.P.A., Jauregui, R., Pieper, D.H., 2015. The microbial community structure of the cotton rat nose. Environmental Microbiology Reports 7(6), 929-935.

Clarke, K.R., Warwick, R.M.R.M., Laboratory, P.M., 2001. Change in marine communities: an approach to statistical analysis and interpretation: Plymouth, U.K.: PRIMER-E Ltd.

Clements, K.D., Angert, E.R., Montgomery, W.L., Choat, J.H. 2014. Intestinal microbiota in fishes: what's known and what's not. Molecular Ecology 23, 1891-98.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Research 42 (Database issue), D633-D642.

Colston, T.J., Jackson, C.R. 2016. Microbiome evolution along divergent branches of the vertebrate tree of life: what is known and unknown. Molecular Ecology 25, 3776-800.

Dehler, C.E., Secombes, C.J., Martin, S.A.M. 2016. Environmental and physiological factors shape the gut microbiota of Atlantic salmon parr (*Salmo salar* L.). Aquaculture 467, 149-157.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., Relman, D.A. 2005. Diversity of the human intestinal microbial flora. Science 308(5728), 1635-1638.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19), 2460-2461.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nature Methods 10, 996-998.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27(16), 2194-2200.

Federici, E., Rossi, R., Fidati, L., Paracucchi, R., Scargetta, S., Montalbani, E., Franzetti, A., La Porta, G., Fagotti, A., Simoncelli, S., Cenci, G., Di Rosa, I. 2015. Characterization of the skin microbiota in Italian stream frogs (*Rana italica*) infected and uninfected by a cutaneous parasitic disease. Microbes and Environment 30, 262-269.

Forberg, T., Sjulstad, E.B., Bakke, I., Olsen, Y., Hagiwara, A., Sakakura, Y., Vadstein, O. 2016. Correlation between microbiota and growth in Mangrove Killifish (*Kryptolebias marmoratus*) and Atlantic cod (*Gadus morhua*). Scientific Reports 6, 21192.

Fouz, B., Toranzo, A.E., Milán, M., Amaro, C. 2000. Evidence that water transmits the disease caused by the fish pathogen *Photobacterium damselae* subsp. *damselae*. Journal of Applied Microbiology 88, 531-535.

Ghanbari, M., Kneifel, W., Domig, K.J. 2015. A new view of the fish gut microbiome: Advances from next-generation sequencing. Aquaculture 448, 464-475.

Gill, S.R., Pop, M., Deboy, R.T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S., Gordon, J.I., Relman, D.A., Fraser-Ligett, C.M., Nelson, K.E. 2006. Metagenomic analysis of the human distal microbiome. Science 312, 1355–1359.

Labella, A.M., Castro, M.D., Manchado, M., Borrego, J.J. 2018. Description of new and amended clades of the genus *Photobacterium*. Microorganisms 6, 24.

Larsen, A.M. 2014. Studies on the microbiota of fishes and the factors influencing their composition. PhD Thesis. Auburn University, Alabama, 255 pp.

Lederberg, J., McCray, A. T. 2001. Ome SweetOmics-A Genealogical Treasury of Words. The Scientist 15(7), 8-8.

Ley, R.E., Hamady, M., Lozupone C., Turnbaugh, P.J., Ramey, R.R., Bircher, J.S., Schlegel, M.L., Tucker, T.A., Schrenzel, M.D., Knight, R., Gordon, J.I. 2008a. Evolution of mammals and their gut microbes. Science 320(5883), 1647-1651.

Ley, R.E., Lozupone, C.A., Hamady, M., Knight, R., Gordon, J.I. 2008b. Worlds within worlds: evolution of the vertebrate gut microbiota. Nature Reviews Microbiology 6(10), 776-788.

Liu, H., Guo, X., Gooneratne, R., Lai, R., Zeng, C., Zhan, F., Wang, W. 2016. The gut microbiome and degradation enzyme activity of wild freshwater fishes influenced by their trophic levels. Science Reports 6, 24340.

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H., Derome, N. 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Frontiers in Microbiology 5, 1-17.

Llewellyn, M.S., McGinnity, P., Dionne, M., Letourneau, J., Thonier, F., Carvalho, G.R., Creer, S., Derome, N. 2016. The biogeography of the Atlantic salmon (*Salmo salar*) gut microbiome. The ISME Journal 10, 1280-84.

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bøgwald, J., Castex, M., Ringø, E., 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture 302, 1-18.

Parris, D.J., Brooker, R.M., Morgan, M.A., Dixson, D.L., Stewart, F.J. 2016. Whole gut microbiome composition of damselfish and cardinalfish before and after reef settlement. PeerJ 4, e2412.

Pérez, T., Balcázar, J.L., Ruiz-Zarzueka, I., Halaihel, N., Vendrell, D., de Blas, I., Múzquiz, J.L., 2010. Host-microbiota interactions within the fish intestinal ecosystem. Mucosal Immunity 3(4), 355-60.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41, D590-D596.

Rajilić-Stojanović, M., Smidt, H., De Vos, W. M. 2007. Diversity of the human gastrointestinal tract microbiota revisited. Environmental microbiology 9(9), 2125-2136.

Ramirez, C., Romero, J. 2017. The microbiome of *Seriola lalandi* of wild and aquaculture origin reveals differences in composition and potential function. Frontiers in Microbiology 8, 1844.

Rivas, A.J., Lemos, M.L., Osorio, C.R. 2013. *Photobacterium damselae* subsp. *damselae*, a bacterium pathogenic from marine animals and humans. Frontiers in Microbiology 4(283), 1-6.

Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K., Rawls, J.F. 2011. Evidence for a core gut microbiota in the zebrafish. The ISME Journal 5, 1595-608.

Romero, J., Ringø, E., Merrifield, D.L. 2014, 'The gut microbiota of fish', in D.L. Merrifield and E. Ringø (eds.), Aquaculture nutrition: gut health, probiotics and prebiotics (John Wiley & Sons, Ltd), pp. 75-100.

Schulze, A.D., Alabi, A.O., Tattersall-Sheldrake, A.R., Miller, K.M., 2006. Bacterial diversity in a marine hatchery: balance between pathogenic and potentially probiotic bacterial strains. Aquaculture 256, 50-73.

Star, B., Haverkamp, T. H., Jentoft, S., and Jakobsen, K. S. 2013. Next generation sequencing shows high variation of the intestinal microbial species composition in Atlantic cod caught at a single location. BMC Microbiology 13, 248.

Stelzmueller, I., Biebl, M., Wiesmayr, S., Eller, M., Hoeller, E., Fille, M., Weiss, G., Lass-Floerl, C., Bonatti, H. 2006. *Ralstonia pickettii* - Innocent by bystander or a potential threat? Clinical Microbiology and Infection 12, 99-101.

Stephens, F.J., Savage, A. 2010. Two mortality events in sea-caged yellowtail kingfish *Seriola lalandi* Valenciennes, 1833 (Nannopercidae) from Western Australia. Australian Veterinary Journal 88, 414-416.

Szafranska, A.K., Oxley, A.P.A., Chaves-Moreno, D., Horst, S.A., Roßlenbrolch, S., Peters, G., Goldmann, O., Rohde, M., Sinha, B., Pleper, D.H., Löffler, B., Jauregul, R., Wos-Oxley, M.L., Medina, E. 2014. High-resolution transcriptomic analysis of the adaptive responses of *Staphylococcus aureus* during acute and chronic phases of osteomyelitis. mBio 5(6), e01775-14.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology 73(16), 5261-5267.

Wang, W., Zhou, Z., He, S., Liu, Y., Cao, Y., Shi, P., Yao, B., Ringo, E. 2010. Identification of the adherent microbiota on gills and skins of poly-cultured gibel carp (*Carassius auratus gibelio*) and bluntnose black bream (*Megalorama mablycephala* Yih). Aquaculture Research, 1-12.

Webster, T.M.U., Consuegra, S., Hitchings, M., de Leaniz, C.G. 2018. Interpopulation variation in the Atlantic Salmon microbiome reflects environmental and genetic diversity. Applied and Environmental Microbiology 84, e00691-18.

Whitaker, S., Herring, P.J., Campbell, A.K., Hill, P.J., Rees, C.E.D. 2006. 'Characterization of a fish symbiont of *Photobacterium phosphoreum* with altered spectral properties', in A.A. Szalay, P.J. Hill, L.J. Kricka and P.E. Stanley (eds.), Bioluminescence and Chemiluminescence: Chemistry, Biology and Applications (World Scientific Publications), San Diego, California, pp. 35-38.

Wu, S., Gao, T., Zheng, Y., Wang, W., Cheng, Y., Wang, G. 2010. Microbial diversity of intestinal contents and mucus in yellow catfish (*Pelteobagrus fulvidraco*). Aquaculture 303, 1-7.

Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30(5), 614-20.

Zhang, M., Sun, Y., Liu, Y., Qiao, F., Chen, L., Liu, W-T, Du, Z., Li, E. 2016. Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference. Aquaculture 454, 72.

Table 3.3.1.1.1. Sample information pertaining to the offshore (sea) vs onshore (land) component.

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
water sample	-	-	-	AB Hatchery	-	-	5/02/2016	55937	1141
water sample	-	-	-	Lincoln	Point Boston	AB16-1	5/02/2016	104217	1218
wild fish	HG	70.5	4180	tuna boats	Young Bay, KI	-	3/02/2016	35748	47
wild fish	MG	70.5	4180	tuna boats	Young Bay, KI	-	3/02/2016	34603	45
wild fish	HG	60.5	2700	tuna boats	Young Bay, KI	-	3/02/2016	52667	54
wild fish	MG	60.5	2700	tuna boats	Young Bay, KI	-	3/02/2016	50074	60
sea	MH	38		Lincoln	Point Boston	AB16-1	5/02/2016	34994	81
sea	MH	42		Lincoln	Point Boston	AB16-1	5/02/2016	18003	40
sea	MH	37		Lincoln	Point Boston	AB16-1	5/02/2016	26264	170
sea	MH	42		Lincoln	Point Boston	AB16-1	5/02/2016	26897	183
sea	MH	41.5		Lincoln	Point Boston	AB16-1	5/02/2016	6959	156
sea	MH	42		Lincoln	Point Boston	AB16-1	5/02/2016	28740	320
sea	MH	40		Lincoln	Point Boston	AB16-1	5/02/2016	15898	24
sea	MH	42		Lincoln	Point Boston	AB16-1	5/02/2016	25556	42
land	MH	35	440	AB Hatchery	-	-	5/02/2016	39035	218
land	MH	30	450	AB Hatchery	-	-	5/02/2016	36903	222
land	MH	40	1300	AB Hatchery	-	-	5/02/2016	36072	23
land	MH	35	940	AB Hatchery	-	-	5/02/2016	53039	81
land	MH	33	450	AB Hatchery	-	-	5/02/2016	49639	37
land	MH	35	450	AB Hatchery	-	-	5/02/2016	58628	326
land	MH	41	1000	AB Hatchery	-	-	5/02/2016	77446	279
land	MH	39	1060	AB Hatchery	-	-	5/02/2016	22747	171
land	MH	41	1040	AB Hatchery	-	-	5/02/2016	44340	204

Abbreviations: AB, Arno Bay; HG, hindgut; KI, Kangaroo Island; MG, midgut; MH, mid- and hindgut.

Table 3.3.1.1.2. Summary of sequenced sample parameters.

Data-set	No. of samples	Total reads	Median library size	Range	Bacterial OTUs post- filtering
Offshore (land) vs onshore (sea)	23	1,088,094	35,715	5,696 - 101,708	776

Table 3.3.1.1.3. Sample information pertaining to the commercial production cycle component.

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
water sample	-	-	-	Arno Bay	AB3-AB Site 2	AB16-7	21/01/2016	94254	363
wild fish 2	MG	70.5	4180	tuna boats	Young Bay, KI	-	3/02/2016	36443	29
wild fish 2	HG	70.5	4180	tuna boats	Young Bay, KI	-	3/02/2016	35321	23
wild fish 3	MG	60.5	2700	tuna boats	Young Bay, KI	-	3/02/2016	54081	30
wild fish 3	HG	60.5	2700	tuna boats	Young Bay, KI	-	3/02/2016	51237	33
T1, 100 g	MH	19.5	~111.4	Arno Bay	AB3-AB Site 2	AB16-7	21/01/2016	65891	81
T1, 100 g	MH	21.0	~111.4	Arno Bay	AB3-AB Site 2	AB16-7	21/01/2016	32593	101
T1, 100 g	MH	20.0	~111.4	Arno Bay	AB3-AB Site 2	AB16-7	21/01/2016	82723	70
T1, 100 g	MH	20.0	~111.4	Arno Bay	AB3-AB Site 2	AB16-7	21/01/2016	66711	115
T1, 100 g	MH	21.0	~111.4	Arno Bay	AB3-AB Site 2	AB16-7	21/01/2016	83875	89
T2, 500 g	MH	34.5	~470.0	Arno Bay	AB3-AB Site 2	AB16-3	21/01/2016	53348	98
T2, 500 g	MH	33.0	~470.0	Arno Bay	AB3-AB Site 2	AB16-3	21/01/2016	72593	67
T2, 500 g	MH	30.0	~470.0	Arno Bay	AB3-AB Site 2	AB16-3	21/01/2016	34821	76
T2, 500 g	MH	34.0	~470.0	Arno Bay	AB3-AB Site 2	AB16-3	21/01/2016	3711	98
T2, 500 g	MH	32.0	~470.0	Arno Bay	AB3-AB Site 2	AB16-3	21/01/2016	53871	66
T2, 500 g	MH	32.0	~470.0	Arno Bay	AB3-AB Site 2	AB16-3	21/01/2016	6820	89
T3, 1000 g	MH	43.0	~930.0	Arno Bay	AB3-AB Site 2	AB16-3	4/03/2016	57712	33
T3, 1000 g	MH	41.0	~930.0	Arno Bay	AB3-AB Site 2	AB16-3	4/03/2016	28534	34
T3, 1000 g	MH	43.0	~930.0	Arno Bay	AB3-AB Site 2	AB16-3	4/03/2016	52458	24
T3, 1000 g	MH	41.0	~930.0	Arno Bay	AB3-AB Site 2	AB16-3	4/03/2016	39262	41
T4, 2000g	MH	49.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	61887	51
T4, 2000g	MH	55.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	47197	107
T4, 2000g	MH	48.5	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	97372	51
T4, 2000g	MH	47.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	58080	122
T4, 2000g	MH	52.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	30323	74
T4, 2000g	MH	53.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	77515	78
T4, 2000g	MH	52.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	87892	45
T4, 2000g	MH	51.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	36958	77

Abbreviations: AB, Arno Bay; HG, hindgut; KI, Kangaroo Island; MG, midgut; MH, mid- and hindgut

Table 3.3.1.1.4. Summary of sequenced sample parameters post-filtering.

Data-set	No. of samples	Total reads	Median library size	Range	Bacterial OTUs post- filtering
Commercial production cycle	28	1,534,702	54,393	4048-97,470	533

Table 3.3.1.1.5. One-way PERMANOVA: Main test to determine if there is any significant difference in microbiome composition between the four size classes (100 g, 500 g, 1000 g and 2000 g).[1]

Source	df	SS	MS	Pseudo-F	P
Size class (g)	3	31249	10416	4.0139	**0.0001**
Residual	19	49306	2592.1		
Total	22	80555			

[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.1.6. One-way PERMANOVA: Pairwise test between the four size classes.[1]

Size class (g)	P	Significant?
100, 500	0.6274	No
100, 1000	**0.0158**	Yes
100, 2000	**0.0024**	Yes
500, 1000	**0.0356**	Yes
500, 2000	**0.0030**	Yes
1000, 2000	**0.0040**	Yes

[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.1.7. The bacterial taxa that have the greatest contribution towards the observed difference between 500 g (Δ) and 1000 g (O) size classes.[1]

Bacterial taxa (RDP similarity [S_ab] score)_OTU no.	Av. abundance 500 g (Δ)	Av. abundance 1000 g (O)	% Contribution	Highest abundant group
Vibrio ichthyoenteri/*V. scophthalmi* (1.000)_OTU 12	0.51	47.96	26.43	O
Photobacterium damselae subsp. *damselae*/*P. leiognathi* (1.000)_OTU 3	24.68	18.95	16.82	Δ
Synechococcus sp. (0.978)_OTU 29	20.65	0.47	11.13	Δ
Synechococcus sp. (1.000)_OTU 23	13.75	0.12	7.51	Δ
Ehrlichia sp. trout isolate (0.853)_OTU 16	10.96	0.08	6.06	Δ
Acinetobacter junii (1.000)_OTU 79	0.02	7.72	4.26	O
Brevinema andersonii (0.632)_OTU 2	7.41	0.99	4.2	Δ
Acinetobacter sp. (0.930)_OTU 6090	0	5.27	2.91	O
Vibrio harveyi/Aliivibrio fischeri (1.000)_OTU 162	0.26	4.39	2.39	O
Acinetobacter johnsonii (0.922)_OTU 5143	0.03	3.93	2.17	O
Acinetobacter sp. (0.963)_OTU 7760	0.07	2.2	1.23	O
alpha *proteobacterium* (1.000)_OTU 10	1.83	0	1.01	Δ
Pseudoalteromonas sp. (1.000)_OTU 140	1.61	0.01	0.88	Δ
Intrasporangium calvum/Terracoccus luteus (0.700)_OTU 194	1.57	0.06	0.84	Δ
Anabaena cylindrica (0.376)_OTU 15	0.09	1.44	0.82	O
Synechococcus sp. (0.978)_OTU 705	1.33	0.03	0.72	Δ
Pseudomonas stutzeri (1.000)_OTU 100	0.16	1.13	0.67	O

[1] The discernible bacterial taxa were derived using the Similarity Percentage (SIMPER) algorithm in the PRIMER program, setting a cut-off at 90% cumulative contribution, in order to give only the top few bacterial taxa that contribute to the difference between the groups.

Table 3.3.1.1.8. The bacterial taxa that have the greatest contribution towards the observed difference between 1000 g (Δ) and 2000 g (O) size classes.

Bacterial (RDP similarity [S_ab] score)_OTU no.	Av. abundance 1000 g (Δ)	Av. abundance 2000 g (O)	% Contribution	Highest abundant group
Vibrio sp. V776/*Aliivibrio finisterrensi* (1.000)_OTU 4	0.01	56.89	28.93	O
Vibrio ichthyoenteri/V. scophthalmi (1.000)_OTU 12	47.96	0.03	24.38	Δ
Photobacterium damselae subsp. *damselae/P. leiognathi* (1.000)_OTU 3	18.95	0.05	9.62	Δ
Acinetobacter junii (1.000)_OTU 79	7.72	0.02	3.93	Δ
Acinetobacter sp. (0.930)_OTU 6090	5.27	0.09	2.71	Δ
Pseudomonas veronii/P. azotoformans/P. chlororaphis subsp. *aureofaciens*_ (1.000) OTU 14	0.28	4.82	2.49	O
Vibrio harveyi/Aliivibrio fischeri (1.000)_OTU 162	4.39	0	2.23	Δ
Geobacillus stearothermophilus/G. thermoparaffinivorans (1.000)_OTU 45	0	4.38	2.23	O
Acinetobacter johnsonii (0.922)_OTU 5143	3.93	0.11	2.03	Δ
Synechococcus sp. (0.978)_OTU 29	0.47	3.99	1.97	O
Synechococcus sp. (1.000)_OTU 23	0.12	3.82	1.92	O
Brevinema andersonii (0.632)_OTU 2	0.99	3.29	1.77	O
Acinetobacter sp. (0.963)_OTU 7760	2.2	0.04	1.13	Δ
Bacillus sp./*Geobacillus stearothermophilus* (0.977)_OTU 234	0.03	2.18	1.11	O
Bacillus smithii (1.000)_OTU 83	0	2.15	1.09	O
Anabaena cylindrica (0.376)_OTU 15	1.44	0.52	0.86	Δ
Fusobacterium mortiferum (0.841)_OTU 86	0	1.56	0.79	O
Lactobacillus delbrueckii subsp. *bulgaricus* (1.000)_OTU 279	0	1.26	0.64	O
Clostridium novyi (1.000)_OTU 402	0	1.19	0.6	O

[1] The discernible bacterial taxa were derived using the Similarity Percentage (SIMPER) algorithm in the PRIMER program, setting a cut-off at 90% cumulative contribution, in order to give only the top few bacterial taxa that contribute to the difference between the groups.

Table 3.3.1.1.9. ANOVA results for diversity indices across size classes comparing 100 g, 500 g, 1000 g and 2000 g fish.

Diversity measure	ANOVA summary[1]	Tukey's posthoc test	Adjusted P-value
Species richness (S)	F=7.151		
	P=0.0021		
		100 v 500	0.9991
		100 v 1000	**0.0033**
		100 v 2000	0.7646
		500 v 1000	**0.0031**
		500 v 2000	0.8147
		1000 v 2000	**0.0104**
Pielou's evenness (J')	F=0.6083		
	P=0.6177		
Shannon's diversity (H')	F=0.9239		
	P=0.4482		
Simpson's diversity (1-λ)	F=1.502		
	P=0.2461		
Delta+ (Δ+)	F=2.323		
	P=0.1075		
Lambda+ (λ+)	F=2.162		
	P=0.1260		

[1] Where a significant difference ($P < 0.05$) is observed then the Tukey's pairwise test was performed.

Table 3.3.1.1.10. Sample information pertaining to the wild vs farmed YTK component.

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
water sample	-	-	-	Hummocks			5/02/2018	55937	213
wild fish	HG	70.5	4180	tuna boats	Young Bay, KI		3/02/2016	35748	31
wild fish	MG	70.5	4180	tuna boats	Young Bay, KI		3/02/2016	34603	35
wild fish	HG	60.5	2700	tuna boats	Young Bay, KI		3/02/2016	52667	40
wild fish	MG	60.5	2700	tuna boats	Young Bay, KI		3/02/2016	50074	37
wild charter	MG	78	5800	Hummocks			5/02/2018	102337	78
wild charter	HG	78	5800	Hummocks			5/02/2018	62313	74
wild charter	MG	92	8900	Hummocks			5/02/2018	139085	56
wild charter	HG	92	8900	Hummocks			5/02/2018	192088	59
wild charter	MG	61	2850	Hummocks			5/02/2018	138972	35
wild charter	HG	61	2850	Hummocks			5/02/2018	148410	52
wild charter	HG	67	5400	Hummocks			5/02/2018	67468	39
wild charter	HG	106	12400	Hummocks			6/02/2018	117813	67
wild charter	MG	78.5	5500	Hummocks			6/02/2018	48523	66
wild charter	HG	78.5	5500	Hummocks			6/02/2018	105240	71
wild charter	MG	76	5190	Hummocks			6/02/2018	91954	65
wild charter	HG	76	5190	Hummocks			6/02/2018	108350	42
sea	MH	38		Lincoln	Point Boston	AB16-1	5/02/2016	34975	44
sea	MH	42		Lincoln	Point Boston	AB16-1	5/02/2016	24593	26
sea	MH	37		Lincoln	Point Boston	AB16-1	5/02/2016	27629	75
sea	MH	42		Lincoln	Point Boston	AB16-1	5/02/2016	28732	73
sea	MH	41.5		Lincoln	Point Boston	AB16-1	5/02/2016	12061	71
sea	MH	42		Lincoln	Point Boston	AB16-1	5/02/2016	28801	108
sea	MH	40		Lincoln	Point Boston	AB16-1	5/02/2016	17832	20
sea	MH	42		Lincoln	Point Boston	AB16-1	5/02/2016	25621	21
land	MH	35	440	AB Hatchery			5/02/2016	38361	81
land	MH	30	450	AB Hatchery			5/02/2016	35128	83
land	MH	40	1300	AB Hatchery			5/02/2016	19964	12
land	MH	35	940	AB Hatchery			5/02/2016	45010	42
land	MH	33	450	AB Hatchery			5/02/2016	49639	18
land	MH	35	450	AB Hatchery			5/02/2016	56237	73
land	MH	41	1000	AB Hatchery			5/02/2016	66447	31
land	MH	39	1060	AB Hatchery			5/02/2016	19677	49
land	MH	41	1040	AB Hatchery			5/02/2016	42489	39
T1, 100 g	MH	19.5	~111.4	Arno Bay	AB3-AB Site 2	AB16-7	21/01/2016	61284	75
T1, 100 g	MH	21.0	~111.4	Arno Bay	AB3-AB Site 2	AB16-7	21/01/2016	38009	81
T1, 100 g	MH	20.0	~111.4	Arno Bay	AB3-AB Site 2	AB16-7	21/01/2016	56858	64
T1, 100 g	MH	20.0	~111.4	Arno Bay	AB3-AB Site 2	AB16-7	21/01/2016	62042	92
T1, 100 g	MH	21.0	~111.4	Arno Bay	AB3-AB Site 2	AB16-7	21/01/2016	72674	72
T2, 500 g	MH	34.5	~470.0	Arno Bay	AB3-AB Site 2	AB16-3	21/01/2016	49682	90
T2, 500 g	MH	33.0	~470.0	Arno Bay	AB3-AB Site 2	AB16-3	21/01/2016	72042	60
T2, 500 g	MH	30.0	~470.0	Arno Bay	AB3-AB Site 2	AB16-3	21/01/2016	46429	70
T2, 500 g	MH	34.0	~470.0	Arno Bay	AB3-AB Site 2	AB16-3	21/01/2016	27098	86
T2, 500 g	MH	32.0	~470.0	Arno Bay	AB3-AB Site 2	AB16-3	21/01/2016	28510	64
T2, 500 g	MH	32.0	~470.0	Arno Bay	AB3-AB Site 2	AB16-3	21/01/2016	20612	86
T3, 1000 g	MH	43.0	~930.0	Arno Bay	AB3-AB Site 2	AB16-3	4/03/2016	58872	30
T3, 1000 g	MH	41.0	~930.0	Arno Bay	AB3-AB Site 2	AB16-3	4/03/2016	31682	36
T3, 1000 g	MH	43.0	~930.0	Arno Bay	AB3-AB Site 2	AB16-3	4/03/2016	53147	24

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
T3, 1000 g	MH	41.0	~930.0	Arno Bay	AB3-AB Site 2	AB16-3	4/03/2016	39885	40
T4, 2000 g	MH	49.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	83260	45
T4, 2000 g	MH	55.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	62027	86
T4, 2000 g	MH	48.5	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	98318	52
T4, 2000 g	MH	47.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	60525	99
T4, 2000 g	MH	52.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	40773	70
T4, 2000 g	MH	53.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	78535	69
T4, 2000 g	MH	52.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	88735	39
T4, 2000 g	MH	51.0	~1950.0	Arno Bay	AB3-AB Site 2	AB16-3	18/07/2016	35429	67

Abbreviations: AB, Arno Bay; HG, hindgut; KI, Kangaroo Island; MG, midgut; MH, mid- and hindgut.

Table 3.3.1.1.11. Summary of sequenced sample parameters.

Data-set	No. of samples	Total reads	Median library size	Range	Bacterial OTUs post- filtering
wild vs farmed	57	3,459,141	60,687	12,061-192,088	397

Table 3.3.1.1.12. ANOVA results for diversity indices comparing wild vs farmed fish.

Diversity measure	ANOVA summary[1]	Tukey's posthoc test	Adjusted P-value
Species richness (S)	F=2.983 ***P*=0.0111**	[2]	
Pielou's evenness (J')	F=2.878 ***P*=0.0136**		
		Wild charter v land	**0.0203**
Shannon's diversity (H')	F=2.836 *P*=0.0148		
		Wild charter v land	**0.0212**
Simpson's diversity (1-λ)	F=3.711 ***P*=0.0028**		
		'Wild' v land	**0.0439**
		Wild charter v land	**0.0023**
Delta+ (Δ+)	F=2.219 *P*=0.050		
Lambda+ (λ+)	F=1.904 *P*=0.0895		

[1] Where a significant difference ($P < 0.05$) is observed then the Tukey's pairwise test was performed.
[2] Significant pairwise comparison not shown – between farmed samples only as presented in Table 3.3.1.1.9.

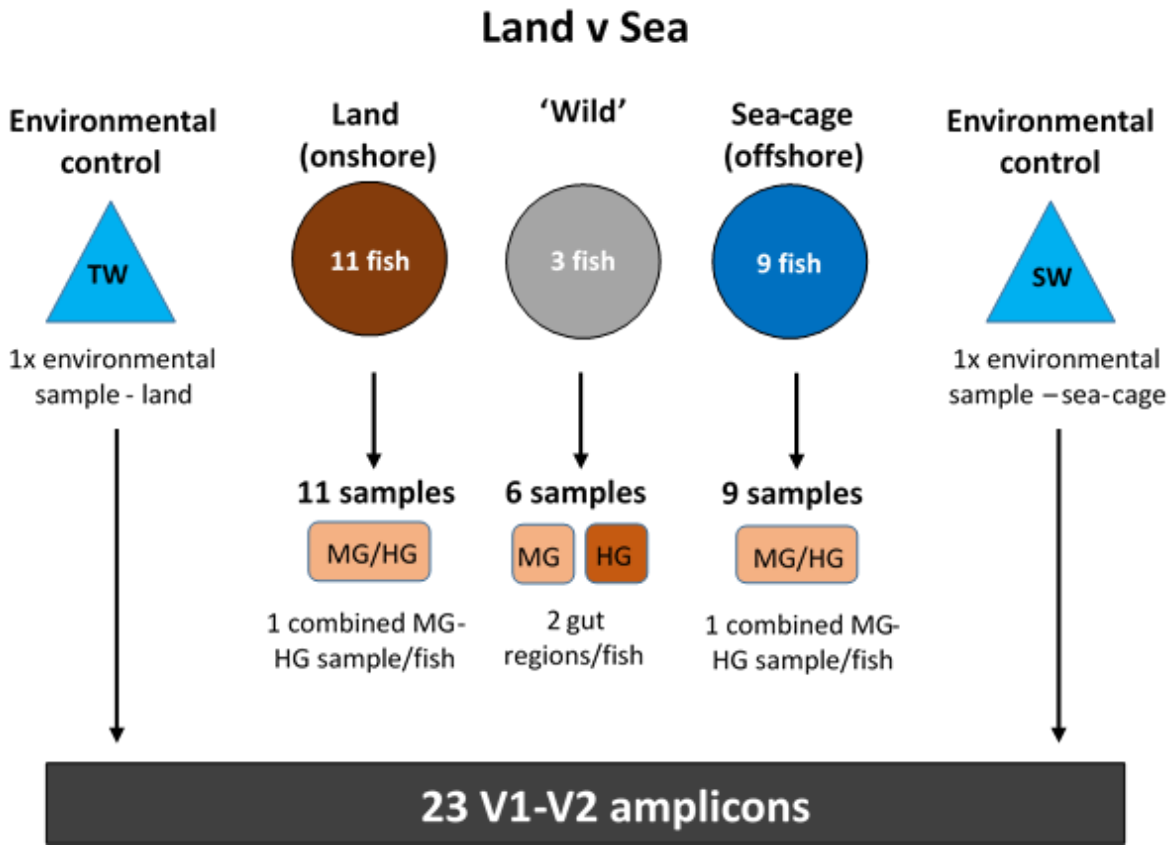


Figure 3.3.1.1.1. Experimental design for onshore (land-based tank system) vs offshore (sea-cage) component.
Abbreviations: HG, hindgut; MG, midgut; SW, seawater; TW, tank water.

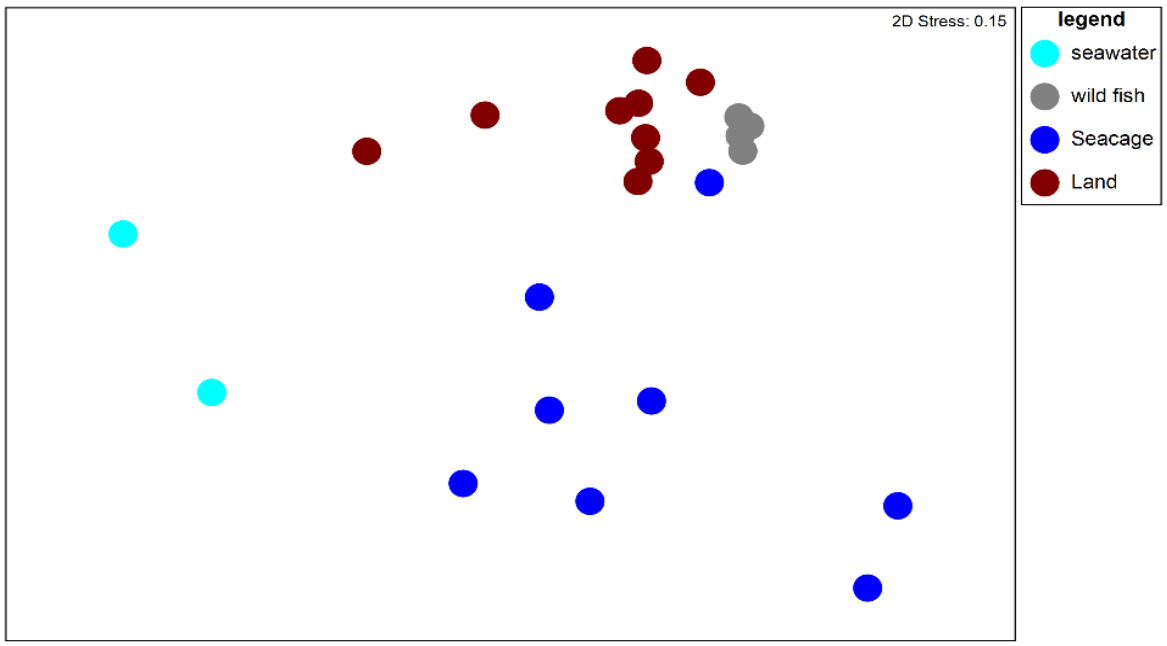


Figure 3.3.1.1.2. Differences between the global bacterial community structures of all 23 samples as analysed by non-metric multidimensional scaling (nMDS).[1]
[1] Gut scrapings from the 2 gut regions midgut and hindgut of 2 'wild' YTK and a combined mid- and hindgut scraping of 8 offshore sea-cage YTK and 9 onshore tank-housed YTK and 2 water samples.

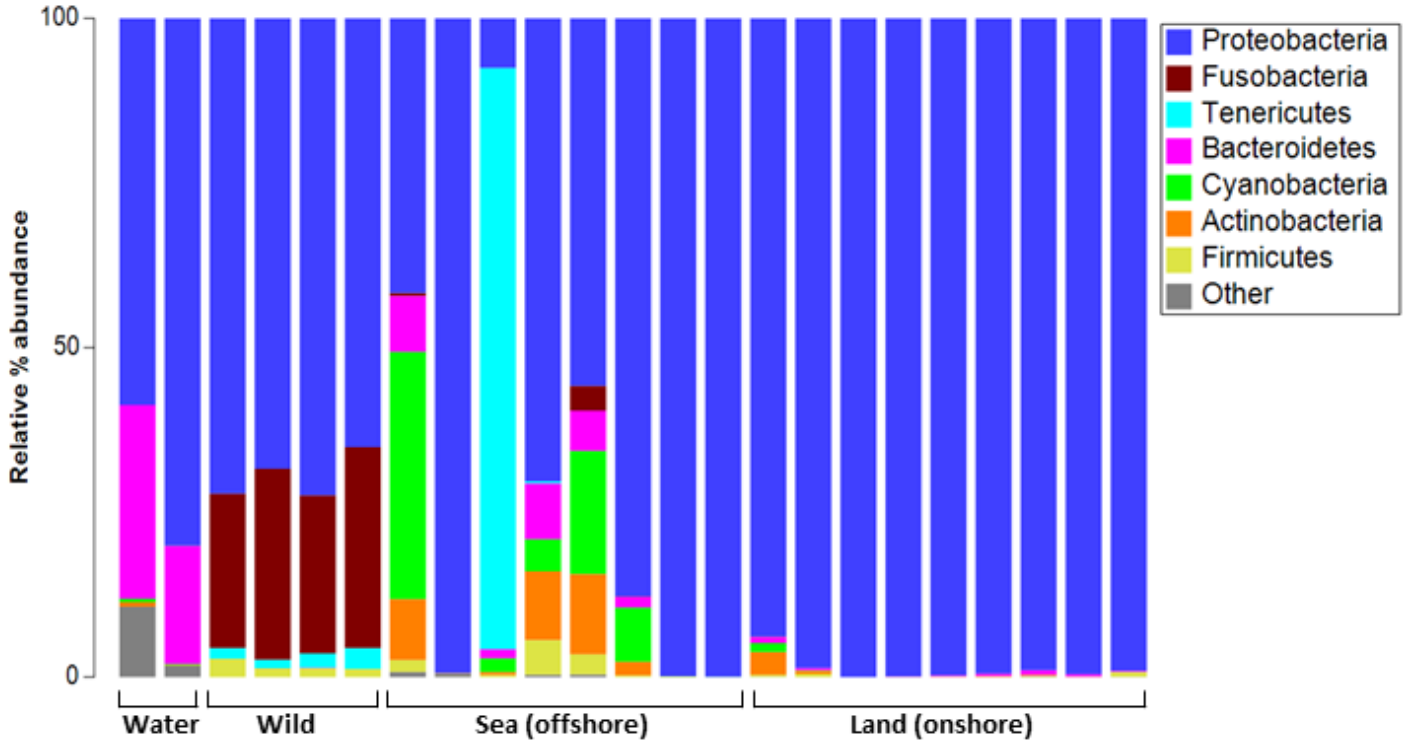


Figure 3.3.1.1.3. Relative percent abundance of bacterial phyla associated with the environmental water samples and gut scrapings of YTK from 'wild', offshore sea-cages and onshore, land-based tank systems.
Abbreviations: SW, seawater; TW, tank water.

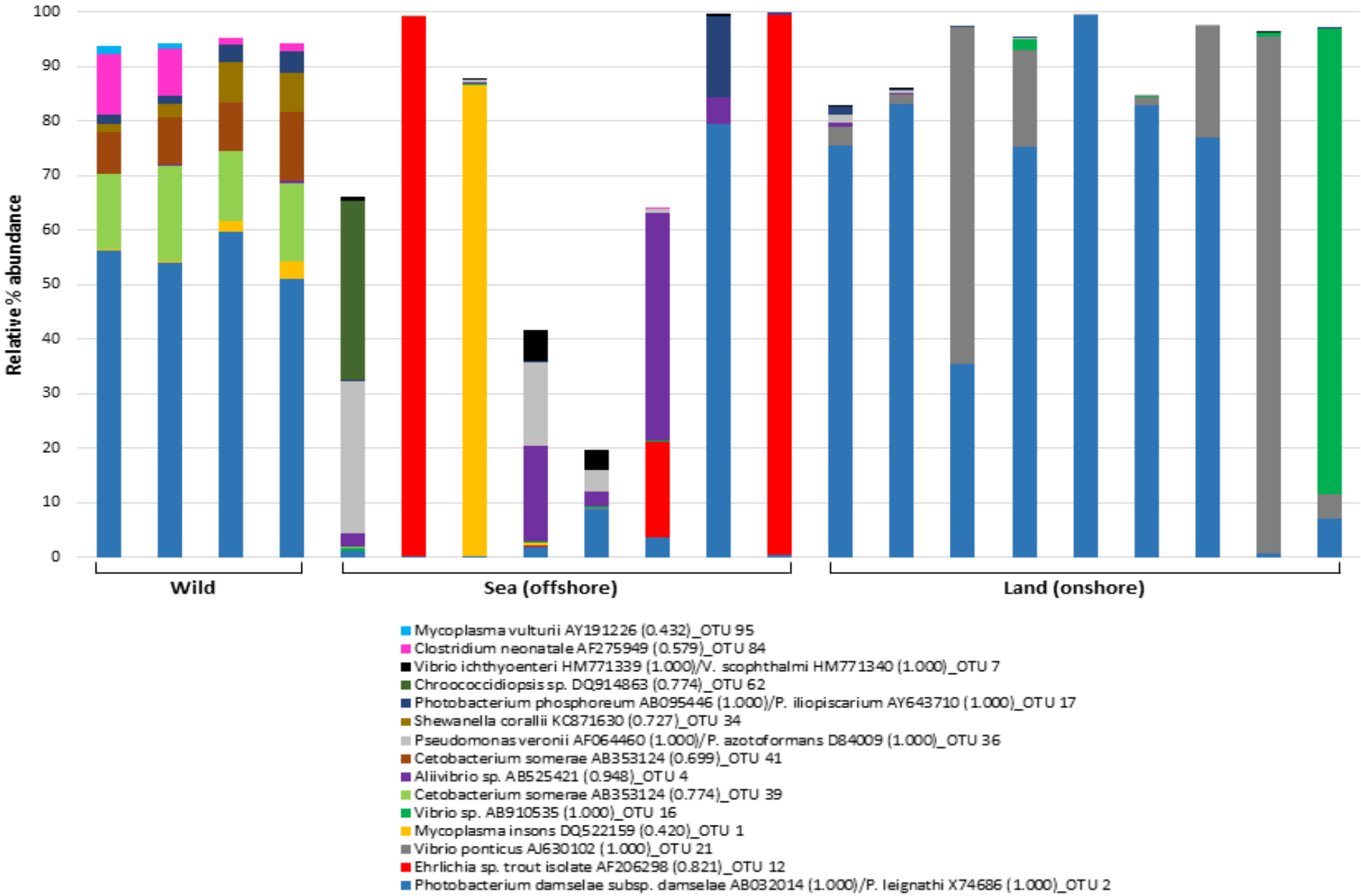


Figure 3.3.1.1.4. Relative percent abundance of the 15 most abundant bacterial OTUs in gut samples from 'wild' YTK and farmed YTK from offshore sea-cages and onshore, land-based tank systems.

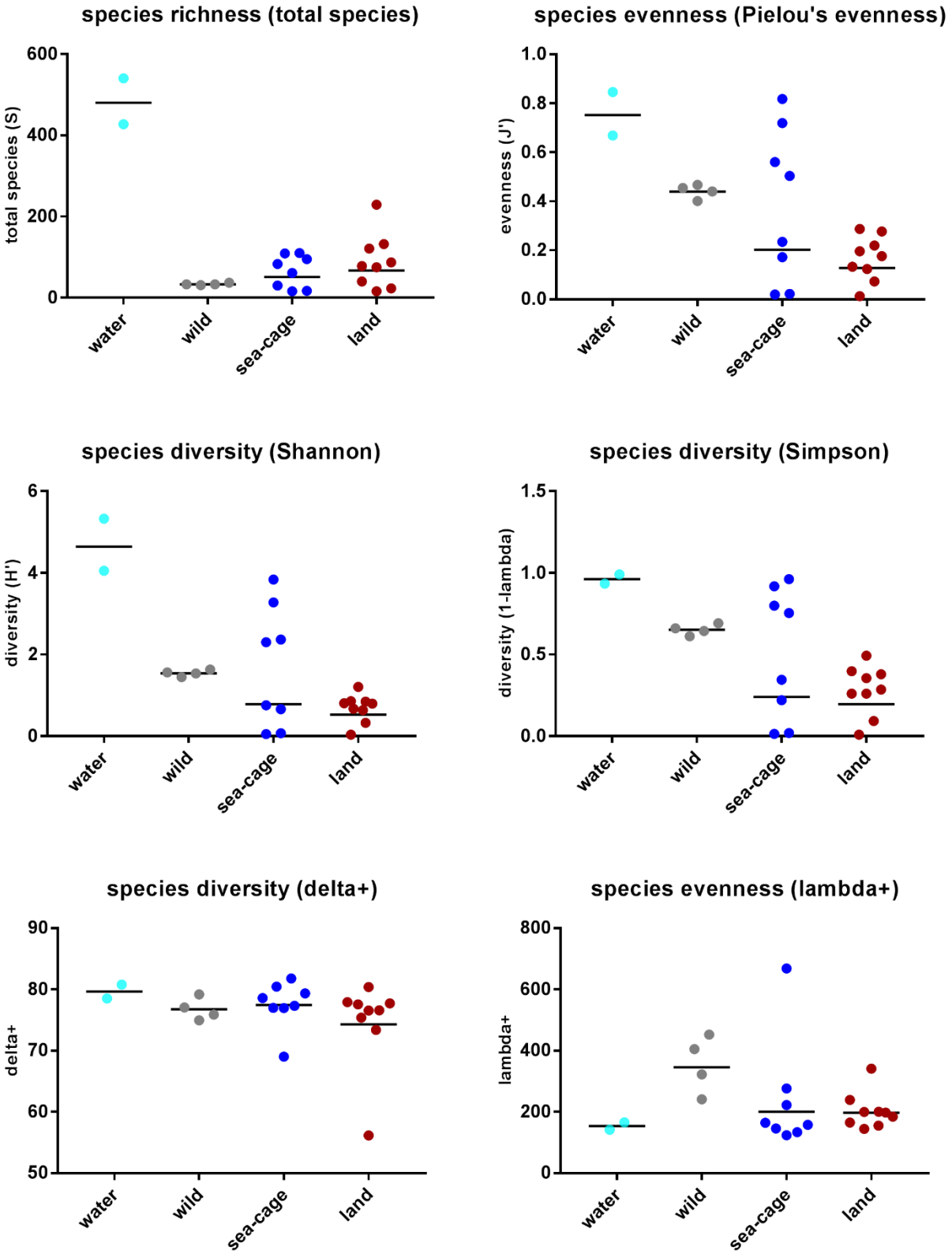


Figure 3.3.1.1.5. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+).[1]

[1] Mean values are plotted for each of the groups of interest (seawater sample vs 'wild' YTK vs farmed sea-cage YTK vs land [tank-reared] YTK).

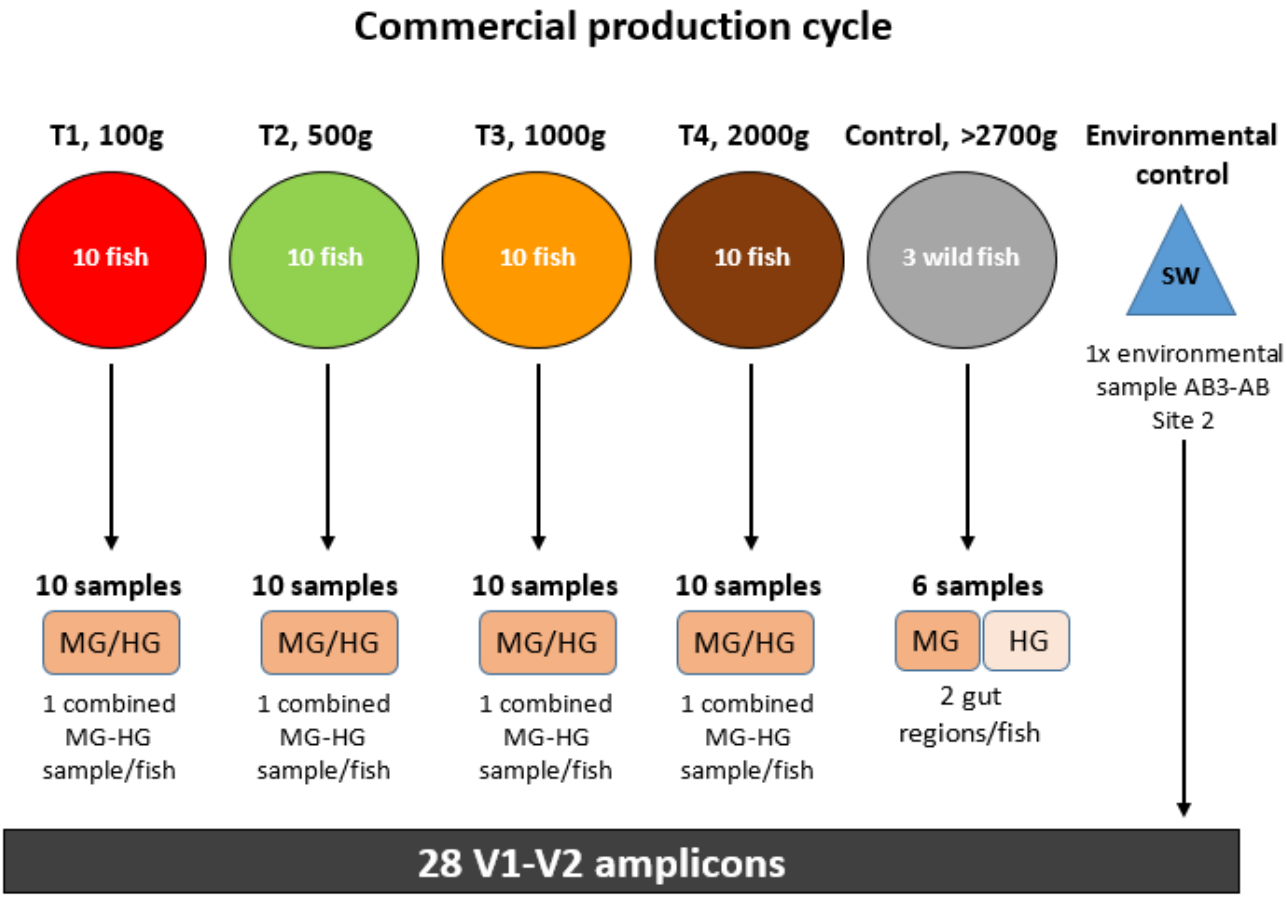


Figure 3.3.1.1.6. Experimental design for the commercial production cycle component comparing major size classes of YTK.
Abbreviations: AB, Arno Bay; FG, foregut; HG, hindgut; MG, midgut; SW, seawater.

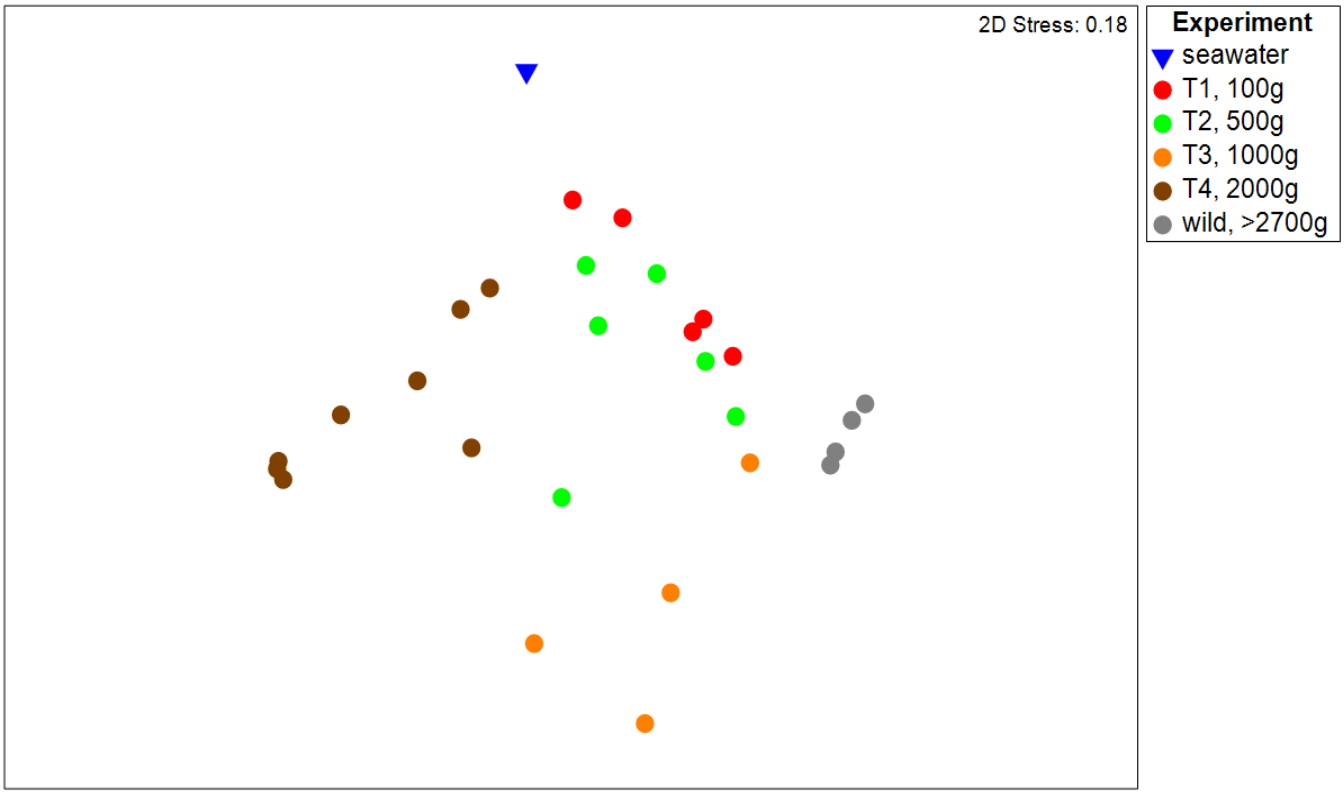


Figure 3.3.1.1.7. Differences between the global bacterial community structures of all 28 samples from the commercial production cycle component comparing major size classes of farmed YTK, as analysed by non-metric multidimensional scaling (nMDS).[1]

[1] Gut scrapings from 2 gut regions midgut and hindgut of 2 'wild' YTK >2700 g and a combined midgut and hindgut scraping of 5x 100 g YTK, 6x 500 g YTK, 4x 1000 g YTK and 8x 2000 g YTK and 1x seawater sample.

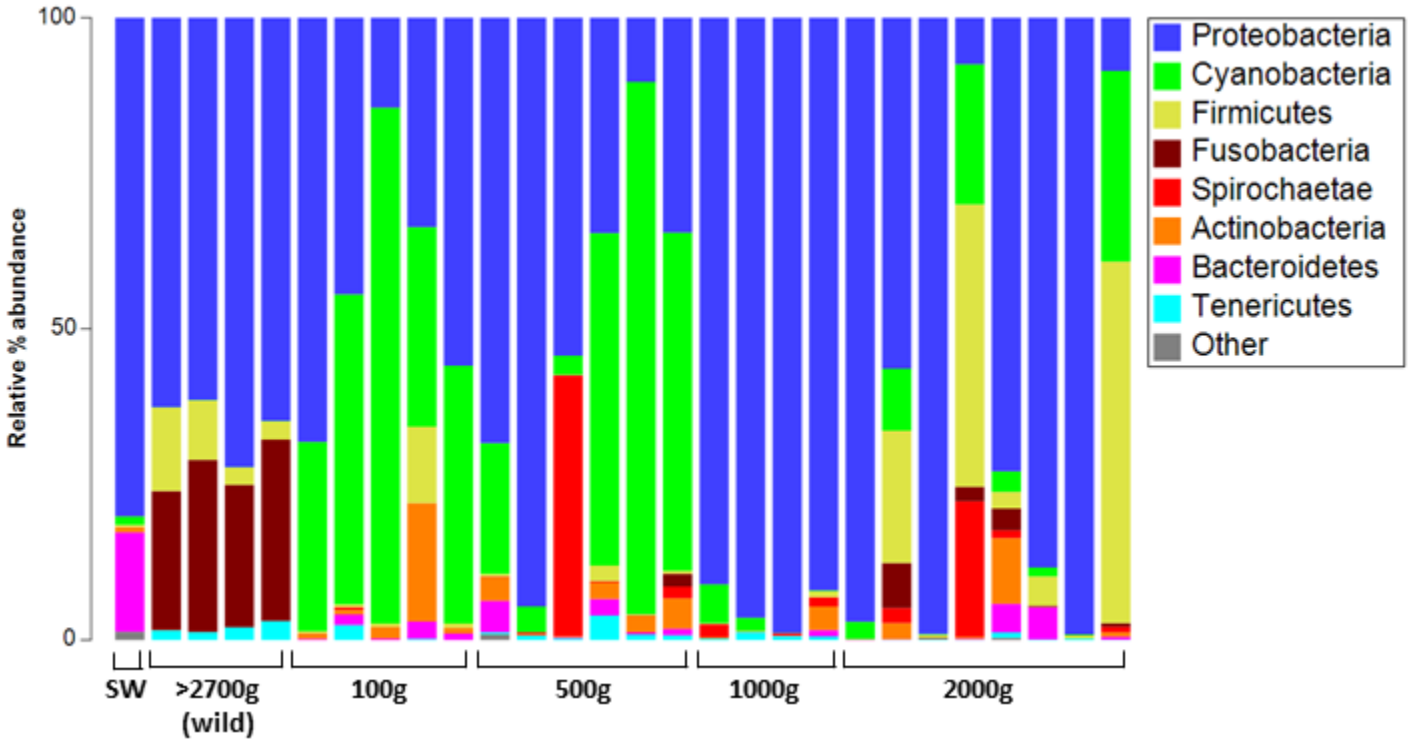


Figure 3.3.1.1.8. Relative percent abundance of bacterial phyla from gut scrapings from farmed YTK across major size classes (100 g, 500 g, 1000 g, 2000 g), gut scrapings from 'wild' YTK (>2700 g) and a seawater sample.
Abbreviations: SW, seawater.

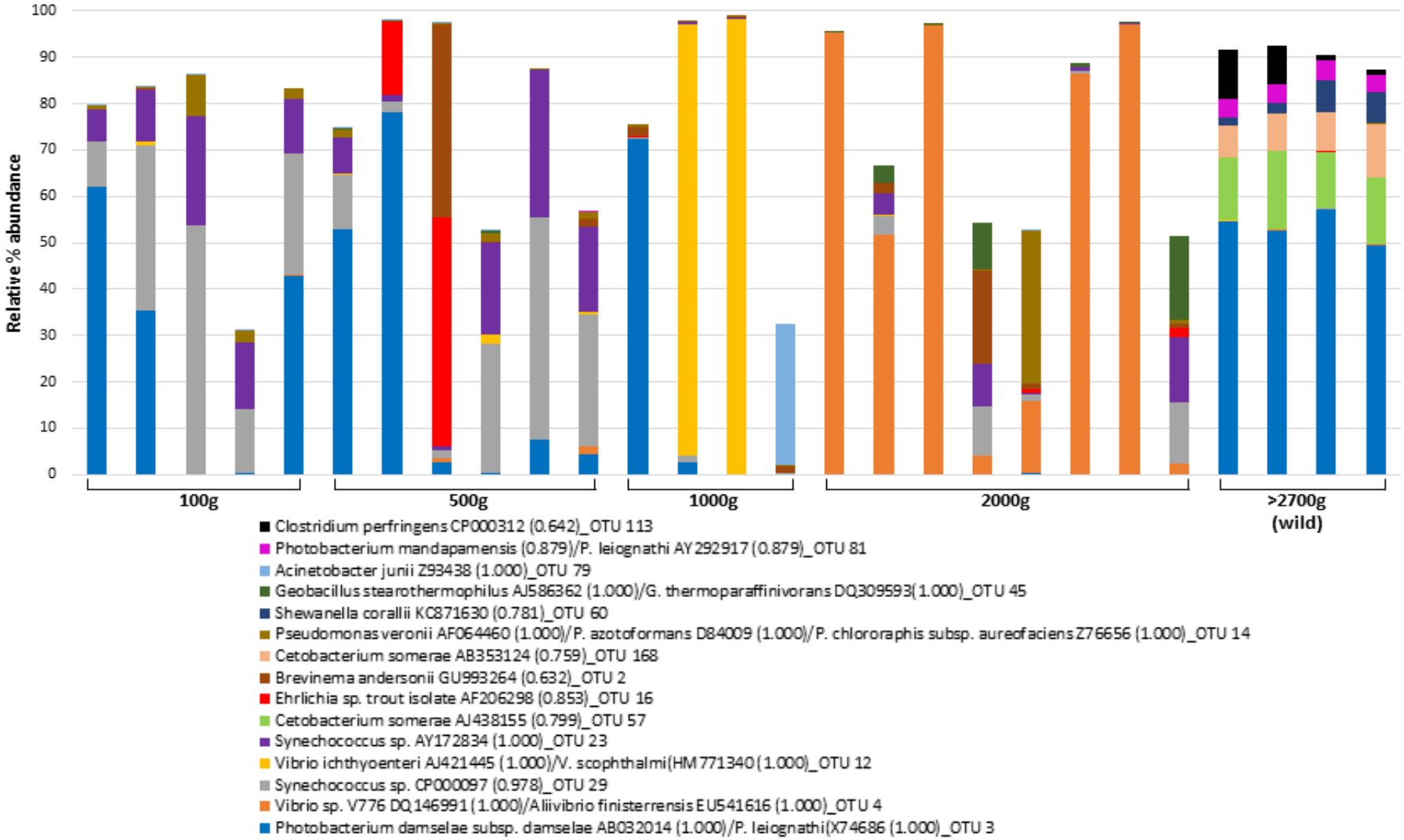


Figure 3.3.1.1.9. Relative percent abundance of the 15 most abundant bacterial OTUs from gut scraping samples collected from farmed YTK across major size classes (100 g, 500 g, 1000 g and 2000 g), and gut scrapings from 'wild' YTK (>2700 g).

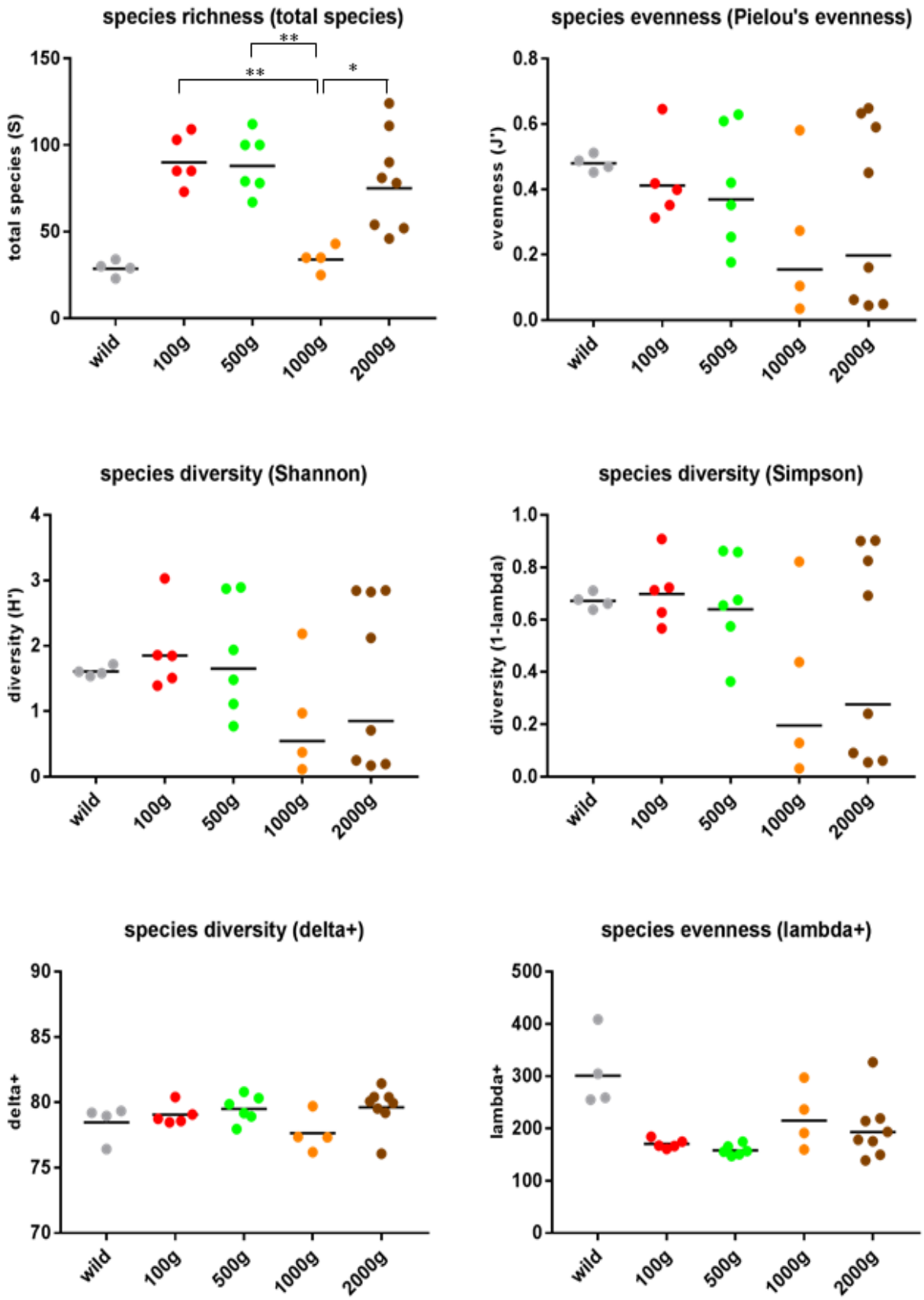


Figure 3.3.1.1.10. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+).[1]

[1] Mean values are plotted for each of the groups of interest – 'wild' YTK (grey) and farmed 100 g (red), 500 g (green), 1000 g (orange) and 2000 g YTK (brown).

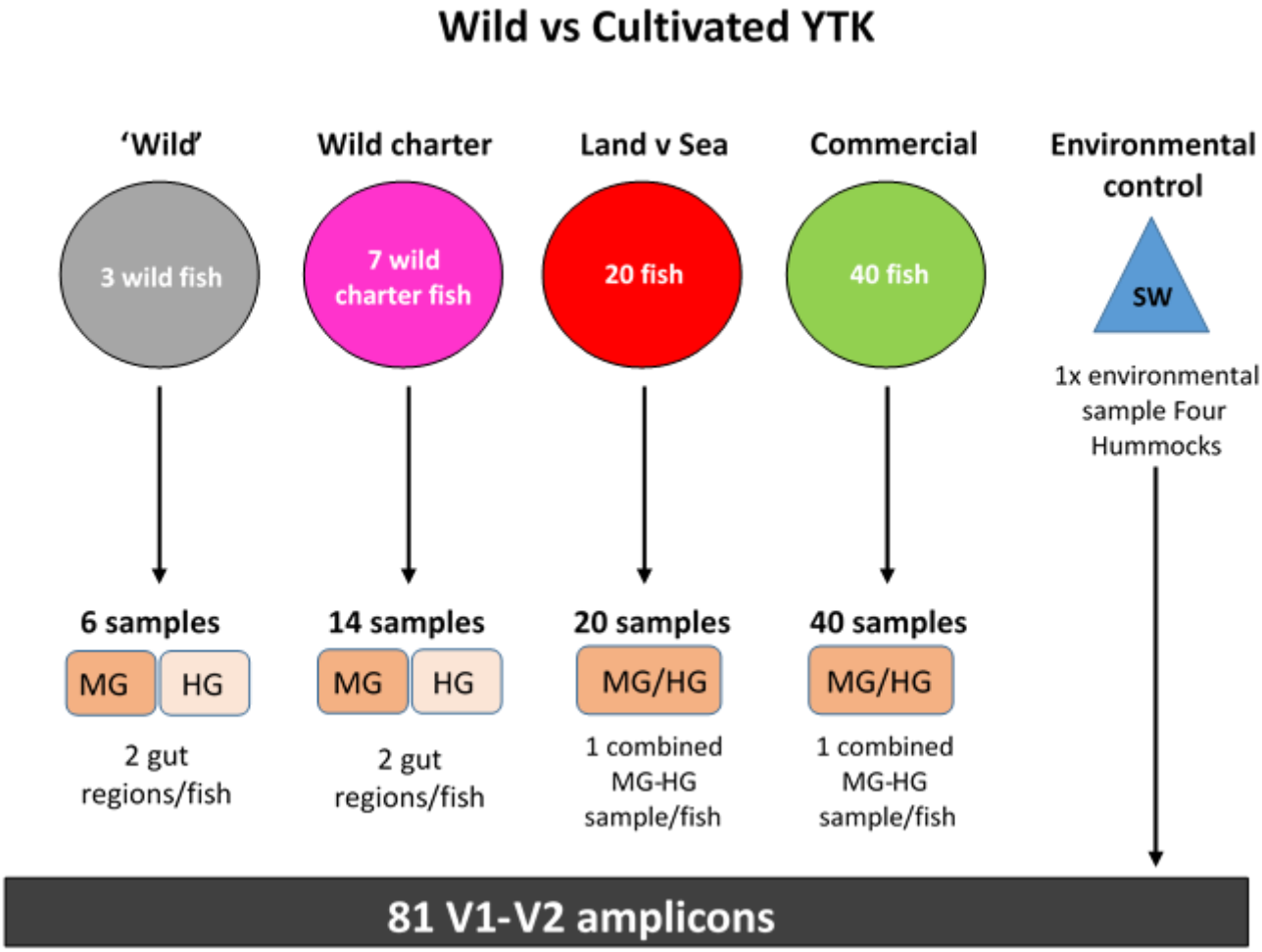


Figure 3.3.1.1.11. Experimental design for the wild vs farmed YTK component.
Abbreviations: HG, hindgut; MG, midgut; SW, seawater.

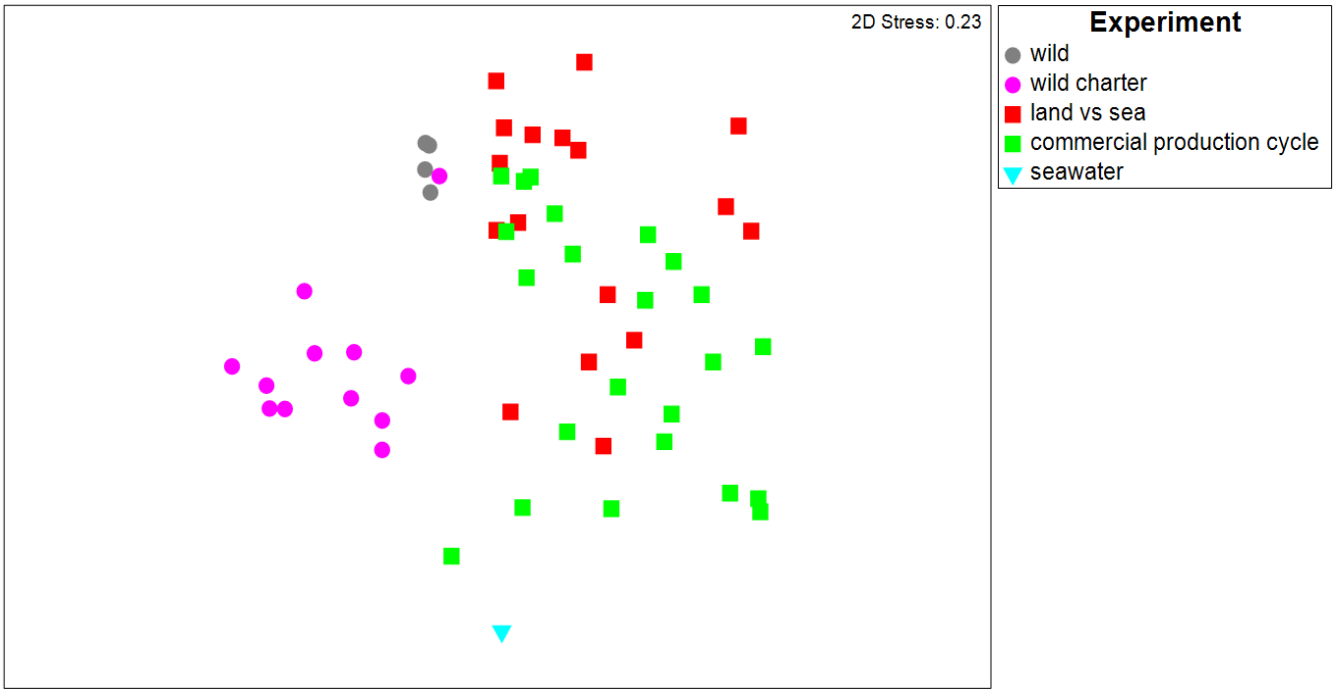


Figure 3.3.1.1.12. Differences between the global bacterial community structures of all 57 samples from wild (circles) and farmed (squares) YTK plus a seawater sample from the wild charter fishing site (triangle) as analysed by non-metric multidimensional scaling (nMDS).

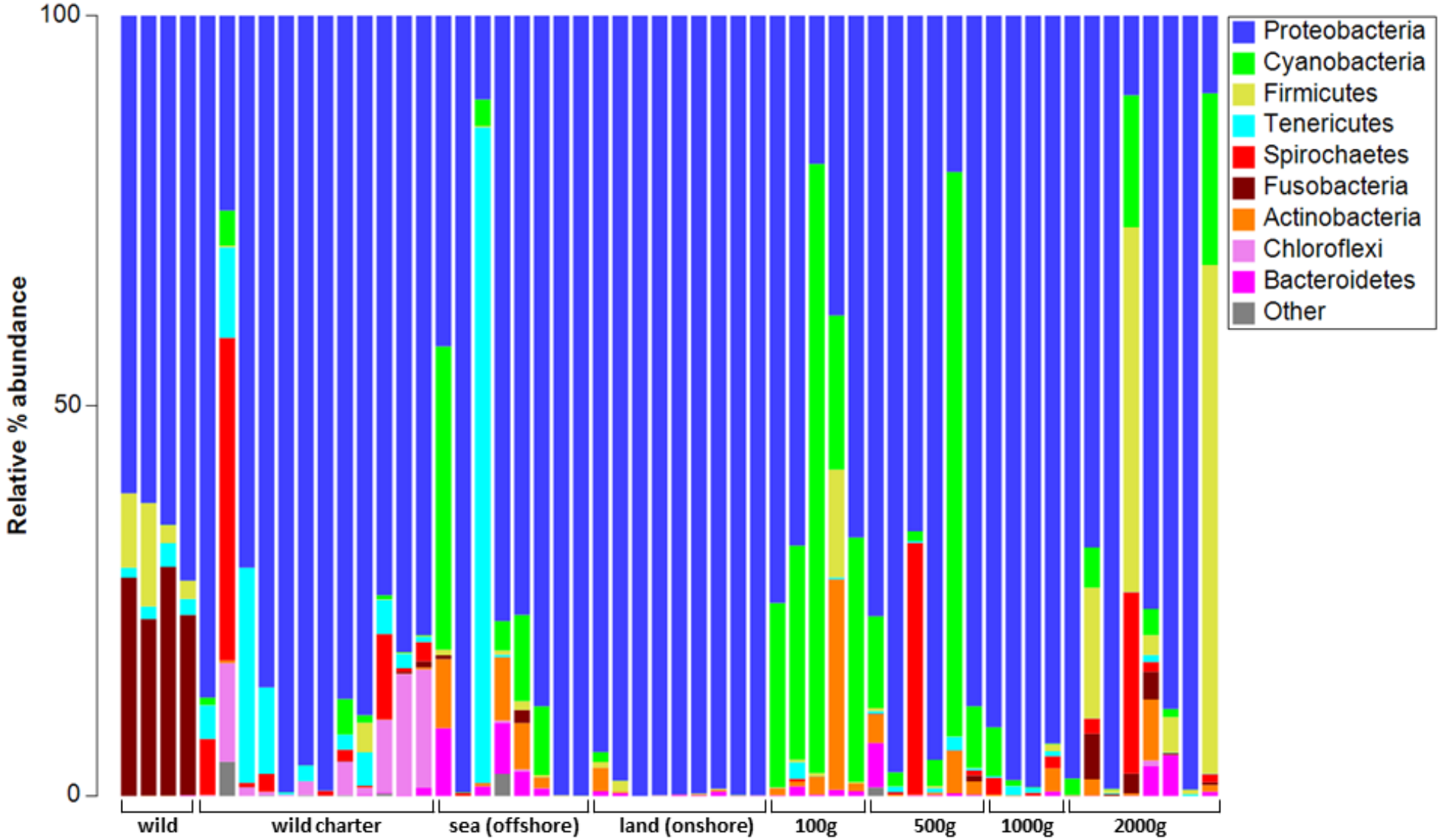


Figure 3.3.1.1.13. Relative percent abundance of bacterial phyla associated with the gut scrapings of YTK from 'wild', wild charter, offshore sea-cages and onshore (land-based) tank systems, and across the major size classes associated with the commercial production cycle (100 g, 500 g, 1000 g and 2000 g).

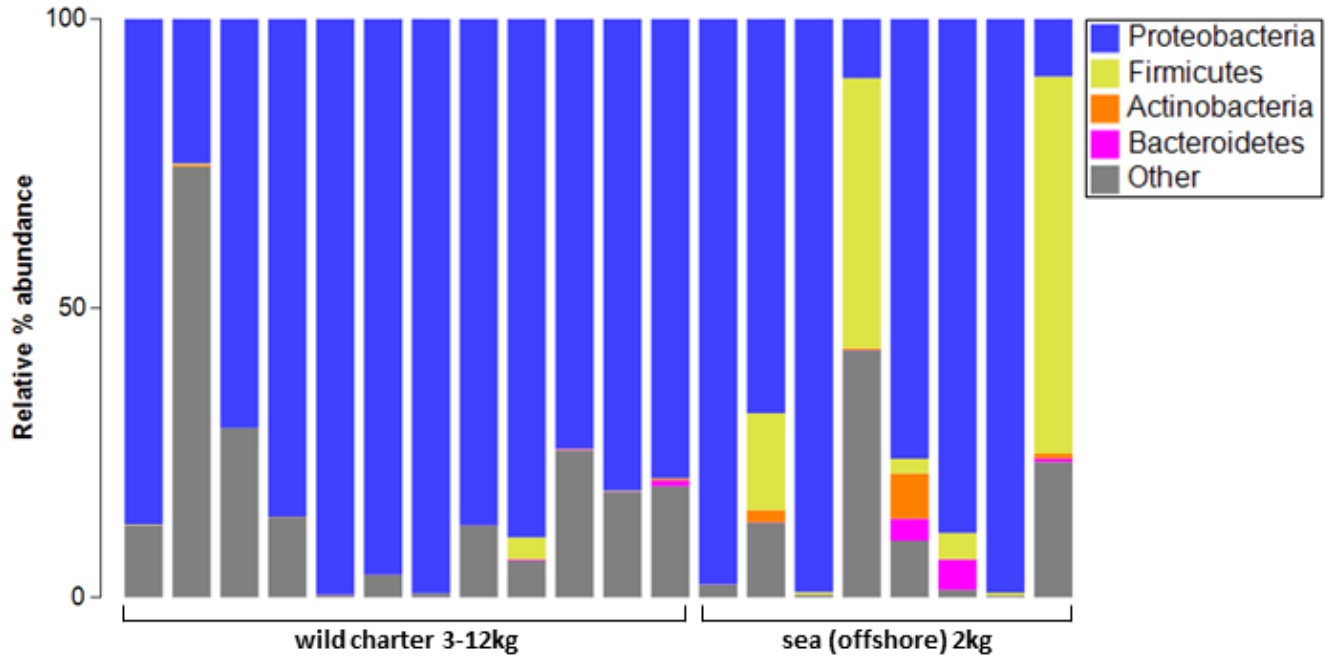


Figure 3.3.1.1.14. Relative percent abundance of bacterial phyla associated with the gut scrapings of wild YTK (obtained from charter operations) vs offshore (sea-cage) farmed 2 kg+ YTK.

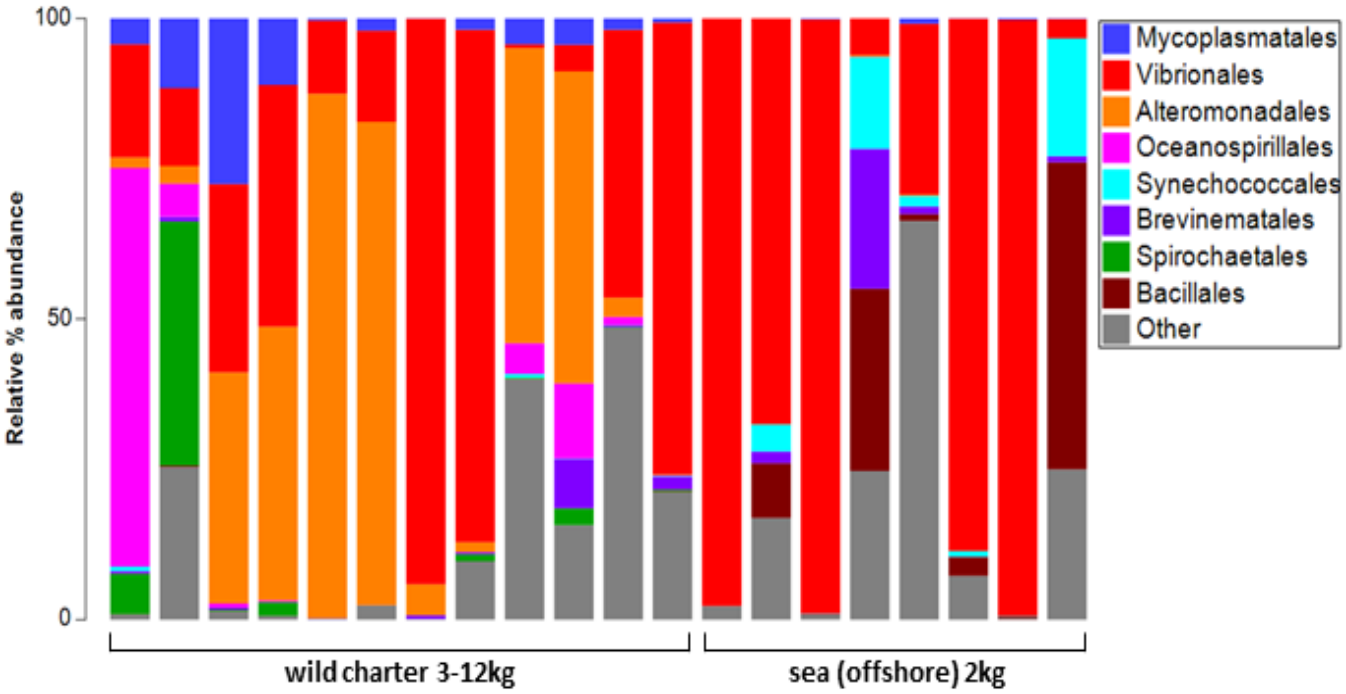


Figure 3.3.1.1.15. Relative percent abundance of bacterial orders associated with the gut scrapings of wild YTK (obtained from charter operations) vs offshore (sea-cage) farmed 2 kg+ YTK.

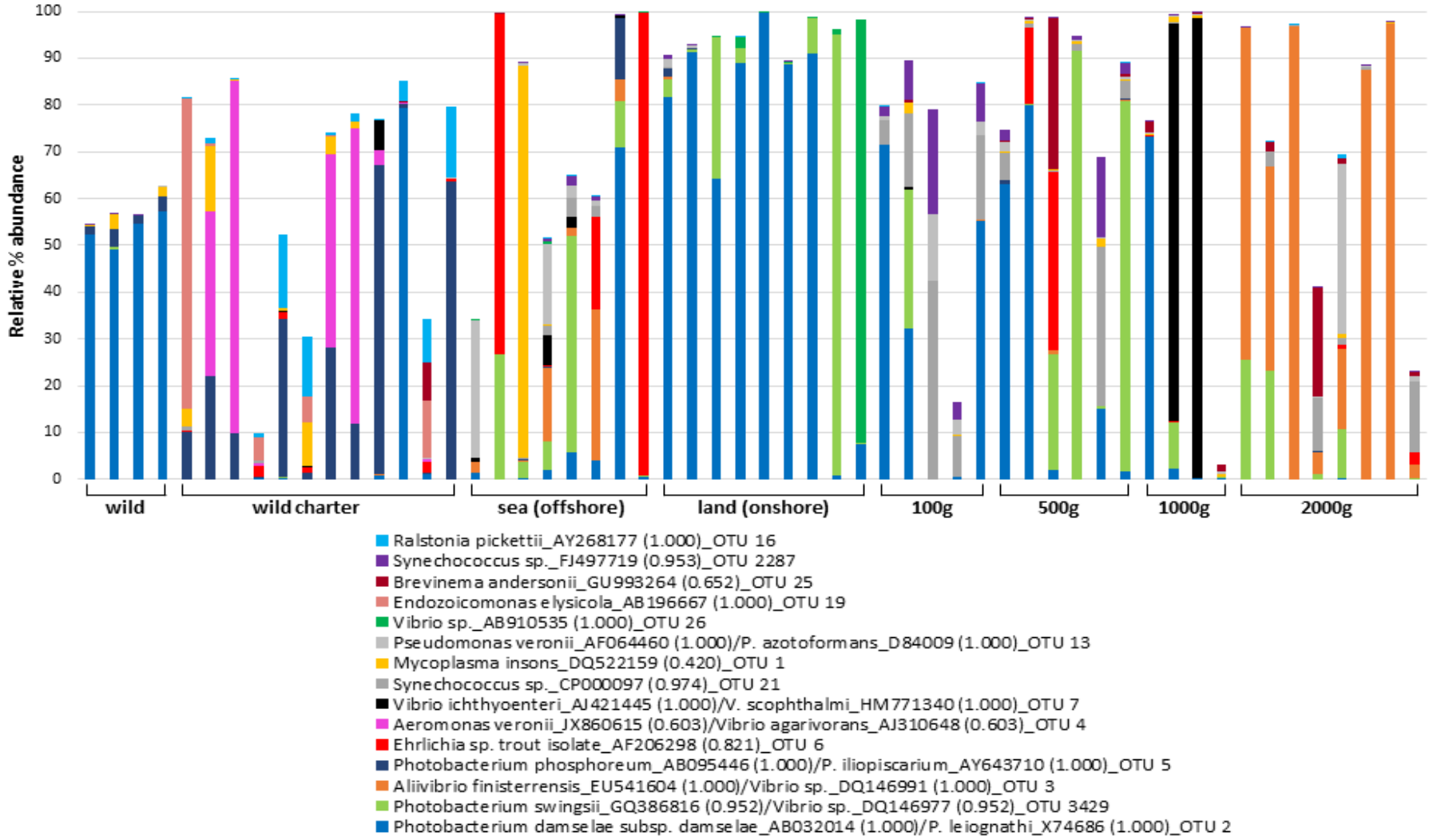


Figure 3.3.1.1.16. Relative percent abundance of the 15 most abundant bacterial OTUs in gut scraping samples from 'wild' and wild charter YTK, farmed YTK from offshore sea-cages and onshore (land-based) tank systems, and farmed YTK across the major commercial production size classes (100 g, 500 g, 1000 g and 2000 g).

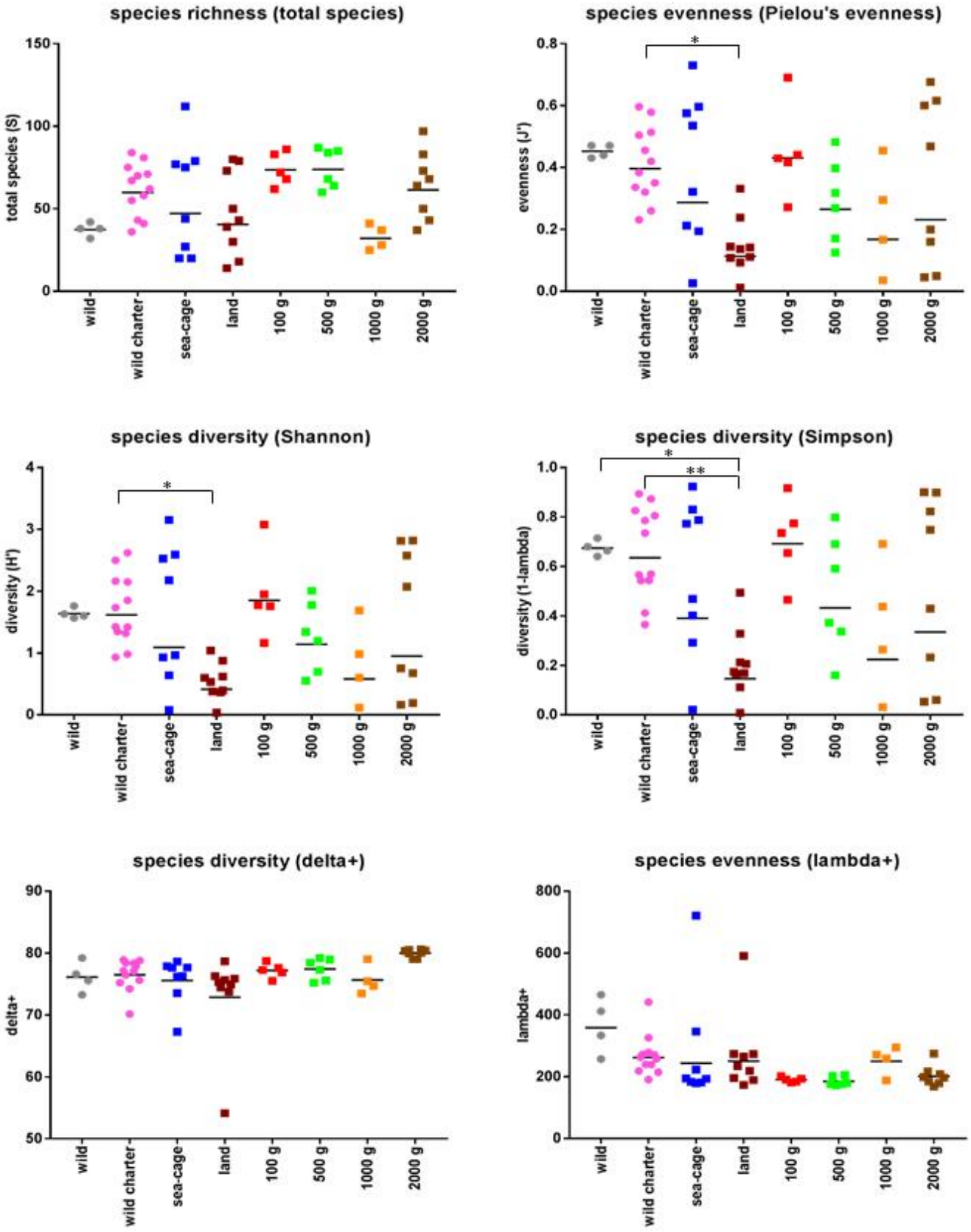
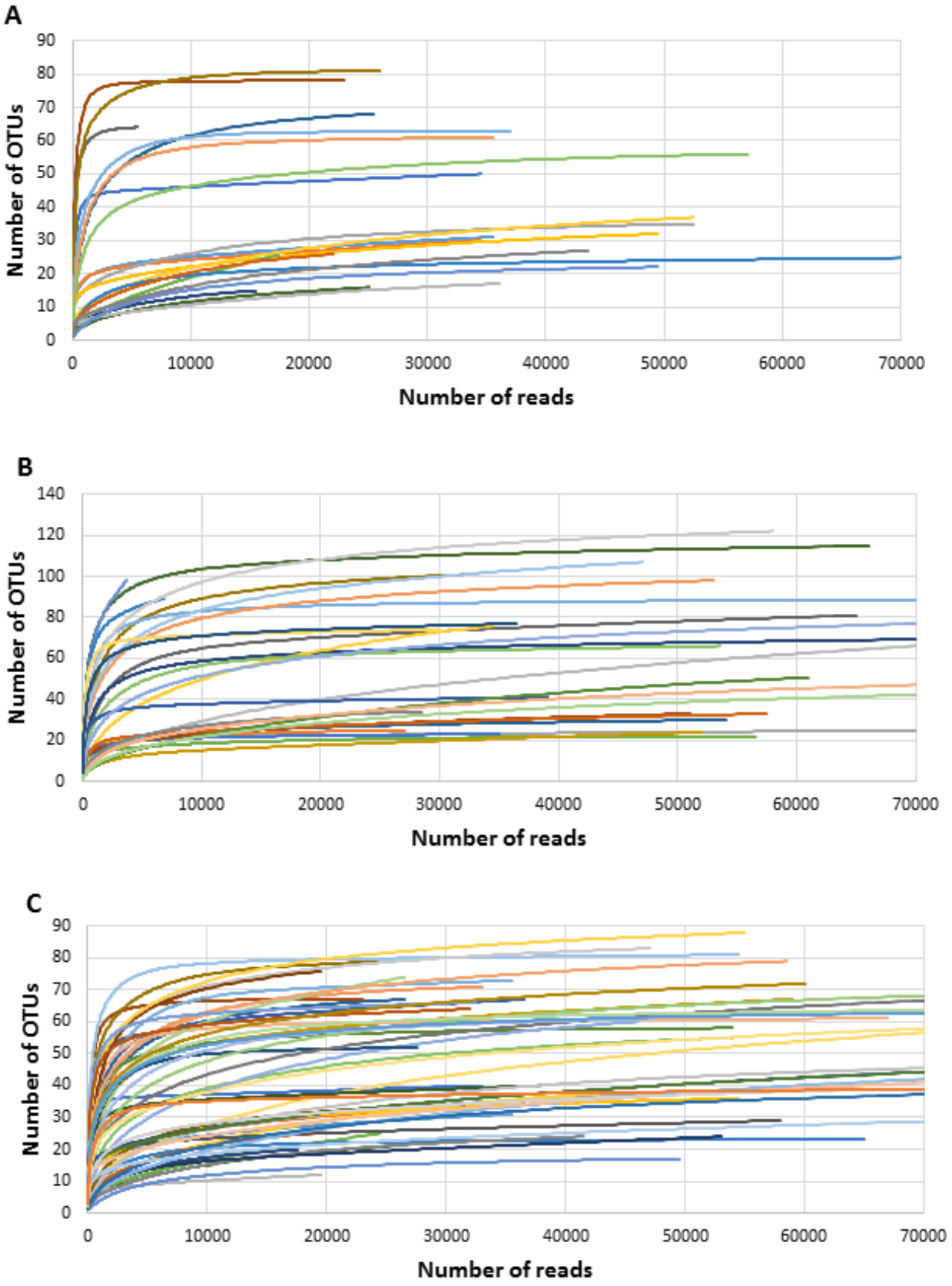


Figure 3.3.1.1.17. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) of samples.[1]

[1] Mean values are plotted for wild YTK ('wild' and wild charter; circles) vs farmed YTK (offshore sea-cages, onshore [land-based] tanks, and 100 g, 500 g, 1000 g and 2000 g size class sea-cage fish; squares).

Appendix 1. Rarefaction curves portraying the number of resolved OTUs against sequencing depth of each sample from component A) land (onshore) vs sea (offshore), B) commercial production cycle, C)



Wild vs farmed YTK.

3.3.1.2. Manuscript - Characterise and understand microbiome changes with diet and their potential implication for health and/or performance for Yellowtail Kingfish (Seriola lalandi).

Sarah R. Catalano [a], Andrew P.A. Oxley [a]

[a] South Australian Research and Development Institute, South Australia Aquatic Sciences Centre, West Beach, SA 5024, Australia.

This manuscript may be referenced as: Catalano, S.R. and Oxley, A.P.A. (2019). 3.3.1.2. Manuscript – Characterise and understand microbiome changes with diet and their potential implication for health and/or performance for Yellowtail Kingfish (*Seriola lalandi*) (Output 4c). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.558-621.

Abstract

To evaluate the effects that specific feeds or dietary formulations may have on the gut microbiome and their potential implication for health and/or performance of farmed YTK, microbiome samples were collected and analysed from four select experimental components. This included 1) comparisons between commercial feed formulations and 'natural' (Australian Sardine [*Sardinops sagax*; Sardine]) diets as part of Clean Seas on-farm and South Australia, SA (Nutrition theme, SARDI pool-farm, see Manuscript 3.2.3.1) and Western Australia, WA (Fremantle facility) tank trials; 2) diets modified to include variable levels of long chain omega 3 fatty acids [LC n-3 PUFA] (Nutrition theme, SARDI pool-farm trial, see Manuscript 3.1.1.1); 3) diets comprising low and high lipid inclusion levels with or without emulsifiers (Nutrition theme, SARDI pool-farm trial, see Manuscript 3.1.2.1); and 4) diets where fish meal (FM) is replaced with alternate protein sources (Nutrition theme, SARDI pool-farm trial, see Manuscript 3.1.3.1).

In the first experimental component comparing commercial dietary formulations and a 'natural' (Sardine) diet as part of tank trials, notable differences in the gut microbiome were observed for certain feeds. The most notable difference being the markedly varied global bacterial community profiles observed between the diets used in the WA (Fremantle facility) trial (Feeds C and D) and the 'natural' (Sardine) and commercial diet (Feed B) used in the SA (SARDI pool-farm) trial. While this may be confounded, in part, by local environmental (system) and/or genetic differences, higher global community diversity and evenness was observed for WA trial Feed C compared to all other diets, with lower total species richness, diversity (Shannon and Simpson) and evenness (Pielou's) observed for both SA trial Feed B and the 'natural' (Sardine) diet. When using different commercial formulations on-farm, differences in the gut microbiome were also observed for certain feeds. Specifically, in comparing three dietary formulations in a Clean Seas trial (Feeds A, B and C), fish fed Feed A appeared to have the highest bacterial diversity (Shannon and Simpson) and a more consistent global community structure among individuals, indicating that it may represent a preferred diet for promoting gut microbial diversity (and possibly gut health). Particularly in comparison to Feed C, which comprised the lowest species diversity and less consistent global structure among individuals. Despite this, however, potentially opportunistic pathogens were observed enriched in Feed A (e.g. *Photobacterium damselae* subsp. *damselae*/*P. damselae* subsp. *piscicida*), with down-regulation of these taxa in Feed B and even more so, in Feed C samples. Feed C therefore presents an interesting prospect for promoting gut microbial diversity (and possibly gut health) in both sea-cage and tank systems, even over more 'natural' diets.

In the second experimental component elucidating dietary formulations comprising long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA), some differences in the gut microbiome were observed. Principally, a change in the global bacterial community composition was apparent in all gut samples collected from fish fed a diet amended with LC n-3 PUFA, regardless of its inclusion at either a high (2.95 g 100 g^{-1}, Diet 1), moderate (2.14 g 100 g^{-1}, Diet 3) or low (0.753 g 100 g^{-1}, Diet 8) level. This difference was attributed to the enrichment of certain phyla (namely *Spirochaetae*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Cyanobacteria*) in the LC n-3 PUFA amended feeds. The inclusion of LC n-3 PUFA also led to the loss of otherwise dominant and potentially opportunistic pathogens (namely *P. damselae* subsp. *damselae*/*P. damselae* subsp. *piscicida* and *Mycoplasma insons*). Within certain gut regions like the midgut, this loss was also accompanied by significantly higher species richness and taxonomic diversity and evenness at a moderate level of LC n-3 PUFA inclusion (2.14 g 100 g^{-1}, Diet 3), with an increase in representation from minor constituents including *Bacteroidetes, Firmicutes* and *Chloroflexi*. With the lack of apparent changes in diversity and evenness observed for the hindgut samples (at any LC n-3 PUFA level), this suggests that the inclusion of LC n-3 PUFA into YTK diets at optimal concentrations may have the capacity to displace potentially opportunistic pathogens by promoting diversity in certain gut regions. With optimal dietary levels of LC n-3 PUFA (based on improvements in SGR/FCR) reported to be between 2.12 and 2.26 g 100 g^{-1} (based on improvements in SGR/FCR, see Manuscript 3.1.1.1), Diet 3 (2.14 g 100g^{-}1) requires further investigation to determine whether higher microbial diversity in the midgut also has the capacity to improve gut health and YTK performance through, e.g. enhanced nutrient (fatty acid) metabolism.

In the third experimental component evaluating lipid inclusion (at 20 and 30%) with or without emulsifiers, only very marginal differences were observed in the gut microbiome. More specifically, like that observed for the previous fatty acid trial, differences in the global bacterial community composition were largely only observed between unamended (pre-trial) and amended (experimental) diets, with increased abundances of select opportunistic taxa such as *Enterovibrio nigricans* in a number of the lipid diet samples likely leading to the observed differences. Among the experimental treatments though, generally conserved patterns of taxonomic diversity (Simpson and delta+) and evenness (Pielou's and lambda+) were observed, with only a marginal increase in the total species richness and diversity (Shannon) observed between Diet 4 (20% lipid with emulsifier) and Diet 1 (30% lipid without emulsifier). The absence of clear differences in changes in diversity was attributed to the occurrence a single dominant organism most closely related to *Brevinema andersonii*, where its high abundance in the pre-trial samples indicated that it had likely established dominance in the fish prior to the commencement of the trial. Despite this shortcoming, further pairwise comparisons revealed significant differences in the global bacterial composition between samples from fish fed diets with or without emulsifiers. Diets amended with emulsifiers appeared to enrich for a number of bacterial species, including a single *Bacteroidetes* species (most closely related to *Aureimarina marisflavi* of the family *Flavobacteriaceae*) with greater abundance in diets with emulsifiers compared to diets without emulsifiers irrespective of lipid level. However, whether the enrichment of this organism is associated with their ability to directly use the emulsifier as a carbon source for growth, or from the improved bioavailability of the lipids, is not clear and requires further investigation.

In the final experimental component investigating the graduated replacement of wild derived FM with alternate protein sources (namely fish meal by-product [FMB-P], digestible poultry meal [PM] and digestible soy protein concentrate [SPC]), some notable differences in the gut microbiome were observed. While this included some distinct differences in the global bacterial community composition between samples collected as part of individual summer and winter-feeding trials (indicating a possible effect due to seasonality), possible confounding effects of diet type and trial duration prevented further detailed assessment. Instead, in comparing among samples collected from the winter-feeding trial only, some (albeit not highly statistically significant) variations were observed in the global bacterial community profiles between individual diets. Notably, in comparison to the control diet (30% FM), there was an enrichment of major taxonomic groups in Diet 3 (10% FM + 21.4% FMB-P), Diet 4 (20% FM + 11.32% PM) and, to a lesser extent, Diet 6 (10% FM, 10.7% FMB-P + 11.32% PM) and included the phyla *Proteobacteria*, *Chloroflexi*, *Cyanobacteria*, *Bacteroidetes* and *Actinobacteria*. Alongside a decrease in otherwise abundant and potentially opportunistic species (most closely related to *Mycoplasma insons*, of the Phyla *Tenericutes*) in these samples, an increase in diversity (Simpson) and

evenness (Pielou's) was also observed for Diet 4. Collectively this highlights that a reduction to 10% wild derived FM content (Diets 3 and 6) or replacement of 10% FM with 11.32% PM (Diet 4) significantly alters the gut community structure and dynamics and may improve the capacity for the displacement of potentially opportunistic species; possibly leading to improved gut function through the selective enrichment of certain taxa like *Actinobacteria* that are well-known for supporting gut homeostasis.

Introduction

Yellowtail Kingfish (*Seriola lalandi*; YTK) is a valuable species in Australia that is in the early stages of aquaculture development. Numerous dietary formulations have been trialed by producers in an effort to optimise growth outcomes while reducing production costs. As the gut microbiota aids in digestion and nutrient absorption (Sullam et al., 2012; Ringo et al., 2016), different dietary formulations have the capacity to cause shifts in the overall community structure and dynamics (Nayak, 2010; Lyons et al., 2017). Certain diets may enrich for specific taxa that are opportunistic organisms, causing a displacement of the microbiome and leading to a dysbiotic state. Other diets may promote the enrichment of key taxa involved in digestion and metabolism of specific dietary components, thereby improving overall species richness, diversity and evenness and allowing for potentially improved gut functionality and possibly improved health outcomes (Heiman and Greenway, 2016). As more complex diets provide a wider range of potential substrates available to the microbes for growth and proliferation, the associated gut bacteria are likely to be far richer than those fed less complex diets (Ghanbari et al., 2015). Different dietary forms (e.g. natural feeds vs formulated pellets), dietary lipids (e.g. lipid levels, lipid sources and polyunsaturated fatty acids), protein sources (e.g. soybean meal, krill meal and other meal products), nutraceuticals (e.g. probiotics, prebiotics, synbiotics) and antibiotics are all known to affect the gut microbiome of fish (Ringø et al., 2016). A balance is therefore desirable in manufacturing diets that are not only sustainable, economically viable, cost effective and have the ability to improve growth rates, but also have the capacity to ensure that optimal diversity and functionality is maintained in the gut microbiome.

This manuscript explores the effect of different dietary formulations on the gut microbiome structure of YTK both on-farm and from tank trial samples. In particular, four experimental components were analysed: A) commercial feed formulations vs 'natural' Australian Sardine [*Sardinops sagax*; Sardine] diets; B) inclusion of varying proportions of LC n-3 PUFA; C) low and high lipid inclusion levels with or without emulsifiers; and D) replacement of wild derived fish meal (FM) with alternate protein sources.

Given the general lack of information pertaining to the effects of commercial dietary formulations on the gut microbiome of YTK, for the first component samples were collected from on-farm (Bickers sea-cages, Port Lincoln, SA) and tank trials (SARDI pool-farm facility, SA and Fremantle facility, WA) with fish fed different feed formulations compared to a 'natural' diet of thawed and diced Sardines. From the SARDI (winter) trial, while feeding Sardines every second day improved growth rates and provided numerically superior FCR, similar to when fish were fed the formulated diet to apparent satiation six days week^{-1}, the fish in fish out (a measure of the sustainable utilisation of marine resources) was 24% higher for YTK fed Sardines compared to the formulated diet (see Manuscript 3.2.3.1 for further details). Hence, the formulated diet was recommended, either fed to apparent satiation two days week^{-1} to maintain fish weight through winter and reduce feed and feeding costs, or fed to apparent satiation six days week^{-1} to increase growth rates and improve FCR through winter (see Manuscript 3.2.3.1). Microbiome samples were collected from the two optimum diets in this trial (i.e. fish fed to apparent satiation six days week^{-1} on the formulated diet, and fish fed Sardines to apparent satiation two days week^{-1}) and were compared with fish fed two formulated diets from WA tank trials (Fremantle facility) and three formulated diets on-farm at Bickers, Port Lincoln, SA, with some overlap between diets (e.g. Feed B on-farm and from the SARDI tank trial and Feed C on-farm and from the WA tank trial).

In the need to determine the optimal dietary LC n-3 PUFA requirements for YTK as part of the broader components of this project, its effect on the gut microbiome was also investigated. Thus, for the second component of this work, three (of a total of eight) experimental diets formulated and evaluated as part of the nutritional trial (see Manuscript 3.1.1.1) were sampled for microbiome analysis and included diets with low (0.753 g 100 g^{-1}), high (2.95 g 100 g^{-1}) and moderate (2.14 g 100 g^{-1}) LC n-3 PUFA levels. The rationale for this was based on the finding that an optimal dietary level of LC n-3 PUFA for YTK (based on SGR/FCR) was between 2.12 and 2.26 g 100 g^{-1} (see Manuscript 3.1.1.1). Thus, the dietary treatments evaluated here were selected to represent high, optimal and sub-optimal concentrations.

For the third component of this work, from the nutritional trial it was suggested that YTK may be fed a 30% dietary lipid level at winter water temperatures to improve growth and feed utilisation, with no apparent benefit to growth or feed utilisation by inclusion of a dietary emulsifier at winter water temperatures (see Manuscript 3.1.2.1). However, the effects of this diet on the gut microbiome of YTK is unknown, particularly in comparison with lower levels of dietary lipid inclusion (either with or without an added dietary emulsifier). Hence microbiome samples were collected from YTK across all four dietary treatments evaluated in the nutritional trial (see Manuscript 3.1.2.1), which comprised both high (30%) and low (20%) lipid levels with and without an emulsifier.

The final dietary study focused on the replacement of wild derived FM with alternate sources, including FM by-product (FMB-P), poultry meal (PM) and soy protein concentrate (SPC). The rationale being that while FM is a common inclusion into the dietary formulation for cultured YTK (typically at a level of 30%), increases in demand may result in substantial increases in price while further demand may result in levels exceeding supply (Gatlin et al., 2007; Merrified et al., 2011). Therefore, the use of alternative ingredients to replace/reduce FM may be required in an effort to reduce diet costs and improve sustainability. From the nutritional trial, it was reported that there was no significant difference in any of the growth, feed utilisation or blood haematology and biochemistry indices measured between the six diets, however in terms of SGR and FCR, fish fed Diet 2 (0% FM + 10.70% FM by-product) and Diet 7 (20% FM + 10.88% SPC) tended to perform slightly better than fish fed other diets (see Manuscript 3.1.3.1). Microbiome samples were thus collected from YTK on all eight of the trial diets to evaluate the effect of reducing/replacing FM on the resultant gut microbiome composition.

Aim

The aim of this experimental work was to characterise and understand the effects that specific feeds or dietary formulations may have on the gut microbiome of YTK and the consequence this may have for health and/or performance, as observed from comparisons between commercial feed formulations and 'natural' (Sardine) diets; diets modified to include variable levels of LC n-3 PUFA; diets comprising low and high lipid inclusion levels (with or without emulsifiers); and diets where FM is replaced with alternate protein sources.

A. Commercial feed formulations vs 'natural' (Sardine) diets

A. Methods

Experimental design

Fish sampled for this experimental component were obtained from both land-based tank trials and offshore sea-cage sites. From the tank trials, a total of 18 fish were sampled from the SARDI winter feeding trial (Feeding Strategies Theme FS-1, optimisation of winter-feeding strategies for large YTK, see Manuscript 3.2.3.1 for further details) on the 17th of September 2015. Fish were held in 5000 L tanks located at the SARDI pool-farm facility in SA with the total duration of the trial being 84 days. This included nine fish fed a formulated diet (Feed B) to apparent satiation six days per week, and nine fish fed an Australian Sardine (*Sardinops sagax*) diet to apparent satiation every second day at winter

water temperatures. For each treatment, three fish were sampled from three replicate tanks (Figure 3.3.1.2.1 A). YTK fed these two diets were similar in growth and feed conversion ratios, however, due to the consumer perception and marketability of feeding YTK Sardines, the formulated diet fed to apparent satiation six days per week was recommended as the most optimal to industry (see Manuscript 3.2.3.1 for nutritional results of this trial). To supplement the numbers of fish examined for this work, and to provide some further insights into the effect that different commercial formulations may have on the gut microbiome in tank trials, a further five YTK were obtained from the Fremantle facility, WA on the 14th July 2016. All fish were fed a formulated diet, with three fish fed Feed D and two fish fed Feed C (Figure 3.3.1.2.1 A). Fish held at the Fremantle facility were on these diets for eight months prior to sampling (from December 2015 to July 2016), with gut scraping samples collected by Dr Gavin Partridge and Dr Lindsey Woolley (South Metropolitan TAFE, Fremantle, WA).

For evaluating commercial formulations on-farm, a total of 15 fish were sampled from three sea-cages at the Bickers site off of Port Lincoln, SA on the 4th February 2016. This included five fish fed Feed A from cage ABK14-10GL, five fish fed Feed B from cage ABK14-10GS and five fish fed Feed C from cage ABK14-10mix (Figure 3.3.1.2.1 B). Fish were fed Feed A for 12 months prior to sampling, Feed B for 15 months prior to sampling and Feed C for three months prior to sampling, with the diet composition/ingredients more similar between Feed A and Feed C compared to Feed B (as indicated by Clean Seas health staff). A seawater sample was also taken and processed in parallel from the Bickers site to control for the influence the environment may have on the structure and composition of the gut bacterial communities (Figure 3.3.1.2.1 B).

Note that although the diet manufacturer and type was the same for fish in the SARDI winter feeding trial and from sea-cage ABK14-10GS (referred to as 'Feed B') as well as for fish held at the Fremantle facility and from sea-cage ABK14-10mix (referred to as 'Feed C'), it should be noted that these feeds may not be identical as they could vary due to batch number, storage conditions and exact quantities of each ingredient type.

Fish sampling

Each fish was euthanised in AQUIS solution, weighed (g) and measured (fork length, cm). For microbiome sampling, the body cavity was opened and the entire GI tract removed. For fish collected as part of the Port Lincoln sea-cage component and Fremantle tank trials, the midgut (MG) and hindgut (HG) were separated from the foregut (FG) using a sterile scalpel blade and placed on a clean surface. Using a clean pair of forceps and sterile scalpel, an incision was made along the length of the MG and HG to expose the inner surfaces, and then a single scraping of the entire region was performed with a sterile glass slide to collect the gut contents/mucosa. For fish collected from the remaining SARDI tank trial component, the GI tract was cut into three sections with a sterile scalpel, representing the fore-, mid- and hindgut regions. A new sterile scalpel was used to open each region and a scraping taken with a sterile glass slide as described above. All scrapings were immediately placed in individual 50 mL falcon tubes containing stabilising buffer (RNAlater™, Ambion), labelled and stored at 4 °C for 1-2 days before being stored for up to a month at -20 °C prior to RNA extraction. Gloves, aluminium foil and scalpel blades were discarded and forceps cleaned with ethanol after sampling each fish to avoid cross contamination.

RNA extraction for gut microbiome samples

RNA was extracted on ice from stabilised samples according to the methods detailed in Szafranska et al. (2014). In brief, the stabilising buffer was removed from each sample and 1 mL of cold (4 °C) RLT buffer supplemented with 1% β-mercaptoethanol was added and transferred to lysing matrix B tubes (MP Biomedicals). Samples were disrupted via bead-beating using the FastPrep-24™ 5G instrument (MP Biomedicals) at an intensity of 5.5 for 45 s, placed on ice for 3 min then disrupted a second time as described above prior to centrifugation at 14,000 × g for 10 min at 4 °C. The supernatant was transferred to 1.5 mL RNase-free Biopur centrifuge tubes (Eppendorf) and the RNeasy minikit (Qiagen)

was used to extract the RNA according to manufacturer's instructions. RNA was eluted in 30 µL of RNase free water, passed through the spin column twice to concentrate each sample and quantified using a NanoDrop 2000 spectrophotometer. To remove any source of potential contaminating gDNA, a routine DNase treatment was performed for all samples using the Turbo DNA-free™ kit (Life Technologies) following the manufacturer's instructions. All samples were precipitated with ethanol using standard procedures, reconstituted in 30 µL of RNase free water and the RNA re-quantified using NanoDrop. Samples were stored at -80 °C prior to use in down-stream procedures.

DNA extraction for environmental samples

One litre of seawater was collected in a sterile Schott bottle from the Bickers sea-cage site. The bottle was labeled with the site location and cage ID and stored at 4 °C prior to filtration and DNA extraction. The seawater sample was filtered onto a sterile 0.22 µM filter (Nalgene®) and the DNA extracted from the filter discs using the FastDNA™ Spin Kit for Soil (MP Biomedicals) following the manufacturer's instructions. In brief, the filter paper was placed in a lysing matrix E tube with sodium phosphate and MT buffer and cells were lysed via bead-beating using the FastPrep-24™ 5G instrument (MP Biomedicals) at an intensity of 5.5 for 45 s. The sample was subsequently centrifuged for 10 min at 14,000 × *g* and the supernatant transferred to a 1.5 mL DNA LoBind tube (Eppendorf). Following the addition of a protein precipitation solution, the sample was mixed and centrifuged to pellet the precipitate before the supernatant was transferred to a clean 15 mL centrifuge tube supplemented with Binding Matrix solution. The DNA was captured on a SPIN filter tube and washed, re-eluted in 100 µL of DES and quantified using a NanoDrop 2000 spectrophotometer followed by precipitation with ethanol using standard procedures. The pelleted DNA was reconstituted in 30 µL of RNase free water, re-quantified using the NanoDrop and stored at 4 °C prior to use in down-stream procedures.

cDNA synthesis, PCR amplification, AMPure bead-purification and Picogreen quantification

For the gut scraping samples only, the RNA extracts were converted to cDNA to assess for the active (and likely resident) bacterial community members using the Superscript™ III First Strand Synthesis System (Life Technologies) following the manufacturer's instructions and stored at -20 °C prior to PCR amplification. The V1-V2 hypervariable region of the 16S rRNA gene was amplified for all samples (DNA and cDNA samples) as described by Camarinha-Silva et al. (2014); though included an initial pre-enrichment of the V1-V2 target region by conducting a 20 cycle PCR reaction with primers 27F and 338R as described by Chaves-Moreno et al. (2015). Specifically, 2 µL of cDNA and 5 µL of each environmental DNA extract was used as template in this first round of PCR, with 1 µL aliquots from this reaction used as template in a second 15 cycle PCR reaction to append sample specific barcodes and reverse adapter sequences complementary to the Illumina platform specific adaptors. One microlitre aliquots of the second PCR reaction were subsequently used as a template in a third 10 cycle PCR to append the Illumina multiplexing sequencing and index primers. PCR amplicons were visualised via agarose gel electrophoresis and products of the expected size (~438 bp) were purified using Agencourt AMPure XP beads (Beckman Coulter). Samples were quantified in duplicate using the Quant-iT™ Picogreen® dsDNA kit (Life Technologies) following the manufacturer's instructions. Approximately 100 samples were pooled for each library in equimolar ratios and sequenced on the MiSeq platform (Illumina, San Diego, CA) using 250 nt paired-end sequencing chemistry through the Australian Genome Research Facility (AGRF). As a sequencing control, amplicons generated from a single bacterial species (*Lactobacillus reuteri*) were included within each Illumina index within each of the libraries. The final list of samples that generated good-quality microbiomic libraries for this component of work are presented in Table 3.3.1.2.1.

Bioinformatics analysis

In total, 2,908,786 million sequence reads were derived from 55 samples (of the 61 that were collected). Six samples failed to amplify enough material to produce good-quality NGS libraries, which was accounted for in the experimental approach by allowing for ample replication of fish. Sequence reads were paired using PEAR (version 0.9.5) (Zhang et al., 2014), where primers were identified and removed. Paired-end reads were quality filtered, with removal of low-quality reads, full-length duplicate sequences (after being counted) and singleton sequences using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010), USEARCH (version 8.0.1623) (Edgar, 2010; Edgar et al., 2011) and UPARSE software (Edgar, 2013). Reads were mapped to Operational Taxonomic Units (OTUs) using a minimum identity of 97%, and putative chimeras removed using the RDP-gold database as a reference (Cole et al., 2014). These OTUs were further filtered as conducted previously (Zhang et al., 2016) where only those that contributed to > 0.01% of the host-associated dataset (gut samples only) or > 0.01% of the environmental water sample were used (see Table 3.3.1.2.2 for a summary of OTUs remaining post-filtering). Rarefaction curves were used to inspect (retrospectively) sampling depth for each gut sample (Appendix 1A). Due to low sequence reads and outlier placement in the non-metric multidimensional scaling (nMDS) plot, four samples were removed from the dataset (see Table 3.3.1.2.1, Appendix 1A). Further interrogation of the resultant OTUs was conducted using the Seqmatch function of the RDP database (Wang et al., 2007) as well as SILVA (Quast et al., 2013), whereby lineages based on the SILVA taxonomy and best hit from RDP were assigned to each OTU alongside the corresponding RDP sequence similarity value (SeqMatch, S_ab score). The S_ab score represents the number of unique 7-base oligomers shared between an OTU and a known sequence contained in the RDP database divided by the lowest number of unique oligos in either of the two sequences. A S_ab score of 1.000 represents an identical match to the nearest database sequence, with values closer to 1.000 providing greater confidence in the identification OTU sequence.

Statistical analysis

In order to explore for patterns across the global bacterial communities, a data matrix comprising the percent standardised abundances of OTUs was used to construct a sample-similarity matrix using the Bray-Curtis algorithm (Bray and Curtis, 1957), where samples were then ordinated using non-metric multidimensional scaling (nMDS) with 50 random restarts (Clarke et al., 2001). Significant differences between *a priori* pre-defined groups of samples (e.g. environmental water samples vs gut scraping samples) were evaluated using both one-way and two-way permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations, allowing for type III (partial) sums of squares, fixed effects sum to zero for mixed terms, and exact p-values generated using unrestricted permutation of raw data (Anderson, 2001). Groups of samples were considered significantly different if the p-value falls < 0.05. Pairwise tests in PERMANOVA were used to determine which *a priori* pre-defined categories (such as diet A vs diet B) were significantly different. The multivariate analyses, relative percent abundance of bacterial phyla, class, order, family and genus along with rarefaction curves were performed and calculated using PRIMER (v.7.0.11), PRIMER-E, Plymouth Marine Laboratory, UK (Clarke et al., 2001).

Conventional measures of species diversity, richness and evenness were calculated using algorithms for total OTUs (S), Pielou's evenness (J'), Shannon diversity (H') and Simpson (1-λ), while taxonomic diversity was calculated using algorithms for taxonomic distinctness: average taxonomic distinctness (avTD - delta+) and variation in taxonomic distinctness (varTD - lambda+) using PRIMER (v.7.0.11) (Clarke et al., 2001). These univariate indicators of diversity (S, J', H', 1-λ, avTD, varTD) were compared between *a priori* groups of samples (such as diet type) using one-way ANOVA and plotted in Prism v. 7.01 (Graphpad Software Inc.). Variables were considered to be significantly different if the p-value falls < 0.05, for which a Tukey's post-hoc multiple comparisons test was then performed (Prism v. 7.01). For further presentation of data, relative abundance plots of the top 15 most abundant OTUs were constructed in Excel. To obtain the identification of the closest cultured species for each of the most abundant OTUs, the corresponding sequence was blasted against the RDP isolate database only.

A similarity (S_ab) score in parenthesis is presented for each OTU in the top 15 OTUs plot and referred to in text.

A. Results

Global community structure

As observed in the baseline data (see Manuscript 3.3.1.1), there was a clear separation and significant difference (PERMANOVA, $P = 0.0169$, table not shown) between the global community structure of the environmental (seawater) sample and all gut samples from YTK fed the different diet types (Figure 3.3.1.2.2 A). The tank trial samples clustered separately from the sea-cage samples, as confirmed by PERMANOVA ($P = 0.0001$, table not shown) (Figure 3.3.1.2.2 A). For this reason, and also due to different fish sizes (fork length, mean 46.3 cm tank vs 67.4 cm sea-cage), the feed dataset was split into two sub-components (tank trials vs sea-cages, Figure 3.3.1.2.2 B, C) for all subsequent analyses.

For the tank-trial diets, the Fremantle facility samples (Feeds C and D, WA) clustered away from the SARDI facility samples (Feed B and Sardines, SA), with a significant difference in the global community structure of tank trial feeds (PERMANOVA, $P = 0.0001$, table not shown). From pairwise comparisons, the two WA tank trial samples were not significantly different to one another ($P = 0.5016$, Table 3.3.1.2.3), but significantly different to the SA formulated Feed B and Sardine samples (Table 3.3.1.2.3). There was also a significant difference between the two SA tank trial samples, Feed B and Sardines (0.0087, Table 3.3.1.2.3). As the two SA tank trial samples contained FG, MG and HG scrapings, a two-way PERMANOVA for each sample type (Feed B or Sardines) crossed with the gut regions (FG, MG, HG) was performed. For the Feed B samples, there was no significant difference between any of the gut regions except FG vs HG ($P = 0.0427$, Table 3.3.1.2.4 A). For the Sardine samples, there was no significant difference between any of the gut regions (Table 3.3.1.2.4 B). As the FG was not sampled from every fish in the SA tank trial, this region was omitted from further analyses. Additionally, as there was no significant difference between the MG and HG scraping samples from either the Feed B ($P = 0.4715$, Table 3.3.1.2.4 A) or Sardine ($P = 0.4783$, Table 3.3.1.2.4 B) diets, only results from the HG scraping samples for this component are presented in the remaining analyses.

For the three sea-cage diets, Feed A samples clustered close together while greater disparity was observed among samples for Feeds B and C (Figure 3.3.1.2.2 C). This was confirmed by PERMANOVA pairwise comparisons with a significant difference in the global community structure occurring between Feed A with Feed B ($P = 0.0079$, Table 3.3.1.2.5) and Feed C ($P = 0.0398$, Table 3.3.1.2.5), but not between Feeds B and C (0.8260, Table 3.3.1.2.5).

Bacterial phyla

In line with the observed variation in the global community structures, differences in the bacterial community composition at the phylum level was observed between the sea-cage and tank trial dietary samples. For the sea-cage trial, despite significant differences occurring among feeds at the global level, samples from all three diets were dominated by the phylum *Proteobacteria* (Figure 3.3.1.2.3). The broader taxonomy in the samples was also conserved at the class (*Gammaproteobacteria*), order (*Vibrionales*) and family (*Vibrionaceae*) levels, though differed with respect to the genera occurring within the *Vibrionaceae* (Figure 3.3.1.2.4). In particular, Feed A was dominated by taxa from the genera *Photobacterium* (with 90% contribution in 3 out of 5 of the samples), whereas Feed B and Feed C also had representation from *Vibrio*, *Aliivibrio* and *Spiroplasma* species (Figure 3.3.1.2.4 D).

For the WA tank trial samples, where fish were fed Feeds C and D, there was representation from additional phyla alongside *Proteobacteria*, including *Actinobacteria*, *Bacteroidetes*, *Fusobacteria* and *Firmicutes* (Figure 3.3.1.2.3). These samples represented those with the greatest (phylum-level) diversity. For the SA tank trial samples, the fish were dominated by the phylum *Spirochaetae*, however, there was also representation from *Proteobacteria* in some of the Sardine diet samples (Figure 3.3.1.2.3).

Top 15 OTUs

For the tank trial samples, fish from the Fremantle facility (WA) that were fed two different diets (Feeds C and D) had greatest taxa diversity, with additional taxa beyond the top 15 contributing to the relative abundance (Figure 3.3.1.2.5). OTU 3, with closest sequence similarity to *Photobacterium damselae* subsp. *damselae*/*P. damselae* subsp. *piscicida/P. leiognathi* (similarity [S_ab] score 1.000), was recorded from all five WA fish, with > 50% relative abundance in two out of the three fish fed Feed D (Figure 3.3.1.2.5). Taxa with closest sequence similarity to *Campylobacter hyointestinalis* subsp. *lawsonii* (OTU 44, S_ab score 0.673), *Cetobacterium somerae* (OTU 25, S_ab score 0.596), *Tenacibaculum soleae* (OTU 58, S_ab score 0.963) and *Propionibacterium acnes* (OTU 34, S_ab score 1.000) were also recorded from these five WA fish (Figure 3.3.1.2.5). In contrast, the SARDI pool-farm facility fish fed the two diets, Feed B and Sardines, were primarily dominated at a high relative abundance by OTU 2, with closest sequence similarity to *Brevinema andersonii* (S_ab score 0.632). In four of the seven Sardine fed fish, there was also representation by *Vibrio* sp. V776/*Allivibrio finisterrensis* (OTU 4, S_ab score 1.000) (Figure 3.3.1.2.5).

For the sea-cage samples, fish fed Feed A were dominated by taxa with closest sequence similarity to various *Photobacterium* species (including *P. damselae* subsp. *damselae*/*P. damselae* subsp. *piscicida/P. leiognathi* [OTU 3, S_ab score 1.000] and *P. phosphoreum/P. iliopiscarium* [OTU 20, S_ab score 1.000]), followed by various *Vibrio* species (including *V. ichthycenteri*/*V. scophthalmi* [OTU 12, S_ab score 1.000], *Vibrio* sp. V776/*Allivibrio finisterrensis* [OTU 4, S_ab score 1.000] and *Vibrio* sp. [OTU 27, S_ab score 1.000]) (Figure 3.3.1.2.6). While *P. damselae* subsp. *damselae*/*P. damselae* subsp. *piscicida/P. leiognathi* (OTU 3, S_ab score 1.000) was also recorded in fish fed Feed B and Feed C, it was less dominant across the five individuals sampled in each group, with lowest representation in fish fed Feed C (Figure 3.3.1.2.6). *Vibrio* species, including *V. ichthycenteri*/*V. scophthalmi* (OTU 12, S_ab score 1.000), *Vibrio* sp. V776/*A. finisterrensis* (OTU 4, S_ab score 1.000) and *Vibrio* sp. (OTU 27, S_ab score 1.000) were recorded from Feed B and C diets, with greater relative abundance in samples compared to Feed A (Figure 3.3.1.2.6).

Diversity indices

Species evenness (Pielou's) and diversity (Shannon and Simpson) was significantly greater for WA Feed C samples compared to SA Feed B and Sardine samples (Table 3.3.1.2.6 A, Figure 3.3.1.2.7). WA Feed D samples also had greater species evenness (Pielou's) and diversity (Simpson) compared to SA Feed B samples (Table 3.3.1.2.6 A, Figure 3.3.1.2.7). No significant differences were observed, however, in any of the diversity indices between WA Feeds C and D, or between SA Feed B and the Sardine diet (Table 3.3.1.2.6 A, Figure 3.3.1.2.7).

For the Bickers sea-cage samples, species diversity (Shannon and Simpson) was significantly higher in Feed A compared to Feed C samples ($P = 0.0208$ and $P = 0.0347$ respectively), with significantly greater diversity (delta+) for Feed B compared to Feed C diets ($P = 0.0255$) (Table 3.3.1.2.6 B, Figure 3.3.1.2.8). Although not significantly different, Feed B and C samples presented a general trend of lower species richness and evenness (Pielou's) compared to Feed A samples (Table 3.3.1.2.6 B, Figure 3.3.1.2.8).

B. Fatty acid inclusion nutritional trial N1

B. Methods

Experimental design

A total of 32 fish were sampled from the SARDI fatty acid trial (Nutrition Theme N-1, understanding the conditional dietary requirements for fatty acids and cholesterol in large YTK at summer water temperatures, see Manuscript 3.1.1.1 for further details), with five fish sampled pre-trial on the 17th of

March 2016 and 27 fish sampled post-trial on the 2nd of June 2016. Fish were held in 5000 L tanks located at the SARDI pool-farm facility and fed different diets with varying proportions of long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA; eicosapentaenoic acid [20:5n-3, EPA], docosapentaenoic acid [22:5n-3, DPA] and docosahexaenoic acid [22:6n-3, DHA]; referred to from here on as LC n-3 PUFA). The total duration of the trial was 84 days where, at its conclusion, a total of 27 fish were collected, including nine fish fed a diet containing 2.95 ∑LC n-3 PUFA (referred to herein as Diet 1), nine fish fed a diet containing 2.14 ∑LC n-3 PUFA (referred to herein as Diet 3) and nine fish fed a diet containing 0.753 ∑LC n-3 PUFA (referred to herein as Diet 8). For each treatment, three fish were sampled from three replicate tanks (Figure 3.3.1.2.9).

Fish sampling

Refer to section A. Methods. For the pre-fatty acid trial samples, the MG and HG were separated from the FG using a sterile scalpel blade and placed on a clean surface. Using a clean pair of forceps and sterile scalpel, an incision was made along the length of the MG and HG to expose the inner surfaces, and then a single scraping of the entire region was performed with a sterile glass slide to collect the gut contents/mucosa. The post-fatty acid trial samples were collected in a similar manner, though the MG and HG were first separated from the FG, opened and scrapings taken from the individual regions.

RNA extraction for gut microbiome samples

Refer to section A. Methods.

DNA extraction for environmental samples

No tank water sample was collected from the fatty acid trial.

cDNA synthesis, PCR amplification, AMPure bead-purification and Picogreen quantification

Refer to section A. Methods. The final list of samples that generated good-quality microbiomic libraries for the components of this work are presented in Table 3.3.1.2.7.

Bioinformatics analysis

In total, 3,669,822 million sequence reads were derived from 46 samples (of the 59 that were collected). Thirteen samples failed to amplify enough material to produce good-quality NGS libraries. This was accounted for in the experimental design approach, by allowing for ample replication of fish. Table 3.3.1.2.8 provides a summary of OTUs remaining post-filtering. Rarefaction curves were used to inspect (retrospectively) sampling depth for each gut sample (Appendix 1B). For detailed methods on quality filtering and mapping reads, refer to section A. Methods.

Statistical analysis

Refer to section A. Methods.

B. Results

Global community structure

There was no significant difference between the MG and HG samples for Diets 1 and 8 ($P = 0.0500$ and $P = 0.1329$ respectively, Table 3.3.1.2.9), therefore for these two diets, only HG results are presented in subsequent analyses. However, there was a significant difference between the MG and HG samples for Diet 3 ($P = 0.0003$, Table 3.3.1.2.9), therefore both the MG and HG results for Diet 3 are presented in subsequent analyses.

The pre-trial samples clustered away from and were significantly different (in terms of global community structure) to the three diet samples (Figure 3.3.1.2.10, Table 3.3.1.2.10). For the HG samples, there was no significant difference in global community structure between the three diets (Figure 3.3.1.2.10, Table 3.3.1.2.10), however, the Diet 3 MG samples were significantly different to all three HG diet samples (Figure 3.3.1.2.10, Table 3.3.1.2.10).

Bacterial phyla

Four out of the five pre-trial samples were dominated by two bacterial phyla, *Tenericutes* and *Proteobacteria*, while the fifth sample was dominated by *Tenericutes* only (Figure 3.3.1.2.11). Over half of the samples collected from the HG of fish on Diets 1, 3 and 8 were also dominated by *Tenericutes* (Figure 3.3.1.2.11). The remaining samples for these diets were represented by additional phyla, including *Proteobacteria*, *Spirochaetae*, *Actinobacteria, Firmicutes*, *Bacteroidetes* and *Cyanobacteria* (Figure 3.3.1.2.11). The diet 3 MG samples had the greatest level of phyla diversity, with high relative abundance from *Proteobacteria* along with decreased abundance of *Tenericutes* and increased representation from minor constituents including *Bacteroidetes, Firmicutes* and *Chloroflexi* (Figure 3.3.1.2.11).

Top 15 OTUs

The pre-trial samples included representation from three main taxa with closest sequence similarity to *Mycoplasma insons* (OTU 1, similarity [S_ab] score 0.428), *Photobacterium damselae* subsp. *damselae*/*P. damselae* subsp. *piscicida/P. leiognathi* (OTU 3, S_ab score 1.000) and *Vibrio ichthycenteri*/*V. scophthalmi* (OTU 12, S_ab score 1.000) (Figure 3.3.1.2.12). Of these three taxa, *M. insons* (OTU 1, S_ab score 0.428) persisted with high relative abundance in the three trial diet HG samples. Taxa with closest sequence similarity to *Brevinema andersonii* (OTU 2, S_ab score 0.632) and *Vibrio* sp. V776/*Allivibrio finisterrensis* (OTU 4, S_ab score 1.000) were also recorded in fish across the three diets (Figure 3.3.1.2.12). For the Diet 3 MG samples, *M. insons* (OTU 1, S_ab score 0.428) was notably decreased in abundance, with additional taxa beyond the top 15 contributing to the total relative abundance (Figure 3.3.1.2.12).

Diversity indices

Total species richness, evenness (Pielou's) and diversity (Shannon) were significantly greater in the Diet 3 MG samples compared to the pre-trial, Diet 1 HG and Diet 8 HG samples. Additionally, for Diet 3 samples, species evenness (Pielou's) and diversity (Shannon and Simpson) were also significantly greater in the MG compared to HG samples (Figure 3.3.1.2.13, Table 3.3.1.2.11). No significant differences were observed, however, in any of the diversity indices between the three diets for the HG scraping samples (Figure 3.3.1.2.13, Table 3.3.1.2.11).

C. Lipid inclusion with and without emulsifier nutritional trial N3

C. Methods

Experimental design

A total of 41 fish were sampled from the SARDI emulsifier trial (Nutrition Theme N-3, evaluation of the effects of emulsifiers on lipid digestibility and gut health in large YTK at winter water temperatures, see Manuscript 3.1.2.1 further details), with five fish sampled pre-trial on the 2nd of September 2016 and 36 fish sampled post-trial on the 25th of November 2016. Fish were held in 5000 L tanks located at the SARDI pool-farm facility and fed different diets with a varying percentage of lipid inclusion with and without emulsifiers. The duration of the trial was 84 days where, at its conclusion, a total of 36 fish were collected, including nine fish fed a diet containing 30% total lipid without emulsifier (referred to herein as Diet 1), nine fish fed a diet containing 30% total lipid with emulsifier (referred to herein as Diet 2), nine fish fed a diet containing 20% total lipid without emulsifier (referred to herein as Diet 3), and nine fish fed a diet containing 20% total lipid with emulsifier (referred to herein as Diet 4). For each treatment, three fish were sampled from three replicate tanks. A seawater sample was also taken post-trial from the system in-flow and processed in parallel to control for the influence the environment may have on the structure and composition of the gut bacterial community (see Figure 3.3.1.2.14).

Fish sampling

Refer to section A. Methods. For all samples collected from this trial, the MG and HG were separated from the FG using a sterile scalpel blade and placed on a clean surface. Using a clean pair of forceps and sterile scalpel, an incision was made along the length of the MG and HG to expose the inner surfaces, and then a single scraping of the entire region was performed with a sterile glass slide to collect the gut contents/mucosa.

RNA extraction for gut microbiome samples

Refer to section A. Methods.

DNA extraction for environmental samples

Refer to section A. Methods.

cDNA synthesis, PCR amplification, AMPure bead-purification and Picogreen quantification

Refer to section A. Methods. The final list of samples that generated good-quality microbiomic libraries for the components of this work are presented in Table 3.3.1.2.12.

Bioinformatics analysis

In total, 1,423,326 million sequence reads were derived from 40 samples (of the 42 that were collected). Two samples (one pre-trial and one Diet 1) failed to amplify enough material to produce good-quality NGS libraries. This is accounted for in the experimental design approach, by allowing for ample replication of fish. Table 3.3.1.2.13 provides a summary of OTUs remaining post-filtering. Rarefaction curves were used to inspect (retrospectively) sampling depth (Appendix 1C). Due to low sequence reads and occurrence as an outlier in the non-metric multidimensional scaling (nMDS) plot, one sample was removed from the dataset (see Table 3.3.1.2.12, Appendix 1C). For detailed methods on quality filtering and mapping reads, refer to section A. Methods.

Statistical analysis

Refer to section A. Methods. In addition, to explore for patterns across the global bacterial communities, the data matrix comprising the percent standardised abundances of OTUs was fourth root transformed and used to construct a sample-similarity matrix using the Bray-Curtis algorithm (Bray and Curtis, 1957), where samples were then ordinated using non-metric multidimensional scaling (nMDS) with 50 random restarts (Clarke et al., 2001). All statistical analyses were performed on the transformed Bray-Curtis similarity matrix, apart from the relative percent abundance of bacterial phyla, class, order, family and genus which were performed on the untransformed Bray-Curtis similarity matrix. For the two-way PERMANOVA design, where significant differences were observed between *a priori* pre-defined categories (emulsifier only diets, lipid only diets, emulsifier × lipid diets), differential abundance analysis on the fourth root transformed dataset was used to seek for OTUs that contribute to the observed differences using STAMP v.2.1.3 (Parks et al., 2014). The 154 OTUs found in the 30% and 20% lipid diets with and without emulsifier inclusion were compared using the Welch's t-test (two-sided) with no correction for p-values. OTUs were considered to be significantly different if the p-value was < 0.05.

C. Results

Global community structure

There was a clear separation in the nMDS plot of the transformed dataset and significant difference recorded on a global community structure level between the seawater and all gut samples (PERMANOVA, $P = 0.0255$, table not shown) (Figure 3.3.1.2.15). A significant difference was also recorded between the diets (PERMANOVA, $P = 0.0020$, table not shown), with pairwise comparisons highlighting a significant difference between pre-trial samples and Diet 2 ($P = 0.0128$), Diet 3 ($P = 0.0025$), and Diet 4 ($P = 0.0037$), as well as between Diet 1 and Diet 4 ($P = 0.0452$) (Table 3.3.1.2.14).

Bacterial phyla

For the majority of samples, including the pre-trial and the four diet types, dominance by a single bacterial phylum, *Spirochaetae*, was observed (Figure 3.3.1.2.16). Correspondingly, conserved taxonomy at the lower class (*Spirochaetes*), order (*Spirochaetales*), family (*Brevinemataceae*) and genus (*Brevinema*) levels was also observed (Figure 3.3.1.2.17). The phylum *Proteobacteria* was, however, also recorded in some of the Diet 1 samples and a single Diet 3 sample (Figure 3.3.1.2.16), with representation at the genera level by *Enterovibrio* (Figure 3.3.1.2.17 D). Due to the generally high levels of dominance from the genus *Brevinema*, it was difficult to discern any clear differences between 30% and 20% lipid inclusion (Diets 1 and 2 vs Diets 3 and 4), or between diets with or without emulsifiers (Diets 2 and 4 vs Diets 1 and 3). Of note, though, was the occurrence of a sample in Diet 1 (30% lipid inclusion without emulsifier) which comprised a high abundance of *Cyanobacteria*; with a low sequence similarity to *Anabaena cyclindrica* (OTU 49, similarity [S_ab] score 0.349) (Figure 3.3.1.2.18).

Top 15 OTUs

In line with the findings above, a single organism, with closest sequence similarity to *Brevinema andersonii* (OTU 1, similarity [S_ab] score 0.704), dominated the samples from the pre-trial and four diet types, with > 99% relative abundance recorded in some fish (Figure 3.3.1.2.18). Three fish on Diet 1 and a single fish on Diet 3 (which correspond to the lipid diets without emulsifiers) were also represented by a taxon with closest sequence similarity to *Enterovibrio nigricans* (OTU 10, S_ab score

1.000), though this organism was not observed in the lipid diets with emulsifiers (Diets 2 and 4) (Figure 3.3.1.2.18).

Diversity indices

There was no significant difference in species evenness (Pielou's and lambda+) or diversity (Simpson and delta+) between samples from the pre-trial or four diet type, although species richness and Shannon's diversity were significantly higher in samples from Diet 4 (20% lipid inclusion with emulsifier) compared to Diet 1 (30% lipid inclusion without emulsifier) (P = 0.0304 and P = 0.0210 respectively, Table 3.3.1.2.15) (Figure 3.3.1.2.19).

Factorial design

As the trial included two distinct factors (i.e. lipids – at 20 and 30% and emulsifier – with and without), we also investigated if there was a difference between: a) 20% and 30% lipid inclusion, irrespective of emulsifier content (combined data from Diets 3 and 4 vs combined data from Diets 1 and 2); b) diets with or without emulsifier (combined data for Diets 2 and 4 vs combined data for Diets 1 and 3); and c) the interaction between lipid diets crossed with emulsifier diets. Although there was no significant difference between lipid diets (20% vs 30%) or the crossed design (lipids × emulsifiers), diets with or without emulsifier were significantly different (P = 0.0268, Table 3.3.1.2.16). In testing for statistically significant OTUs contributing to the observed differences between diets with or without emulsifier, a total of 10 were observed, of which two were found to be more abundant in 30% lipid diets + emulsifier and six in 20% lipid diets + emulsifier (Tables 3.3.1.2.17 and 3.3.1.2.18). Of these, only one (OTU 149) occurred in diets with emulsifier, irrespective of lipid content, and was most closely related to *Aureimarina marisflavi* (S_ab score 0.835), a species belonging to the *Flavobacteriaceae* (Phylum *Bacteroidetes*).

D. Fish meal replacement nutritional trial N2/N5

D. Methods

Experimental design

A total of 72 fish were sampled from the SARDI land animal protein trial (Nutrition Theme N-5, utilise land animal protein to reduce fish meal in commercial diets for large YTK during summer and N-2, during winter, see Manuscript 3.1.3.1 for further details), with 18 fish sampled from two treatments on the 6th of June 2017 as a modified version of the summer component and 54 fish sampled from six treatments on the 22nd of November 2017 as a 12 week extension of the winter trial. The six treatments in the winter trial extension were carried on from the 12 week summer component, resulting in a trial duration of 252 days. Fish were held in 5000 L tanks located at the SARDI pool-farm facility and fed different diets where FM content was reduced (to levels of 20% and 10%) and replaced with commercially relevant alternate protein sources including digestible fish meal by-product protein (FMB-P), digestible poultry meal protein (PM) and/or digestible soy protein concentrate (SPC). For full details on each dietary formulation and inclusions, refer to Manuscript 3.1.3.1.

For each treatment, a total of nine fish were collected from three tanks (three fish per tank), with treatments representing diets comprising: 30% FM (control, referred to herein as Diet 1), 20% FM + 10.7% FMB-P (referred to herein as Diet 2), 10% FM + 21.4% FMB-P (referred to herein as Diet 3), 20% FM + 11.32% PM (referred to herein as Diet 4), 10% FM + 22.64% PM (referred to herein as Diet 5), 10% FM, 10.7% FMB-P + 11.32% PM (referred to herein as Diet 6), 20% FM + 10.88% SPC (referred to herein as Diet 7) and 10% FM, 10.7% FMB-P + 10.88% SPC (referred to herein as Diet 8) (Figure 3.3.1.2.20). Diets 5 and 8 were the two treatments that concluded in June after 84 days, while the remaining six diets continued on into the winter trial component and 12 week extension. Two

seawater samples were taken from the system in-flow and processed in parallel to control for the influence the environment may have on the structure and composition of the gut bacterial community (Figure 3.3.1.2.20).

Fish sampling

Refer to section A. Methods. For all samples collected from this trial, the MG and HG were separated from the FG using a sterile scalpel blade and placed on a clean surface. Using a clean pair of forceps and sterile scalpel, an incision was made along the length of the midgut and hindgut to expose the inner surfaces, and then a single scraping of the entire region was performed with a sterile glass slide to collect the gut contents/mucosa.

RNA extraction for gut microbiome samples

Refer to section A. Methods.

DNA extraction for environmental samples

Refer to section A. Methods.

cDNA synthesis, PCR amplification, AMPure bead-purification and Picogreen quantification

Refer to section A. Methods. The final list of samples that generated good-quality microbiomic libraries for the components of this work are presented in Table 3.3.1.2.19.

Bioinformatics analysis

In total, 3,378,921 million sequence reads were derived from 69 samples (of the 74 that were collected). Five samples failed to amplify enough material to produce good-quality NGS libraries. This was accounted for in the experimental design approach, by allowing for ample replication of fish. Table 3.3.1.2.20 provides a summary of OTUs remaining post-filtering. Rarefaction curves were used to inspect (retrospectively) sampling depth (Appendix 1D). Due to low sequence reads and occurrence as outliers in the non-metric multidimensional scaling (nMDS) plot, two samples (one from Diet 4 and one from Diet 7) were removed from the dataset (Table 3.3.1.2.19, Appendix 1D). For detailed methods on quality filtering and mapping reads, refer to section A. Methods.

Statistical analysis

Refer to section A. Methods.

D. Results

Global community structure

In the nMDS plot, there was a clear pattern of separation between the seawater samples and all gut samples, along with clustering of the summer trial samples (two diets) away from the winter trial samples (six diets) (Figure 3.3.1.2.21 A). This was confirmed by PERMANOVA, with a significant difference in global community structure between the seawater and summer ($P = 0.0054$) and winter ($P = 0.0008$) trial components, as well as between the summer and winter trial samples ($P = 0.0001$) (Table

3.3.1.2.21). The capacity to delineate seasonal effects, however, is likely confounded by possible variations in the formulation of feeds and/or the differences in trial duration (with summer samples collected 84 days after trial commencement and winter samples collected 252 days after trial commencement). For the latter reason, and that two summer diets (i.e. Diet 5 and Diet 8) were not recommended by the Nutrition Team (see Manuscript 3.1.3.1), these components were removed from all further analyses with the focus instead being on the six winter trial diets.

In the nMDS plot comprising samples from the six winter diets, no clear separation between diets were observed (Figure 3.3.1.2.21 B). Only samples from the control diet (Diet 1) and Diet 7 appeared to group closely together, with Diet 2 following a similar pattern apart from two 'outliers' (Figure 3.3.1.2.21 B). Despite this, a significant difference in the global bacterial community composition among diets was observed among diets (PERMANOVA, $P = 0.0263$, table not shown). This included some, though not so highly significant differences between Diet 1 and Diet 3 ($P = 0.0291$), Diet 1 and Diet 4 ($P = 0.0451$), Diet 2 and Diet 3 ($P = 0.0139$), Diet 2 and Diet 4 ($P = 0.0267$), and Diet 4 and Diet 6 ($P = 0.0299$) (Table 3.3.1.2.22).

Bacterial phyla

The patterns between diets observed at the global community level (Figure 3.3.1.2.21 B) were further highlighted in the phyla plot, with samples from the control diet (Diet 1) and Diets 2 and 7 primarily dominated by taxa from the phyla *Tenericutes*, while samples from Diet 3, Diet 4 and Diet 6 had greater diversity with higher abundance of *Proteobacteria* along with representation of *Chloroflexi*, *Cyanobacteria*, *Actinobacteria* and *Bacteroidetes*, with Diet 4 having a notable increase in abundance of *Actinobacteria* (Figure 3.3.1.2.22). This indicates that replacing 10% wild derived FM with either 10.7% FMB-P (Diet 2) or 10.88% SPC (Diet 7) does not significantly change the global bacterial community structure (Figure 3.3.1.2.21 B, Table 3.3.1.2.22) or the phyla composition (Figure 3.3.1.2.22) from what is observed when YTK are fed the control 30% FM diet (Diet 1). However, a reduction to 10% wild derived FM content (Diets 3 and 6) or replacing with 11.32% PM (Diet 4) does significantly alter the global community structure (Figure 3.3.1.2.21 B, Table 3.3.1.2.22) and phyla composition (Figure 3.3.1.2.22).

Top 15 OTUs

An OTU with closest sequence similarity to *Mycoplasma insons* (OTU 1, similarity [S_ab] score 0.420), was observed in almost all the samples from the six winter trial diets, and was particularly dominant in samples from the control diet (Diet 1) and Diets 2 and 7 (Figure 3.3.1.2.23). Samples from Diet 3, 4 and 6 comprised the greatest diversity of taxa (and inter-individual variation), with additional taxa beyond the top 15 OTUs contributing to the total relative abundance of these samples. In particular, samples from Diet 3 were enriched with taxa with closest sequence similarity to *Vibrio ichthyoenteri* (OTU 4, S_ab score 1.000) and *Anabaena cylindrica* (OTU 11, S_ab score 0.349), while Diet 4 samples were enriched with taxa with closest sequence similarity to *Ralstonia picketti* (OTU 2, S_ab score 1.000), *Pseudomonas pseudoalcaligenes* (OTU 6, S_ab score 0.973) and *A. cylindrica* (OTU 11, S_ab score 0.349), and Diet 6 samples with *V. ichthyoenteri* (OTU 4, S_ab score 1.000) and *Vibrio* sp. V776/*Allivibrio finisterrensis* (OTU 5, S_ab score 1.000) (Figure 3.3.1.2.23).

Diversity indices

For the diversity indices, there was no significant differences in total species richness, evenness (Pielou's and lambda+) and diversity (Shannon and delta+) between any of the six FM replacement winter trial diets (Figure 3.3.1.2.24, Table 3.3.1.2.23), although Diet 4 had significantly greater species diversity (Simpson) compared to Diet 2 ($P = 0.0273$, Table 3.3.1.2.23) (Figure 3.3.1.2.24).

Discussion

The aim of this experimental work was to characterise and understand the effects that specific feeds or dietary formulations may have on the gut microbiome of YTK and the consequence this may have for health and/or performance. To elucidate this, samples from various trials were collected for comparing between commercial feed formulations and 'natural' (Sardine) diets; diets modified to include variable levels of LC n-3 PUFA; diets comprising low and high lipid inclusion levels (with or without emulsifiers); and diets where wild derived fish meal (FM) is replaced with alternate protein sources. Compositional comparisons were also evaluated in relation to those of the surrounding seawater to determine if environmentally-independent gut community assemblages are selected for in the gut. Indeed, as previously observed in the baseline data (Manuscript 3.3.1.1), the global bacterial community composition between the environmental samples (surrounding seawater) and the gut samples was markedly different, irrespective of diet formulation. While the local habitat is thought to shape the gut microbiome of fish, this further supports the notion that YTK are able to regulate and maintain their own environmentally-independent bacterial communities in the gut.

A. Commercial feed formulations v 'natural' (Sardine) diets

In comparing between commercial feed formulations and 'natural' (Sardine) diets as part of Clean Seas on-farm and SA (SARDI pool-farm) and WA (Fremantle facility) tank trials, a number of differences were observed in the gut microbiome analyses. Firstly, while common diets were fed to fish contained in both sea-cages and tanks (e.g. Feed B and Feed C), markedly different gut bacterial communities were observed, with higher bacterial diversity observed in samples from fish from sea-cages compared to tanks. This supports the earlier observations reported in the baseline dataset (see Manuscript 3.3.1.1) and indicates a possible role for environment in the formation of these communities. Indeed, considering that the environment and management practices associated with onshore (tank-based) and offshore (sea-cage) systems are notably different, such differences are not surprising and support evidence in the literature to suggest that while fish are able to support environmentally independent gut bacterial communities, local environment plays a role in how these assemblages are shaped (Dehler et al., 2017). Of course, differences in size/age and background genetics may also be contributing features, particularly considering that many of the sea-cage and tank trial fish varied in size and were likely from different stocks (e.g. SA vs WA YTK). However, when investigating fish of the same size class (of likely mixed genetics), who were fed three different dietary formulations and who were contained in three separate sea-cages from the same site as part of an on-farm Clean Seas trial, markedly conserved gut bacterial assemblages were also observed. Specifically, fish from sea-cages from the same site (Bickers, Port Lincoln, SA) who were fed either Feed A, B or C, were all dominated by taxa from a single phyla (*Proteobacteria*), with conserved taxonomy at the lower class (*Gammaproteobacteria*), order (*Vibrionales*) and family (*Vibrionaceae*) levels. Only at the genus level were differences observed between the three on-farm feeds, with Feed A being dominated by taxa from unresolved *Photobacterium* species clades, including one with closest sequence similarity to *P. damselae* subsp. *damselae*, *P. damselae* subsp. *piscicida* and *P. leiognathi* (OTU 3). An increased level of diversity (and less variation among individuals) was also observed for fish fed Feed A, indicating that while environment may be a strong driver, diet still has some capacity to influence these communities. Nevertheless, with samples from fish fed Feed B and C also observed to comprise the same *Photobacterium* species clade (though at a lower abundance), it appears that certain organisms may be more generally widespread throughout fish cultivated in these systems, and may occur irrespective of diet. For organisms like *Photobacterium damselae* subsp. *piscicida*, which are a known pathogen of marine fish with the capacity to cause severe stock losses in *Seriola* species (Romalde, 2002; Kawanishi et al., 2006), this is of particular concern and warrants further investigation. This may include the need to undertake more structured (diagnostic) population and site surveys to establish the occurrence and prevalence of these organisms from other related taxa (e.g. *P. phosphoreum*, *P. iliopiscarium*, *P. leiognathi*) which were also observed in these samples and which may occur as commensals in fish and other marine species (Urbanczyk et al., 2011).

Despite a role for environment/cultivation practice in the types of bacterial taxa occurring within the gut, notable dietary specific effects were also observed in this experimental component. Specifically, alongside select diets used on-farm (e.g. Feed A at Clean Seas), some of the diets used in the WA tank trials were observed to enhance microbial diversity within the gut. Most strikingly was that of Feed C which, in contrast to WA Feed D and the diets used in the SA trial (i.e. Feed B and Sardines), was associated with considerably higher diversity and more even representation of bacterial taxa within the samples. This observation may be confounded by local environmental (system) and/or genetic differences between the trials, a more diverse taxa (over a greater range of lineages) is likely to support a greater array of (genetically encoded) metabolic functions (Heiman and Greenway, 2016). Such diets may thus represent an interesting prospect for promoting gut microbial diversity (and possibly gut health), even over more 'natural' diets, and requires further elucidation. In a recent study by Walburn et al. (2018), which investigated the gut microbiome associated with the early developmental stages (larvae and juveniles) of YTK in a larviculture production facility, the greatest change in the microbiome occurred as fish moved from a diet of live feeds to formulated pellets, as characterised by a transition from *Proteobacteria* to *Firmicutes* as the dominant phylum. They therefore suggest that diet is a major contributor to the early microbiome development of farmed YTK. However, for adult YTK, other factors also appear to be playing a role in shaping the gut community dynamics, with similarities between YTK fed Feed B and a 'natural' (Sardine) diet recorded from the SA trial. Furthermore, while the role of diet on influencing the gut microbiome of fish is well-known, the primary focus has largely been on the association of select feed ingredients or additives (for a review see Llewellyn et al., 2014; Ringø et al., 2016). Promoting optimal microbial diversity through the use of appropriate diets (or therapeutic supplements) may, in part, improve the fish's resilience and robustness to opportunistic organisms through the competitive interactions that inherently occur among microbes or through the indirect stimulation of the host's immune response and/or nutrient uptake (Bruijn et al., 2017; Dimitroglou et al., 2011; Llewellyn et al., 2014). This may be particularly important where reduced diversity and/or dominance from select groups like *Photobacterium* or *Brevinema* (as observed for a number of tank and on-farm trial diets) may result from the use of specific commercial fed formulations; a prospect that may lead to altered or diminished microbiome functionality (and possibly health) (Piazzon et al., 2017). However, with nutritional results showing improved growth rates and optimal FCR values for both diets used in the SA trial, irrespective of dominance by these organisms, further investigation is required to establish the broader metabolic or health effects posed by these altered communities, particularly if challenged.

B. Fatty acid inclusion trial N1

In comparing the effects of specific dietary formulations such as the use of diets supplemented to include variable levels of long chain omega 3 fatty acids (LC n-3 PUFA), some differences in the gut microbiome were also observed. Specifically, in comparison to the control (pre-trial) diet, samples collected from fish fed diets amended with LC n-3 PUFA had an altered global bacterial community structure, irrespective of the inclusion of LC n-3 PUFA at either a high (2.95 g 100 g^{-1}, Diet 1), moderate (2.14 g 100 g^{-1}, Diet 3) or low (0.753 g 100 g^{-1}, Diet 8) level. This change was associated with the enrichment of certain phyla (including *Spirochaetae*, *Actinobacteria, Firmicutes*, *Bacteroidetes* and *Cyanobacteria*) and the loss of otherwise dominant OTUs representing potentially opportunistic species like *P. damselae* subsp. *damselae*/*P. damselae* subsp. *piscicida* and *Mycoplasma insons*. Within certain gut regions like the MG, this loss was also accompanied by significantly higher species richness and taxonomic diversity and evenness at a moderate level of LC n-3 PUFA inclusion (2.14 g 100 g^{-1}, Diet 3), with increased representation from minor constituents including *Bacteroidetes*, *Firmicutes* and *Chloroflexi*. With the lack of apparent changes in diversity and evenness observed for the HG samples (at any LC n-3 PUFA level), this suggests that the inclusion of LC n-3 PUFA into YTK diets at optimal concentrations may have the capacity to displace potentially opportunistic pathogens by promoting diversity in certain gut regions. Considering that microbial structure (and function) may differ along the gastrointestinal tract and be associated with specific gut regions (Llewellyn et al., 2014; Egerton et al., 2018; Feng et al., 2018), such a result is not surprising. However, while the effect of omega-3 fatty acids on the gut microbiota in fish is largely unknown, within humans and animal models it has been

demonstrated that diets supplemented with omega-3 PUFAs can exert positive actions by reverting altered microbiome compositions during disease through the production of anti-inflammatory compounds (e.g. short-chain fatty acids); whereby certain groups like *Bacteroidetes* may be key players (Costantini et al., 2017). Though it is tempting to postulate then that the decrease of potentially opportunistic pathogens here was associated with the concomitant increase of diversity due to LC n-3 PUFA inclusion, further investigation is required. Nevertheless, with marginal improvements in SGR/FCRs observed for Diet 3 in the broader Nutrition theme trial (see Manuscript 3.1.1.1), such a prospect is intriguing and requires further elucidation to determine whether higher microbial diversity in the MG also has the capacity to improve gut health and YTK performance through, e.g. enhanced nutrient (fatty acid) metabolism.

C. Lipid inclusion with and without emulsifier trial N3

Similar to the experimental components for the fatty acid trial detailed above, in comparing the effects of diets amended with lipids (with and without emulsifier), differences in the gut microbiome were largely observed between the experimental treatments and the unamended (pre-trial) samples. This was largely due to the increased abundance of an OTU in the lipid diet treatment samples that was most closely related to *Enterovibrio nigricans* (OTU 10) (family *Vibrionaceae*). Considering that this organism was previously isolated and described from the internal organs (head kidney) of diseased farmed Gilthead Sea Bream (*Sparus aurata*) and Common Dentex (*Dentex dentex*) (Pascaul et al., 2009) its occurrence here is intriguing and may be suggestive of possible negative health impacts associated with lipid inclusion in the diet. From the nutritional trial, a 30% dietary lipid level was suggested to improve YTK growth and feed utilisation, hence its relevance here requires further elucidation.

Among the dietary treatments, the overall diversity and evenness of the samples were similar, with only a marginal (though significant) increase in total species richness and diversity in Diet 4 (20% lipid with emulsifier) compared to Diet 1 (30% lipid without emulsifier); a feature likely due to the higher abundance of *Enterovibrio* and *Cyanobacteria* in one or more of the samples from Diet 1. Further variation among the treatments, however, was difficult to discern due to the occurrence of a single, dominant OTU most closely related to *Brevinema andersonii* (OTU 1) within the phylum *Spirochaetae*. This organism was widespread among both the pre-trial and experimental treatments, where it occurred at an abundance of $\geq$ 99% in some samples. Its presence within the pre-trial samples (rather than solely within the treatments), indicated that the organism had likely established dominance within the gut on-farm prior to the commencement of the trial. Interestingly, this organism was also observed as one of the dominant taxa in the earlier SA (SARDI pool-farm) tanks trials assessing commercial feeds and 'natural' (Sardine) diets (see Part A above), where fish came from the same farm, were also sampled in spring (Part A, sampled September 2015; and Part C, November 2016) and belonged to the same size class (~1.4-1.7 kg). However, while it was not possible to discern whether this organism had established dominance in the fish prior to the commencement of the earlier trial (due to the absence of pre-trial samples), in a consecutive trial comparing fatty acids from fish obtained from the same farm (though in winter and from a larger size class) the organism only occurred in the post-trial samples, and was not considered to be overly dominant (see Part B above and Figure 3.3.1.2.12). Given its occurrence also in wild (SA) YTK as well as healthy fish sampled as part of the baseline datasets at low relative abundances (Manuscript 3.3.1.1), it is likely then that *Brevinema* represents a common constituent of the YTK gut microbiome, with seasonal, diet and/or cultivation system-specific effects leading to its dominance, possibly as an opportunistic species. To the best of our knowledge, however, *Brevinema* species have not have been previously observed from diseased fish, though is an infectious spirochaete of mammals (Defosse et al., 1995) and has been shown to occur alongside other opportunistic pathogens in disease sensitive lines of farmed Rainbow Trout (*Oncorhynchus mykiss*) (Salinas et al., 2018). In this regard, its relevance in the gut of YTK (either as a commensal or opportunistic species) requires further elucidation, along with the broader (functional) effects associated with a diminished gut microbial community.

Despite a conserved level of taxonomy and similar levels of species diversity and evenness across the experimental treatments, in comparing diets with or without emulsifiers (irrespective of lipid level),

significant differences in the abundances of select (minor) community constituents were observed. Of the 10 OTUs observed to be associated with diets comprising an emulsifier, one was found to be common across both experimental lipid inclusion diets and was most closely related to *Aureimarina marisflavi*, a species belonging to the Family *Flavobacteriaceae*. To the best of our knowledge, this organism has not been previously described to occur in association with fish or other marine animals but, instead, has been described as an environmental organism occurring as part of marine bacterioplanktonic assemblages (Brettar et al., 2012). While it is possible then that this organism may represent a transient (rather than a strictly resident) species within the gut of YTK, the microbiomic assay used in this study seeks to delineate these assemblages by examining the active bacterial constituents from total community RNA (rather than DNA). Its occurrence here, albeit at a low abundance, may thus suggest that it is being enriched in the gut from the surrounding environment, possibly due to the inclusion of the addition of emulsifier in the diet. Given the wide range of natural and synthetic emulsifiers used in the manufacture of animal feeds (Siyal et al., 2017) and the plethora of unique metabolic pathways supported by microbes for metabolising an array of different substrates, including synthetic compounds, such a prospect is not implausible. Interestingly, some of the metabolic traits exhibited by members of the *Flavobacteriaceae* include the ability to metabolise common synthetic additives used in feed manufacture such as polyethylene glycol (Willetts, 1981). Considering this, and the broader effects dietary emulsifiers have been recently shown to impart on the gut microbiome and health in other animal (rodent) model systems (Chassaing et al., 2015), their influence on the gut microbiota of YTK warrants consideration. However, whether the enrichment of this (or other organisms) is associated with their ability to directly use the emulsifier as a carbon source for growth, or from the improved bioavailability of the lipids, is not clear and requires further investigation.

D. Fish meal replacement trial N2/N5

In the last experimental component investigating the graduated replacement of wild derived FM with alternate protein sources, eight diets were investigated, with two diets sampled after 84 days and constituting the modified summer component, while the remaining six diets were continued on for a further 12 weeks before being sampled (as the winter extension component). For each diet, FM content was reduced and replaced with commercially relevant alternate protein sources including digestible FMB-P, PM and/or SPC. In evaluating the gut microbiome from samples collected from these treatments, some notable differences were observed and included some distinct differences in the global bacterial community composition between samples collected as part of the individual summer and winter-feeding trials. While this may indicate an effect due to seasonality, confounding effects due to variations in the formulation of feeds and/or differences in the duration of the trials existed (whereby summer samples were collected 84 days after trial commencement; and 252 days after winter trial commencement). With the two summer diets not recommended by the Nutrition Team (see Manuscript 3.1.3.1) and consequently not carried on through to the winter component of the trial, such a finding could not be delineated. Thus, further analysis of the summer samples was not conducted, and is not included in this report discussion. Instead, in comparing among samples collected from the winter-feeding trial only, some (albeit not highly statistically significant) variations were observed in the global bacterial community profiles between individual diets. Notably, in comparison to the control diet (30% FM), there was an enrichment of major taxonomic groups in Diet 3 (10% FM + 21.4% FMB-P), Diet 4 (20% FM + 11.32% PM) and, to a lesser extent, Diet 6 (10% FM, 10.7% FMB-P + 11.32% PM) and included the phyla *Proteobacteria*, *Chloroflexi*, *Cyanobacteria*, *Bacteroidetes* and *Actinobacteria*. Despite this, among the control and treatment groups, a high relative abundance of a single OTU was observed with closest sequence similarity to *Mycoplasma insons*, OTU 1 (belonging to class *Mollicutes;* phyla *Tenericutes*). Having evolved over long co-evolutionary periods with a range of different hosts, mycoplasma are typically parasitic species that are characterised by their uniquely small genomes and lack of a cell wall, where they are able to evade the hosts immunological responses by infecting the cellular membranes (Razin et al., 1998). For this reason, infections arising from mycoplasma are thus often difficult to treat with antibiotics. Though *Mycoplasma insons* has been specifically described to occur in association from Iguanas (May et al., 2007), only a weak sequence identity to this species was observed (similarity [S_ab] score 0.420), and thus this OTU likely represents a related taxon within the

broader family *Mycoplasmataceae* (or Order *Mycoplasmatales*); as is also the case where it was previously detected within the earlier fatty acid experimental component (see Section B. Results) and on-farm samples collected as part of the earlier baseline datasets (see Manuscript 3.3.1.1). Considering that this organism was also detected in wild YTK samples, though in low abundances, it seems reasonable to assume then that this organism is generally more widespread in YTK where effects of cultivation and diet may be drivers of its dominance in the gut of farmed fish. To the best of our knowledge, this organism has not been previously documented in *Seriola* species. However, with various related *Mycoplasma* species having been associated with infectious diseases and other conditions in fish (though not exclusively) (Ahmed and Hady, 2008; Burns et al., 2018; Romero et al., 2014), further work is required to determine its relevance, including whether it is an invasive pathogen, or occurs opportunistically as a secondary feature of a primary pathology or an immunosuppressed state in YTK.

Despite the high relative abundance of the *M. insons* related OTU among the control and experimental treatments, it occurred at notably lower abundances in Diets 3 (10% FM + 21.4% FMB-P), 4 (20% FM + 11.32% PM) and 6 (10% FM, 10.7% FMB-P + 11.32% PM). Alongside the enrichment of the major taxonomic (phyla level) lineages noted in these dietary treatments (as indicated above), an increase in diversity and evenness was also apparent for Diet 4 (20% FM + 11.32% PM) and likely corresponded to the increase in abundance of particular taxa like *Actinobacteria* in these samples. Higher abundances of *Actinobacteria* have been previously reported in healthy compared to diseased farmed fish (Atlantic Salmon, *Salmo salar*) (Wang et al., 2018) and are thought to play a role as a relevant (though often minor constituent) of the microbiome in regulating gut homeostasis (Binda et al., 2018). Collectively, this highlights that a reduction to 10% wild derived FM content (Diets 3 and 6) or replacement of 10% FM with 11.32% PM (Diet 4) significantly alters the gut community structure and dynamics and may improve the capacity for the displacement of potentially opportunistic species; possibly leading to improved gut function through the selective enrichment of certain taxa like *Actinobacteria* that are well-known for supporting gut health. Interestingly, however, this group appeared to be in reduced abundance in wild compared to farmed YTK in the baseline microbiome dataset (see Manuscript 3.3.1.1) and is a feature that has also been observed for Chilean wild and farmed YTK which, as part of the broader microbiome, was purported to be a due to the differences in the 'natural' or formulated feeds consumed by these fish (Ramírez and Romero, 2017). The prospective role for diet in the enrichment of these select (perhaps beneficial) constituents thus appears likely and requires further elucidation to determine their functional association and contribution to YTK health and/or performance, particularly considering that no significant differences were observed in any of the growth, feed utilisation or blood haematology and biochemistry indices measured between the dietary treatments in the broader nutritional trial (see Manuscript 3.1.3.1).

This work demonstrates that specific feeds or dietary formulations have varied impacts on the structure and dynamics of the gut microbiome of farmed YTK, with some diets leading to improved gut microbiome diversity and the enrichment of potentially beneficial taxa. The emergence of single, dominant bacterial taxa in the gut of fish fed certain diets, however, is likely to be linked to poor performance and disease. Evaluation of the drivers of changes in bacterial communities and the involvement of identified bacterial taxa as pathogens would be valuable.

Conclusion and Recommendation

We provide an analysis of the influence and effects that various commercial feeds and select dietary formulations have on the gut microbiome of YTK, establishing the role for certain feeds in promoting microbial diversity (and possibly functionality) through the enrichment of potentially beneficial taxa. Considerable variation in the gut microbiome was observed associated with different commercial feeds within and between individual farms, with some formulations appearing to increase microbial diversity even over more 'natural' diets. Dominant, potentially pathogenic species were also observed in association with the use of certain feeds though, in some instances, these pathogens also occurred irrespective of diet, so other factors (e.g. host size class/age, environment/seasonality, cultivation practice and host genetics) also contribute to the emergence of these organisms within the gut of farmed

YTK. While from the current findings it is not clear whether such taxa represent primary pathogens or opportunistic species, their occurrence as a dominant feature is indicative of a microbiome with depleted diversity and probable diminished gut function. Further investigation would establish the effects they exert (if any) on the health and/or performance of YTK. Despite a clear role of diet in their emergence, for some amended diets comprising improved levels of select feed ingredients, these taxa occurred in lower abundances and were likely displaced from hosts with improved diversity. While such feeds may represent prospects for promoting diversity and gut health, some additives used to promote bioavailability and nutritional uptake of specific feed ingredients were found to enrich environmental organisms (albeit in low abundances). Formulas that select for 'optimal' gut microbiomes should be developed by conducting assessments of the underlying gene functions contributing to varied health and/or performance in YTK.

Findings

This component of the work found marked differences in the global bacterial community structure and diversity between certain commercial formulations used on-farm and as part of tank trials, along with differences arising from their use on individual farms. Some formulations promoted microbial diversity in the gut while others promoted the enrichment of potentially opportunistic species. Notably, Feed C from the formulated feeds vs 'natural' Sardine diet component occurred in association with lower abundance of potentially opportunistic pathogens (in particular OTU 3 with closest sequence similarity to *Photobacterium damselae* subsp. *damselae*/*P. damselae* subsp. *piscicida*/*P. leiognathi*) and increased diversity in both the sea-cage and tank trial samples. In contrast, lower diversity and taxonomic evenness was observed in the other formulated feeds and the more 'natural' Sardine diets. Feed C thus represents an interesting prospect for promoting gut microbial diversity in farmed YTK. In evaluating select dietary formulations such as the inclusion of LC n-3 PUFA, moderate levels of inclusion (at 2.14 g $100g^{-1}$, Diet 3) significantly increased species richness, evenness and diversity within certain gut regions (namely the MG) and was associated with a greater representation of additional phyla (including potentially beneficial taxa) as well as a decrease in otherwise dominant pathogens including *Photobacterium damselae* subsp. *damselae*/*P. damselae* subsp. *piscicida* and *Mycoplasma insons*. In contrast, high or low levels of lipids (with and without the addition of an emulsifier) did not significantly alter the gut microbiome structure or composition, although all samples including the controls were dominated by a single taxon (with closest sequence similarity to *Brevinema andersonii*) which may have established dominance in the fish prior to commencement of the trial. *Brevinema andersonii* also appeared to be more widespread in samples from other treatments and was also observed in the earlier baseline datasets (Manuscript 3.3.1.1), though not as a dominant feature, and should be investigated as a likely opportunistic pathogen. The use of emulsifiers (irrespective of lipid inclusion level), however, resulted in the enrichment (albeit in low abundances) of select environmental microorganisms which may have the capacity to use the emulsifier as a carbon source for growth and highlights the need to assess the broader impacts that dietary components have on the gut microbiome. When reducing or replacing fish meal content in formulated diets a reduction to 10% wild derived FM content (Diets 3 and 6) or replacing with 11.32% PM (Diet 4) promoted a richer and more diverse microbiome composition with enrichment of potentially beneficial taxa leading to the displacement of potentially opportunistic taxa such as *M. insons*. Given its broader occurrence in other treatments and earlier samples collected as part of the baseline datasets (Manuscript 3.3.1.1), further work is required to determine its relevance in YTK.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit program, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds. The authors would also like to acknowledge the support of SARDI and provision of the Molecular Sciences Laboratory facilities at West Beach, South Australia. Thanks to Dr Trent D'Antignana of Nutrisea, Dr Jay Dent and Dr Erin Bubner of Clean Seas, and Clean Seas health team staff for coordinating and assisting with sample collections on-farm. Thanks to the SARDI nutrition team (Dr David Stone, Dr Matt Bansemer, Paul Skordas and volunteers) and Dr Gavin Partridge and Dr Lindsey Woolley from South Metropolitan TAFE, Fremantle, WA for access to tank-trial samples and assistance with sample collection and Dr Melissa Wos-Oxley of Flinders University for supporting and guiding the statistical analyses.

References

Ahmed, H., El-Hady, M., 2008. Antigenic variations and pathogenicity between *Mycoplasma* spp isolated from fishes and shellfishes. 8th International Symposium on Tilapia Aquaculture.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology 26, 32-46.

Binda, C., Lopetuso, L.R., Rizzatti, G., Gibiino, G., Cennamo, V., Gasbarrini, A., 2018. Actinobacteria: a relevant minority for the maintenance of gut homeostasis. Digestive and Liver Disease 50, 421-428.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. Ecological monographs 27(4), 325-349.

Brettar, I., Christen, R., Hofle, M.G., 2012. Analysis of bacterial core communities in the central Baltic by comparative RNA-DNA-based fingerprinting provides links to structure-function relationships. ISME J 6, 195-212.

Bruijin, I., Liu, Y., Wiegertjes, G.F., Raaijmakers, J.M., 2017. Exploring fish microbial communities to mitigate emerging diseases in aquaculture. FEMS Microbiology Ecology 94, 1-12.

Burns, A.R., Watral, V., Sichel, S., Spagnoli, S., Banse, A.V., Mittge, E., Sharpton, T.J., Guillemin, K., Kent, M.L., 2018. Transmission of a common intestinal neoplasm in zebrafish by cohabitation. Journal of Fish Disease 41, 569-579.

Camarinha-Silva, A., Jáuregui, R., Chaves-Moreno, D., Oxley, A.P.A., Schaumburg, F., Becker, K., Wos-Oxley, M.L., Pieper, D.H., 2014. Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. Environmental Microbiology 16(9), 2939-2952.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7(5), 335-336.

Chassaing, B., Koren, O., Goodrich, J., Poole, A.C., 2015. Dietary emulsifiers impacts the mouse gut microbiota promoting colitis and metabolic syndrome. Nature 519, 92-96.

Chaves-Moreno, D., Plumeier, I., Kahl, S., Krismer, B., Peschel, A., Oxley, A.P.A., Jauregui, R., Pieper, D.H., 2015. The microbial community structure of the cotton rat nose. Environmental Microbiology Reports 7(6), 929-935.

Clarke, K.R., Warwick, R.M.R.M., Laboratory, P.M., 2001. Change in marine communities: an approach to statistical analysis and interpretation: Plymouth, U.K.: PRIMER-E Ltd.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Research 42 (Database issue), D633-D642.

Costantini, L., Molinari, R., Farinon, B., Merendino, N., 2017. Impact of omega-3 fatty acids on the gut microbiota. International Journal of Molecular Sciences 18, 1-12.

Dehler, C.E., Secombes, C.J., Martin, S.A.M. 2017. Environmental and physiological factors shape the gut microbiota of Atlantic salmon parr (*Salmo salar* L.). Aquaculture 467, 149-157.

Defosse, D.L., Johnson, R.C., Paster, B.J., Dewhirst, F.E., Fraser, G.J., 1995. *Brevinema andersonii* gen. nov., sp. nov., an infectious spirochete isolated from the short-tailed shrew (*Blarina brevicauda*) and the white-footed mouse (*Peromyscus leucopus*). International Journal of Systematic Bacteriology 45, 78-84.

Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C., Guroy, D., Davies, S.J., 2011. Microbial manipulations to improve fish health and production – A Mediterranean perspective. Fish and Shellfish Immunology 30, 1-16.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19), 2460-2461.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nature Methods 10, 996-998.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27(16), 2194-2200.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., Ross, R.P., 2018. The gut microbiota of marine fish. Frontiers in Microbiology 9, 1-17.

Feng, W., Zhang, J., Jakovlic, I., Xiong, F., Wu, S., Zou, H., Li, W., Li, M., Wang, G., 2018. Gut segments outweigh the diet in shaping the intestinal microbiome composition in grass carp *Ctenopharyngodon idellus*. bioRxiv, 1-30.

Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krofdahl, A., Nelson, R., Overturf, K., Rust, K., Sealey, W., Skonberg, D., Souza, E.J., Stone, D., Wilson, R., Wurtelel, E. 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquaculture Research 38, 551-579.

Ghanbari, M., Kneifel, W., Domig, K.J. 2015. A new view of the fish gut microbiome: Advances from next-generation sequencing. Aquaculture 448, 464-475.

Heinman, M.L., Greenway, F.L., 2016. A healthy gastrointestinal microbiome is dependent on dietary diversity. Molecular Metabolism 5, 317-320.

Kawanishi, M., Kijima, M., Kojima, A., Ishihara, K., Esaki, H., Yagyu, K., Takahashi, T., Suzuki, S., Tamura, Y. 2006. Drug resistance and random amplified polymorphic DNA analysis of *Photobacterium damselae* ssp. *piscicida* isolates from cultured *Seriola* (yellowtail, amberjack and kingfish) in Japan. Letters in Applied Microbiology 42, 648-653.

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H., Derome, N., 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Frontiers in Microbiology 5, 1-17.

Lyons, P.P., Turnbull, J.F., Dawson, K.A., Crumlish, M. 2017. Effects of low-level dietary microalgae supplementation on the distal intestinal microbiome of farmed rainbow trout *Oncorhynchus mykiss* (Walbaum). Aquaculture Research 48, 2438-2452.

May, M., Ortiz, G.J., Wendland, L.D., Rotstein, D.S., Relich, R.F., Balish M.F., Brown, D.R., 2007. *Mycoplasma insons* sp. nov., a twisted mycoplasma from green iguanas (*Iguana iguana*). FEMS Microbiology Letter 274, 298-303.

Merrifield, D.L., Olsen, R.E., Myklebust, R., Ringø, E. 2011. Dietary effect of soybean (Glycine max) products on gut histology and microbiota of fish. Soybean and Nutrition, El-Shemy, H. (ed), InTech, 476pp.

Nayak, S.K. 2010. Role of gastrointestinal microbiota in fish. Aquaculture Research 41, 1553-1573.

Parks, D.H., Tyson, G.W., Hugenholtz, P., Beiko, R.G. 2014. STAMP: statistical analysis of taxonomic and functional profiles. Bioinformatics 30, 3123-3124.

Pascual, J., Macian, M.C., Arahal, D.R., Garay, E., Pujalte, M.J., 2009. Description of *Enterovibrio nigricans* sp. nov., reclassification of *Vibrio calviensis* as *Enterovibrio calviensis* comb. nov. and emended description of the genus *Enterovibrio* Thompson *et al.* 2002. International Journal of Systematic and Evolutionary Microbiology 59, 698-704.

Piazzon, M.C., Calduch-Giner, J.A., Fouz, B., Estensoro, I., Simo-Mirabet, P., Puyalto, M., Karalazos, V., Palenzuela, O., Sitha-Bobadilla, A., Perez-Sanchez, J., 2017. Under control: how a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. Microbiome 5, 164.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41, D590-D596.

Ramirez, C., Romero, J. 2017. The microbiome of *Seriola lalandi* of wild and aquaculture origin reveals differences in composition and potential function. Frontiers in Microbiology 8, 1844.

Razin, S., Yogev, D., Naot, Y., 1998. Molecular biology and pathogenicity of *Mycoplasmas*. Microbiology and Molecular Biology Reviews 62, 1094-1156.

Ringø, E., Zhou, Z., Vecino, J.L.G., Wadsworth, S., Romero, J., Krogdahl, A., Olsen, R.E., Dimitroglou, A., Foey, A., Davies, S., Owen, M., Lauzon, H.L., Martinsen, L.L., De Schryver, P., Bossier, P., Sperstad, S., Merrifield, D.L. 2016. Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? Aquaculture Nutrition 22, 219-282.

Romalde, J.L. 2002. *Photobacterium damselae* subsp. *piscicida*: an integrated view of a bacterial fish pathogen. International Microbiology 5, 3-9.

Romero, J., Ringø, E., Merrifield, D.L., 2014, 'The gut microbiota of fish', in D.L. Merrifield and E. Ringø (eds.), Aquaculture nutrition: gut health, probiotics and prebiotics (John Wiley & Sons, Ltd), pp. 75-100.

Salinas, I., Brown, R.M., Wiens, G.D., 2018. Analysis of the gut and gill microbiome of resistant and susceptible lines of rainbow trout (*Oncorhynchus mykiss*). bioRxiv.

Siyal, F., Babazadeh, D., Wang, C., Arain, M.A., 2017. Emulsifiers in the poultry industry. World's Poultry Science Journal 73, 1-9.

Sullam, K.E., Essinger, S.D., Lozupone, C.A., O'Conner, M.P., Rosen, G.L., Knight, R., Kilham, S.S., Russell, J.A. 2012. Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. Molecular Ecology 21(13).

Szafranska, A.K., Oxley, A.P.A., Chaves-Moreno, D., Horst, S.A., Roßlenbrolch, S., Peters, G., Goldmann, O., Rohde, M., Sinha, B., Pleper, D.H., Löffler, B., Jauregul, R., Wos-Oxley, M.L., Medina, E., 2014. High-resolution transcriptomic analysis of the adaptive responses of *Staphylococcus aureus* during acute and chronic phases of osteomyelitis. mBio 5(6), e01775-14.

Urbanczyk, H., Ast, J.C., Dunlap, P.V., 2011. Phylogeny, genomics, and symbiosis of *Photobacterium*. FEMS Microbiology Reviews 35, 324-342.

Walburn, J.W., Wemheuer, B., Thomas, T., Copeland, E., O'Connor, W., Booth, M., Fielder, S., Egan, S., 2018. Diet and diet-associated bacteria shape early microbiome development in Yellowtail Kingfish (*Seriola lalandi*). Microbial Biotechnology, 1-14.

Wang, C., Sun, G., Li, S., Li, X., Liu, Y., 2018. Intestinal microbiota of healthy and unhealthy Atlantic salmon *Salmo salar* L. in a recirculating aquaculture system. Journal of Oceanology and Limnology 36, 414-426.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology 73(16), 5261-5267.

Willetts, A., 1981. Bacterial metabolism of ethylene glycol. Biochimica et Biophysica Acta 677, 194-199.

Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30(5), 614-20.

Zhang, M., Sun, Y., Liu, Y., Qiao, F., Chen, L., Liu, W-T, Du, Z., Li, E., 2016. Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference. Aquaculture 454, 72.

Table 3.3.1.2.1. Sample information pertaining to the Commercial Feed Formulations vs 'Natural' (Sardine) Diet component.

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
water sample	-	-	-	Lincoln	Bickers	ABK14-10GS	4/02/2016	104107	477
Feed A	MH	69.0	-	Lincoln	Bickers	ABK14-10GL	4/02/2016	57311	117
Feed A	MH	68.0	-	Lincoln	Bickers	ABK14-10GL	4/02/2016	75808	186
Feed A	MH	68.0	-	Lincoln	Bickers	ABK14-10GL	4/02/2016	36158	18
Feed A	MH	66.0	-	Lincoln	Bickers	ABK14-10GL	4/02/2016	49663	22
Feed A	MH	65.0	-	Lincoln	Bickers	ABK14-10GL	4/02/2016	21280	30
Feed B	MH	71.0	-	Lincoln	Bickers	ABK14-10GS	4/02/2016	60718	37
Feed B	MH	61.0	-	Lincoln	Bickers	ABK14-10GS	4/02/2016	75479	33
Feed B	MH	69.0	-	Lincoln	Bickers	ABK14-10GS	4/02/2016	72469	27
Feed B	MH	67.0	-	Lincoln	Bickers	ABK14-10GS	4/02/2016	70905	23
Feed B	MH	69.0	-	Lincoln	Bickers	ABK14-10GS	4/02/2016	53122	24
Feed C	MH	66.0	-	Lincoln	Bickers	ABK14-10mix	4/02/2016	74088	27
Feed C	MH	67.0	-	Lincoln	Bickers	ABK14-10mix	4/02/2016	54631	21
Feed C	MH	68.0	-	Lincoln	Bickers	ABK14-10mix	4/02/2016	52299	18
Feed C	MH	68.0	-	Lincoln	Bickers	ABK14-10mix	4/02/2016	53096	22
Feed C	MH	69.0	-	Lincoln	Bickers	ABK14-10mix	4/02/2016	50061	15
Feed C	MH	45.0	1312	Fremantle	Pool-farm facility	-	14/07/2016	73414	60
Feed C	MH	46.0	1335	Fremantle	Pool-farm facility	-	14/07/2016	17533	54
Feed D	MH	45.0	1568	Fremantle	Pool-farm facility	-	14/07/2016	22968	60
Feed D	MH	47.0	1451	Fremantle	Pool-farm facility	-	14/07/2016	60792	54
Feed D	MH	44.0	1393	Fremantle	Pool-farm facility	-	14/07/2016	13316	53
Feed B	FG	47.6	1462	SARDI	Pool-farm facility	Tank 7	17/09/2015	66770	83
Feed B	MG	47.6	1462	SARDI	Pool-farm facility	Tank 7	17/09/2015	38344	94
Feed B	HG	47.6	1462	SARDI	Pool-farm facility	Tank 7	17/09/2015	47022	92
Feed B	FG	48.1	1649	SARDI	Pool-farm facility	Tank 7	17/09/2015	68923	65
Feed B	HG	48.1	1649	SARDI	Pool-farm facility	Tank 7	17/09/2015	36420	46
Feed B	MG	47.0	1552	SARDI	Pool-farm facility	Tank 7	17/09/2015	72876	40
Feed B	HG	47.0	1552	SARDI	Pool-farm facility	Tank 7	17/09/2015	80124	40
Feed B	HG	45.5	1462	SARDI	Pool-farm facility	Tank 13	17/09/2015	59119	24
Feed B	MG	47.8	1457	SARDI	Pool-farm facility	Tank 13	17/09/2015	45692	56
Feed B	HG	47.8	1457	SARDI	Pool-farm facility	Tank 13	17/09/2015	48456	42
Feed B	MG	48.5	1636	SARDI	Pool-farm facility	Tank 13	17/09/2015	64678	55
Feed B	HG	48.5	1636	SARDI	Pool-farm facility	Tank 13	17/09/2015	51733	39
Feed B	MG	46.4	1420	SARDI	Pool-farm facility	Tank 20	17/09/2015	53534	55
Feed B	HG	46.4	1420	SARDI	Pool-farm facility	Tank 20	17/09/2015	44645	69
Feed B	MG	47.3	1630	SARDI	Pool-farm facility	Tank 20	17/09/2015	39669	100
Feed B	HG	47.3	1630	SARDI	Pool-farm facility	Tank 20	17/09/2015	33787	28
Sardines	FG	48.1	1752	SARDI	Pool-farm facility	Tank 5	17/09/2015	4642	43
Sardines	MG	48.1	1752	SARDI	Pool-farm facility	Tank 5	17/09/2015	6506	30
Sardines	HG	48.1	1752	SARDI	Pool-farm facility	Tank 5	17/09/2015	23890	17
Sardines	MG	46.9	1577	SARDI	Pool-farm facility	Tank 5	17/09/2015	55074	43
Sardines	HG	46.9	1577	SARDI	Pool-farm facility	Tank 5	17/09/2015	67305	35
Sardines	MG	48.6	1613	SARDI	Pool-farm facility	Tank 5	17/09/2015	56521	55
Sardines	HG	48.6	1613	SARDI	Pool-farm facility	Tank 5	17/09/2015	48416	36
Sardines[1]	MG	46.0	1445	SARDI	Pool-farm facility	Tank 10	17/09/2015	319	23
Sardines[1]	HG	46.0	1445	SARDI	Pool-farm facility	Tank 10	17/09/2015	407	23
Sardines	MG	49.0	1689	SARDI	Pool-farm facility	Tank 10	17/09/2015	39160	34
Sardines	HG	49.0	1689	SARDI	Pool-farm facility	Tank 10	17/09/2015	41197	31
Sardines	MG	45.7	1489	SARDI	Pool-farm facility	Tank 10	17/09/2015	21779	38

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Sardines	HG	45.7	1489	SARDI	Pool-farm facility	Tank 10	17/09/2015	18593	48
Sardines[1]	MG	47.5	1501	SARDI	Pool-farm facility	Tank 21	17/09/2015	263	23
Sardines[1]	HG	47.5	1501	SARDI	Pool-farm facility	Tank 21	17/09/2015	661	32
Sardines	MG	43.0	1516	SARDI	Pool-farm facility	Tank 21	17/09/2015	61363	39
Sardines	HG	43.0	1516	SARDI	Pool-farm facility	Tank 21	17/09/2015	48197	44
Sardines	HG	49.4	1840	SARDI	Pool-farm facility	Tank 21	17/09/2015	30253	27

Abbreviations: FG, foregut; HG, hindgut; MG, midgut; MH, combined mid- and hindgut scraping.
[1] Samples removed due to low sequence reads.

Table 3.3.1.2.2. Sample information pertaining to the Commercial Feed Formulations vs 'Natural' (Sardine) Diet component.

Experiment data-set	No. of samples	Total reads	Median library size	Range	Bacterial OTUs post- filtering
Formulated feeds and 'natural' diets	55	2,908,786	50,758	263-104,107	616

Table 3.3.1.2.3. One-way PERMANOVA: Pairwise test between the five tank trial feeds.[1]

Feed_tank trial location	P	Significant?
Feed C_WA, Feed D_WA	0.5016	No
Feed C_WA, Feed B_SA	**0.0068**	Yes
Feed C_WA, Sardines_SA	**0.1010**	Yes
Feed D_WA, Feed B_SA	**0.0018**	Yes
Feed D_WA, Sardines_SA	**0.0014**	Yes
Feed B_SA, Sardines_SA	**0.0087**	Yes

Abbreviations: SA, South Australian (SARDI pool-farm facility); WA, Western Australia (Fremantle facility).
[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.2.4. Two-way PERMANOVA: Pairwise test between the three gut regions for SARDI pool-farm facility tank trial samples: A) feed B and B) Sardines.[1]

Diet type	P	Significant?
A) Feed B		
FG, MG	0.0729	No
FG, HG	**0.0427**	Yes
MG, HG	0.4715	No
B) Sardines		
FG, MG	0.5650	No
FG, HG	0.6291	No
MG, HG	0.4783	No

Abbreviations: FG, foregut; HG, hindgut; MG, midgut.
[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.2.5. One-way PERMANOVA: Pairwise test between the three Bickers sea-cage feeds.[1]

Feed (sea-cages)	P	Significant?
Feed A, Feed B	**0.0079**	Yes
Feed A, Feed C	**0.0398**	Yes
Feed B, Feed C	0.8260	No

[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.2.6. ANOVA results for diversity indices for A) tank trial and B) sea-cage samples.

Diversity measure	ANOVA summary[1]	Tukey's posthoc test	Adjusted P-value
A) Tank trial samples (WA & SA)			
Species richness (S)	F=1.564		
	P=0.2369		
Species evenness (J')	F=8.452		
	***P*=0.0013**		
		feed C_WA vs feed D_WA	0.6374
		feed C_WA vs feed B_SA	**0.0035**
		feed C_WA vs Sardine_SA	**0.0079**
		feed D_WA vs feed B_SA	**0.0224**
		feed D_WA vs Sardine_SA	0.0543
		feed B_SA vs Sardine_SA	0.9525
Shannon's diversity (H')	F=6.878		
	***P*=0.0035**		
		feed C_WA vs feed D_WA	0.6907
		feed C_WA vs feed B_SA	**0.0092**
		feed C_WA vs Sardine_SA	**0.0139**
		feed D_WA vs feed B_SA	0.0519
		feed D_WA vs Sardine_SA	0.0797
		feed B_SA vs Sardine_SA	0.9953
Simpson's diversity (1-λ)	F=7.948		
	***P*=0.0018**		
		feed C_WA vs feed D_WA	0.8986
		feed C_WA vs feed B_SA	**0.0066**
		feed C_WA vs Sardine_SA	**0.0270**
		feed D_WA vs feed B_SA	**0.0118**
		feed D_WA vs Sardine_SA	0.0589
		feed B_SA vs Sardine_SA	0.7536
Delta+ (Δ+)	F=3.36		
	P=0.0510		
Lambda+ (λ+)	F=3.126		
	P=0.0551		
B) Sea-cage samples (SA)			
Species richness (S)	F=2.542		
	P=0.1201		
Species evenness (J')	F=2.389		
	P=0.1339		
Shannon's diversity (H')	F=5.271		
	***P*=0.0228**		
		feed A vs feed B	0.1703
		feed A vs feed C	**0.0208**
		feed B vs feed C	0.6281
Simpson's diversity (1-λ)	F=4.541		
	***P*=0.0340**		
		feed A vs feed B	0.1078
		feed A vs feed C	**0.0347**
		feed B vs feed C	0.7937
Delta+ (Δ+)	F=5.322		
	***P*=0.0222**		
		feed A vs feed B	0.8673
		feed A vs feed C	0.0631
		feed B vs feed C	**0.0255**
Lambda+ (λ+)	F=1.213		
	P=0.3313		

Abbreviations: SA, South Australia; WA, Western Australia.

[1] Where a significant difference ($P < 0.05$) is observed then the Tukey's pairwise test was performed.

Table 3.3.1.2.7. Sample information pertaining to the Fatty Acid Inclusion trial component.

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Pre-trial	MH	52.8	2018	SARDI	Pool-farm facility	reserve	17/03/2016	129538	46
Pre-trial	MH	55.8	2465	SARDI	Pool-farm facility	reserve	17/03/2016	105189	54
Pre-trial	MH	51.2	1878	SARDI	Pool-farm facility	reserve	17/03/2016	108129	55
Pre-trial	MH	55.5	2432	SARDI	Pool-farm facility	reserve	17/03/2016	84669	64
Pre-trial	MH	58.8	2728	SARDI	Pool-farm facility	reserve	17/03/2016	78406	83
Diet 1 – 2.95	MG	58.5	3495	SARDI	Pool-farm facility	Tank 2	2/06/2016	144108	127
Diet 1 – 2.95	HG	58.5	3495	SARDI	Pool-farm facility	Tank 2	2/06/2016	90393	94
Diet 1 – 2.95	HG	60.5	3554	SARDI	Pool-farm facility	Tank 2	2/06/2016	71403	143
Diet 1 – 2.95	HG	62.5	4012	SARDI	Pool-farm facility	Tank 2	2/06/2016	14754	59
Diet 1 – 2.95	HG	60.5	3700	SARDI	Pool-farm facility	Tank 10	2/06/2016	71135	49
Diet 1 – 2.95	MG	60.0	3604	SARDI	Pool-farm facility	Tank 10	2/06/2016	83524	131
Diet 1 – 2.95	HG	60.0	3604	SARDI	Pool-farm facility	Tank 10	2/06/2016	80882	59
Diet 1 – 2.95	MG	57.5	3177	SARDI	Pool-farm facility	Tank 10	2/06/2016	65356	152
Diet 1 – 2.95	HG	57.5	3177	SARDI	Pool-farm facility	Tank 10	2/06/2016	101660	18
Diet 1 – 2.95	MG	60.0	3579	SARDI	Pool-farm facility	Tank 23	3/06/2016	97552	112
Diet 1 – 2.95	HG	60.0	3579	SARDI	Pool-farm facility	Tank 23	3/06/2016	99177	75
Diet 1 – 2.95	MG	59.0	3460	SARDI	Pool-farm facility	Tank 23	3/06/2016	51665	105
Diet 1 – 2.95	HG	64.5	4156	SARDI	Pool-farm facility	Tank 23	3/06/2016	61346	71
Diet 3 – 2.14	MG	64.5	4341	SARDI	Pool-farm facility	Tank 1	2/06/2016	40457	147
Diet 3 – 2.14	HG	64.5	4341	SARDI	Pool-farm facility	Tank 1	2/06/2016	74026	98
Diet 3 – 2.14	MG	60.0	3933	SARDI	Pool-farm facility	Tank 1	2/06/2016	42513	108
Diet 3 – 2.14	HG	60.0	3933	SARDI	Pool-farm facility	Tank 1	2/06/2016	76062	71
Diet 3 – 2.14	MG	60.0	3717	SARDI	Pool-farm facility	Tank 1	2/06/2016	61285	91
Diet 3 – 2.14	HG	60.0	3717	SARDI	Pool-farm facility	Tank 1	2/06/2016	77186	105
Diet 3 – 2.14	MG	59.5	3815	SARDI	Pool-farm facility	Tank 13	3/06/2016	44228	96
Diet 3 – 2.14	HG	59.5	3815	SARDI	Pool-farm facility	Tank 13	3/06/2016	50822	93
Diet 3 – 2.14	HG	60.0	4066	SARDI	Pool-farm facility	Tank 13	3/06/2016	73344	105
Diet 3 – 2.14	MG	61.0	3702	SARDI	Pool-farm facility	Tank 13	3/06/2016	27777	102
Diet 3 – 2.14	HG	61.0	3702	SARDI	Pool-farm facility	Tank 13	3/06/2016	72236	91
Diet 3 – 2.14	MG	58.5	3591	SARDI	Pool-farm facility	Tank 20	3/06/2016	26559	111
Diet 3 – 2.14	HG	58.5	3591	SARDI	Pool-farm facility	Tank 20	3/06/2016	39784	56
Diet 3 – 2.14	HG	62.0	3695	SARDI	Pool-farm facility	Tank 20	3/06/2016	61376	47
Diet 3 – 2.14	MG	60.5	3837	SARDI	Pool-farm facility	Tank 20	3/06/2016	34840	96
Diet 3 – 2.14	HG	60.5	3837	SARDI	Pool-farm facility	Tank 20	3/06/2016	51305	62
Diet 8 – 0.753	MG	60.0	3625	SARDI	Pool-farm facility	Tank 7	2/06/2016	72481	113
Diet 8 – 0.753	HG	60.0	3625	SARDI	Pool-farm facility	Tank 7	2/06/2016	61460	75
Diet 8 – 0.753	HG	60.0	3575	SARDI	Pool-farm facility	Tank 7	2/06/2016	70910	83
Diet 8 – 0.753	MG	62.0	3505	SARDI	Pool-farm facility	Tank 7	2/06/2016	51400	119
Diet 8 – 0.753	HG	62.0	3505	SARDI	Pool-farm facility	Tank 7	2/06/2016	73312	62
Diet 8 – 0.753	MG	60.0	3503	SARDI	Pool-farm facility	Tank 15	3/06/2016	39960	54
Diet 8 – 0.753	HG	60.0	3503	SARDI	Pool-farm facility	Tank 15	3/06/2016	103717	78
Diet 8 – 0.753	HG	61.0	3320	SARDI	Pool-farm facility	Tank 15	3/06/2016	75523	82
Diet 8 – 0.753	MG	63.0	4104	SARDI	Pool-farm facility	Tank 22	3/06/2016	35052	47
Diet 8 – 0.753	HG	63.0	4104	SARDI	Pool-farm facility	Tank 22	3/06/2016	87556	89
Diet 8 – 0.753	MG	60.5	3591	SARDI	Pool-farm facility	Tank 22	3/06/2016	67609	86
Diet 8 – 0.753	HG	60.5	3591	SARDI	Pool-farm facility	Tank 22	3/06/2016	80588	59

Abbreviations: HG, hindgut; MG, midgut; MH, combined mid- and hindgut scraping.

Table 3.3.1.2.8. Sample information pertaining to the Fatty Acid Inclusion trial component.

Experiment data-set	No. of samples	Total reads	Median library size	Range	Bacterial OTUs post- filtering
Fatty Acid Inclusion trial	46	3,669,822	71,605	14,754-144,108	299

Table 3.3.1.2.9. Two-way PERMANOVA: Pairwise test between the two gut regions sampled (MG, HG) from each fatty acid inclusion diet.[1]

Diet (gut region)	P	Significant?
Diet 1 (MG, HG)	0.0500	No
Diet 3 (MG, HG)	**0.0003**	Yes
Diet 8 (MG, HG)	0.1329	No

Abbreviations: HG, hindgut; MG, midgut.
[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.2.10. One-way PERMANOVA: Pairwise test between the pre-trial samples and three fatty acid inclusion diets.[1]

Diet	P	Significant?
pre-trial, diet 1 HG	**0.0081**	Yes
pre-trial, diet 3 MG	**0.0014**	Yes
pre-trial, diet 3 HG	**0.0014**	Yes
pre-trial, diet 8 HG	**0.0107**	Yes
diet 1 HG, diet 3 HG	0.6152	No
diet 1 HG, diet 8 HG	0.8390	No
diet 3 HG, diet 8 HG	0.6399	No
diet 3 MG, diet 1 HG	**0.0026**	Yes
diet 3 MG, diet 3 HG	**0.0002**	Yes
diet 3 MG, diet 8 HG	**0.0017**	Yes

Abbreviations: HG, hindgut; MG, midgut.
[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.2.11. ANOVA results for diversity indices for fatty acid inclusion trial samples.

Diversity measure	ANOVA summary[1]	Tukey's posthoc test[2]	Adjusted P-value
Species richness (S)	F=4.433		
	***P*=0.0060**		
		pre-trial vs diet 3_MG	**0.0068**
		diet 1_HG vs diet 3_MG	**0.0264**
		diet 3_MG vs diet 8_HG	**0.0238**
Species evenness (J')	F=8.175		
	***P*=0.0001**		
		pre-trial vs diet 3_MG	**0.0034**
		diet 1_HG vs diet 3_MG	**0.0010**
		diet 3_MG vs diet 3 HG	**0.0003**
		diet 3_MG vs diet 8_HG	**0.0005**
Shannon's diversity (H')	F=9.652		
	***P*<0.0001**		
		pre-trial vs diet 3_MG	**0.0010**
		diet 1_HG vs diet 3_MG	**0.0003**
		diet 3_MG vs diet 3 HG	**0.0001**
		diet 3_MG vs diet 8_HG	**0.0002**
Simpson's diversity (1-λ)	F=5.312		
	***P*=0.0022**		
		diet 1_HG vs diet 3_MG	**0.0051**
		diet 3_MG vs diet 3 HG	**0.0040**
		diet 3_MG vs diet 8_HG	**0.0066**
Delta+ (Δ+)	F=1.371		
	P=0.2666		
Lambda+ (λ+)	F=1.279		
	P=0.2996		

Abbreviations: HG, hindgut; MG, midgut.

[1] Where a significant difference ($P < 0.05$) is observed then the Tukey's pairwise test was performed.

[2] Only significant pairwise comparisons are shown

Table 3.3.1.2.12. Sample information pertaining to the Lipid and Emulsifier trial.

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Tank ID	Date sample collected	library size	# bacterial OTUs
water sample	-	-	-	SARDI	Pool-farm facility	Tank 26	25/11/2016	51258	465
Pre-trial	MH	43	1144	SARDI	Pool-farm facility	reserve	2/09/2016	3071	22
Pre-trial	MH	44	1145	SARDI	Pool-farm facility	reserve	2/09/2016	50797	44
Pre-trial	MH	45.5	1318	SARDI	Pool-farm facility	reserve	2/09/2016	14939	27
Pre-trial	MH	42.5	1186	SARDI	Pool-farm facility	reserve	2/09/2016	49620	89
Diet 1	MH	46	1387	SARDI	Pool-farm facility	Tank 28	25/11/2016	33275	67
Diet 1	MH	47	1544	SARDI	Pool-farm facility	Tank 28	25/11/2016	49203	46
Diet 1	MH	45.5	1523	SARDI	Pool-farm facility	Tank 28	25/11/2016	44921	87
Diet 1	MH	45	1365	SARDI	Pool-farm facility	Tank 32	25/11/2016	3714	35
Diet 1	MH	45	1475	SARDI	Pool-farm facility	Tank 32	25/11/2016	28848	33
Diet 1	MH	46	1486	SARDI	Pool-farm facility	Tank 33	25/11/2016	26597	37
Diet 1	MH	44	1571	SARDI	Pool-farm facility	Tank 33	25/11/2016	14642	31
Diet 1	MH	45	1439	SARDI	Pool-farm facility	Tank 33	25/11/2016	40885	22
Diet 2	MH	47.5	1789	SARDI	Pool-farm facility	Tank 27	25/11/2016	34575	48
Diet 2[1]	MH	47	1703	SARDI	Pool-farm facility	Tank 27	25/11/2016	3454	101
Diet 2	MH	44.5	1383	SARDI	Pool-farm facility	Tank 27	25/11/2016	50836	55
Diet 2	MH	45.5	1453	SARDI	Pool-farm facility	Tank 30	25/11/2016	47146	36
Diet 2	MH	44	1363	SARDI	Pool-farm facility	Tank 30	25/11/2016	34412	62
Diet 2	MH	46.5	1619	SARDI	Pool-farm facility	Tank 30	25/11/2016	9486	96
Diet 2	MH	44.5	1348	SARDI	Pool-farm facility	Tank 34	25/11/2016	30346	91
Diet 2	MH	46	1390	SARDI	Pool-farm facility	Tank 34	25/11/2016	30550	48
Diet 2	MH	45	1514	SARDI	Pool-farm facility	Tank 34	25/11/2016	39407	39
Diet 3	MH	46	1530	SARDI	Pool-farm facility	Tank 25	25/11/2016	53779	62
Diet 3	MH	44	1447	SARDI	Pool-farm facility	Tank 25	25/11/2016	53609	66
Diet 3	MH	45	1348	SARDI	Pool-farm facility	Tank 25	25/11/2016	41485	68
Diet 3	MH	44.5	1214	SARDI	Pool-farm facility	Tank 31	25/11/2016	32662	47
Diet 3	MH	45.5	1423	SARDI	Pool-farm facility	Tank 31	25/11/2016	43515	66
Diet 3	MH	45.5	1459	SARDI	Pool-farm facility	Tank 31	25/11/2016	44401	50
Diet 3	MH	45.5	1521	SARDI	Pool-farm facility	Tank 35	25/11/2016	42004	47
Diet 3	MH	45.5	1469	SARDI	Pool-farm facility	Tank 35	25/11/2016	43439	53
Diet 3	MH	45.5	1456	SARDI	Pool-farm facility	Tank 35	25/11/2016	46880	22
Diet 4	MH	44	1240	SARDI	Pool-farm facility	Tank 26	25/11/2016	38937	49
Diet 4	MH	44.5	1374	SARDI	Pool-farm facility	Tank 26	25/11/2016	30263	79
Diet 4	MH	44.5	1348	SARDI	Pool-farm facility	Tank 26	25/11/2016	26651	85
Diet 4	MH	46.5	1594	SARDI	Pool-farm facility	Tank 29	25/11/2016	41778	66
Diet 4	MH	46.5	1568	SARDI	Pool-farm facility	Tank 29	25/11/2016	38803	54
Diet 4	MH	44.5	1444	SARDI	Pool-farm facility	Tank 29	25/11/2016	45380	154
Diet 4	MH	47	1687	SARDI	Pool-farm facility	Tank 36	25/11/2016	27099	93
Diet 4	MH	45.5	1364	SARDI	Pool-farm facility	Tank 36	25/11/2016	33956	81
Diet 4	MH	47	1713	SARDI	Pool-farm facility	Tank 36	25/11/2016	17960	58

Abbreviations: combined mid- and hindgut scraping.
[1] Sample removed due to low sequence reads

Table 3.3.1.2.13. Sample information pertaining to the Lipid and Emulsifier trial component.

Experiment data-set	No. of samples	Total reads	Median library size	Range	Bacterial OTUs post- filtering
Emulsifier trial	40	1,423,326	39,262	3,454-51,258	570

Table 3.3.1.2.14. One way PERMANOVA: Pairwise test between the pre-trial samples and four lipid and emulsifier trial diets.[1]

Diet[2]	P	Significant?
pre-trial, diet 1	0.0732	No
pre-trial, diet 2	**0.0128**	Yes
pre-trial, diet 3	**0.0025**	Yes
pre-trial, diet 4	**0.0037**	Yes
diet 1, diet 2	0.5282	No
diet 1, diet 3	0.2960	No
diet 1, diet 4	**0.0452**	Yes
diet 2, diet 3	0.1190	No
diet 2, diet 4	0.5268	No
diet 3, diet 4	0.0542	No

[1] Significant difference denoted by $P < 0.05$, bolded if significant.
[2] Diet 1 = 30% L –E, Diet 2 = 30% L +E, Diet 3 = 20% L –E, Diet 4 = 20% L +E.

Table 3.3.1.2.15. ANOVA results for diversity indices for lipid and emulsifier trial samples.

Diversity measure	ANOVA summary[1]	Tukey's posthoc test[2]	Adjusted P-value
Species richness (S)	F=2.957 *P*=0.0341		
		diet 1 vs diet 4	0.0304
Species evenness (J')	F=2.049 *P*=0.1102		
Shannon's diversity (H')	F=3.088 *P*=0.0289		
		diet 1 vs diet 4	0.0210
Simpson's diversity (1-λ)	F=2.215 *P*=0.1068		
Delta+ (Δ+)	F=2.416 *P*=0.0859		
Lambda+ (λ+)	F=0.9348 *P*=0.4360		

[1] Where a significant difference ($P < 0.05$) is observed then the Tukey's pairwise test was performed.
[2] Only significant pairwise comparisons are shown

Table 3.3.1.2.16. Two-way PERMANOVA: Main test to determine if there is any significant difference in microbiome composition between lipid diets (low 20% vs high 30%), emulsifier diets (with and without inclusion) and crossed design of lipid × emulsifier diets.[1]

Source	df	SS	MS	Pseudo-F	P-value
Lipid	1	2164.8	2164.8	1.2981	0.1398
Emulsifier	1	2870.3	2870.3	1.7212	**0.0268**
Lipid × Emulsifier	1	1245.6	1245.6	0.74697	0.8138
Res	30	50028	1667.3		
Total	33	56363			

[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.2.17. Relative abundances of the statistically significant OTUs (P-values <0.05) from the 30% lipid diet types (diet 1 and diet 2) with (Δ) and without (O) emulsifier, where the particular symbol denotes in which diet (with or without emulsifier) the OTU was more abundant.

Bacterial Taxa (S_ab score)_OTU no.	Mean relative frequency (%) with emulsifier (Δ)	Mean relative frequency (%) without emulsifier (O)	Highest abundant group
Aureimarina marisflavi (0.835)_OTU 149	0.67	0.11	Δ
Marinitoga camini (0.423)_OTU 370	0.87	0.09	Δ
Pseudophaeobacter arcticus (1.000)/*Roseobacter* sp. (1.000)_OTU 898	0.59	1.49	O

Table 3.3.1.2.18. Relative abundances of the statistically significant OTUs (P-values <0.05) from the 20% lipid diet types (diet 3 and diet 4) with (Δ) and without (O) emulsifier, where the particular symbol denotes in which diet (with or without emulsifier) the OTU was more abundant.

Bacterial Taxa (S_ab score)_OTU no.	Mean relative frequency (%) with emulsifier (Δ)	Mean relative frequency (%) without emulsifier (O)	Highest abundant group
Aestuariibacter salexigens (0.679) _OTU 144	0.74	0.09	Δ
Aureimarina marisflavi (0.835)_OTU 149	0.89	0.00	Δ
Jonesia sp. (0.422) _OTU 166	0.67	1.66	O
Polaribacter irgensii (0.756) _OTU 181	1.37	0.45	Δ
Polaribacter huanghezhanensis (0.709)_OTU 227	0.91	0.14	Δ
Paraglaciecola mesophila (0.840) _OTU 234	0.81	0.00	Δ
Fusobacterium mortiferum (0.905)/*Clostridium rectum* (0.905) _OTU 3	1.36	2.00	O
Gangjinia marincola (0.500)_OTU 365	1.03	0.00	Δ

Table 3.3.1.2.19. Sample information pertaining to the Fish Meal Replacement trial.

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Tank ID	Date sample collected	library size	# bacterial OTUs
water sample	-	-	-	SARDI	Pool-farm facility	21	22/11/2017	69775	435
water sample	-	-	-	SARDI	Pool-farm facility	23	22/11/2017	50668	409
Diet 5	MH	590	3353	SARDI	Pool-farm facility	4	06/06/2017	38193	74
Diet 5	MH	605	3391	SARDI	Pool-farm facility	4	06/06/2017	48779	100
Diet 5	MH	540	2660	SARDI	Pool-farm facility	4	06/06/2017	43669	66
Diet 5	MH	580	2976	SARDI	Pool-farm facility	12	06/06/2017	118925	19
Diet 5	MH	595	3166	SARDI	Pool-farm facility	12	06/06/2017	72228	100
Diet 5	MH	590	3870	SARDI	Pool-farm facility	12	06/06/2017	81256	113
Diet 5	MH	595	3680	SARDI	Pool-farm facility	24	06/06/2017	49046	78
Diet 5	MH	585	3259	SARDI	Pool-farm facility	24	06/06/2017	58059	34
Diet 5	MH	585	3356	SARDI	Pool-farm facility	24	06/06/2017	74652	50
Diet 8	MH	530	3097	SARDI	Pool-farm facility	5	06/06/2017	50087	31
Diet 8	MH	570	3127	SARDI	Pool-farm facility	5	06/06/2017	104621	48
Diet 8	MH	610	3316	SARDI	Pool-farm facility	5	06/06/2017	63222	30
Diet 8	MH	590	3511	SARDI	Pool-farm facility	16	06/06/2017	89992	79
Diet 8	MH	610	3387	SARDI	Pool-farm facility	16	06/06/2017	72413	34
Diet 8	MH	590	3455	SARDI	Pool-farm facility	16	06/06/2017	96618	70
Diet 8	MH	575	2955	SARDI	Pool-farm facility	21	06/06/2017	102094	73
Diet 8	MH	590	3491	SARDI	Pool-farm facility	21	06/06/2017	99047	96
Diet 8	MH	600	3436	SARDI	Pool-farm facility	21	06/06/2017	48052	101
Diet 1	MH	645	4411	SARDI	Pool-farm facility	2	22/11/2017	98863	20
Diet 1	MH	620	4421	SARDI	Pool-farm facility	2	22/11/2017	32060	27
Diet 1	MH	610	4238	SARDI	Pool-farm facility	9	22/11/2017	71153	75
Diet 1	MH	645	4478	SARDI	Pool-farm facility	9	22/11/2017	36064	34
Diet 1	MH	625	4275	SARDI	Pool-farm facility	9	22/11/2017	47983	48
Diet 1	MH	670	5186	SARDI	Pool-farm facility	21	22/11/2017	26323	19
Diet 1	MH	640	4430	SARDI	Pool-farm facility	21	22/11/2017	54363	31
Diet 2	MH	590	3387	SARDI	Pool-farm facility	3	22/11/2017	63162	30
Diet 2	MH	650	4304	SARDI	Pool-farm facility	3	22/11/2017	33321	33
Diet 2	MH	610	4257	SARDI	Pool-farm facility	3	22/11/2017	30153	37
Diet 2	MH	645	4276	SARDI	Pool-farm facility	11	22/11/2017	75606	40
Diet 2	MH	635	4311	SARDI	Pool-farm facility	11	22/11/2017	91325	21
Diet 2	MH	620	4122	SARDI	Pool-farm facility	11	22/11/2017	47939	21
Diet 2	MH	615	3832	SARDI	Pool-farm facility	18	22/11/2017	70285	17
Diet 2	MH	660	4954	SARDI	Pool-farm facility	18	22/11/2017	48206	19
Diet 2	MH	655	3985	SARDI	Pool-farm facility	18	22/11/2017	37814	22
Diet 3	MH	665	5048	SARDI	Pool-farm facility	6	22/11/2017	28459	23
Diet 3	MH	610	4598	SARDI	Pool-farm facility	6	22/11/2017	28067	46
Diet 3	MH	665	5039	SARDI	Pool-farm facility	6	22/11/2017	36546	21
Diet 3	MH	635	4249	SARDI	Pool-farm facility	15	22/11/2017	18494	27
Diet 3	MH	600	3925	SARDI	Pool-farm facility	15	22/11/2017	54536	19
Diet 3	MH	590	3375	SARDI	Pool-farm facility	15	22/11/2017	70899	27
Diet 3	MH	665	4448	SARDI	Pool-farm facility	9	22/11/2017	36476	21
Diet 3	MH	595	4105	SARDI	Pool-farm facility	9	22/11/2017	44875	36
Diet 3	MH	595	3618	SARDI	Pool-farm facility	9	22/11/2017	45016	30
Diet 4	MH	620	4574	SARDI	Pool-farm facility	7	22/11/2017	47857	29
Diet 4	MH	660	4868	SARDI	Pool-farm facility	7	22/11/2017	19846	48
Diet 4[1]	MH	650	4693	SARDI	Pool-farm facility	7	22/11/2017	353	46
Diet 4	MH	610	3650	SARDI	Pool-farm facility	14	22/11/2017	49070	26
Diet 4	MH	640	4273	SARDI	Pool-farm facility	14	22/11/2017	6517	17

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Tank ID	Date sample collected	library size	# bacterial OTUs
Diet 4	MH	655	4822	SARDI	Pool-farm facility	14	22/11/2017	75424	17
Diet 4	MH	640	4134	SARDI	Pool-farm facility	17	22/11/2017	39720	26
Diet 4	MH	625	4031	SARDI	Pool-farm facility	17	22/11/2017	100203	20
Diet 4	MH	615	3439	SARDI	Pool-farm facility	17	22/11/2017	13117	17
Diet 6	MH	630	4087	SARDI	Pool-farm facility	5	22/11/2017	56199	21
Diet 6	MH	620	4428	SARDI	Pool-farm facility	5	22/11/2017	50115	19
Diet 6	MH	635	4915	SARDI	Pool-farm facility	5	22/11/2017	48656	18
Diet 6	MH	670	4768	SARDI	Pool-farm facility	10	22/11/2017	55138	16
Diet 6	MH	640	4353	SARDI	Pool-farm facility	10	22/11/2017	55729	19
Diet 6	MH	615	4004	SARDI	Pool-farm facility	22	22/11/2017	47948	25
Diet 6	MH	645	4329	SARDI	Pool-farm facility	22	22/11/2017	39235	26
Diet 6	MH	650	4856	SARDI	Pool-farm facility	22	22/11/2017	49625	17
Diet 7[1]	MH	660	4754	SARDI	Pool-farm facility	1	22/11/2017	11901	43
Diet 7	MH	620	4230	SARDI	Pool-farm facility	1	22/11/2017	58170	29
Diet 7	MH	600	3552	SARDI	Pool-farm facility	13	22/11/2017	76822	67
Diet 7	MH	640	4062	SARDI	Pool-farm facility	13	22/11/2017	51668	20
Diet 7	MH	675	5082	SARDI	Pool-farm facility	23	22/11/2017	42103	17
Diet 7	MH	635	4408	SARDI	Pool-farm facility	23	22/11/2017	31327	29
Diet 7	MH	625	4703	SARDI	Pool-farm facility	23	22/11/2017	6053	25

Abbreviations: combined mid- and hindgut scraping.
[1] Sample removed due to low sequence reads.

Table 3.3.1.2.20. Sample information pertaining to the Fish Meal Replacement trial component.

Experiment data-set	No. of samples	Total reads	Median library size	Range	Bacterial OTUs post- filtering
Fish meal replacement trial	69	3,758,921	50,206	353-118,925	639

Table 3.3.1.2.21. One-way PERMANOVA: Pairwise test between the tank water, summer trial and winter trial samples.[1]

Sample type	P	Significant?
water, FMR summer	**0.0054**	Yes
water, FMR winter	**0.0008**	Yes
FMR summer, FMR winter	**0.0001**	Yes

Abbreviations: FMR, fish meal replacement.
[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.2.22. One-way PERMANOVA: Pairwise test between the diet types for the fish meal replacement winter trial samples (diet 1 control, diet 2, diet 3, diet 4, diet 6 and diet 7).[1]

Diet type	P	Significant?
diet 1, diet 2	0.4927	No
diet 1, diet 3	**0.0291**	Yes
diet 1, diet 4	**0.0451**	Yes
diet 1, diet 6	0.3407	No
diet 1, diet 7	0.7622	No
diet 2, diet 3	**0.0139**	Yes
diet 2, diet 4	**0.0267**	Yes
diet 2, diet 6	0.1670	No
diet 2, diet 7	0.4862	No
diet 3, diet 4	0.4522	No
diet 3, diet 6	0.4517	No
diet 3, diet 7	0.1886	No
diet 4, diet 6	**0.0299**	Yes
diet 4, diet 7	0.0730	No
diet 6, diet 7	0.6379	No

[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.2.23. ANOVA results for diversity indices for fish meal replacement winter trial samples.

Diversity measure	ANOVA summary[1]	Tukey's posthoc test[2]	Adjusted P-value
Species richness (S)	F=1.560 *P*=0.1930		
Species evenness (J')	F=2.631 *P*=0.0594		
Shannon's diversity (H')	F=2.253 *P*=0.0671		
Simpson's diversity (1-λ)	F=2.789 *P*=0.0294		
		diet 2 vs diet 4	0.0273
Delta+ (Δ+)	F=1.996 *P*=0.0995		
Lambda+ (λ+)	F=0.4807 *P*=0.7886		

[1] Where a significant difference ($P < 0.05$) is observed then the Tukey's pairwise test was performed.
[2] Only significant pairwise comparisons are shown.

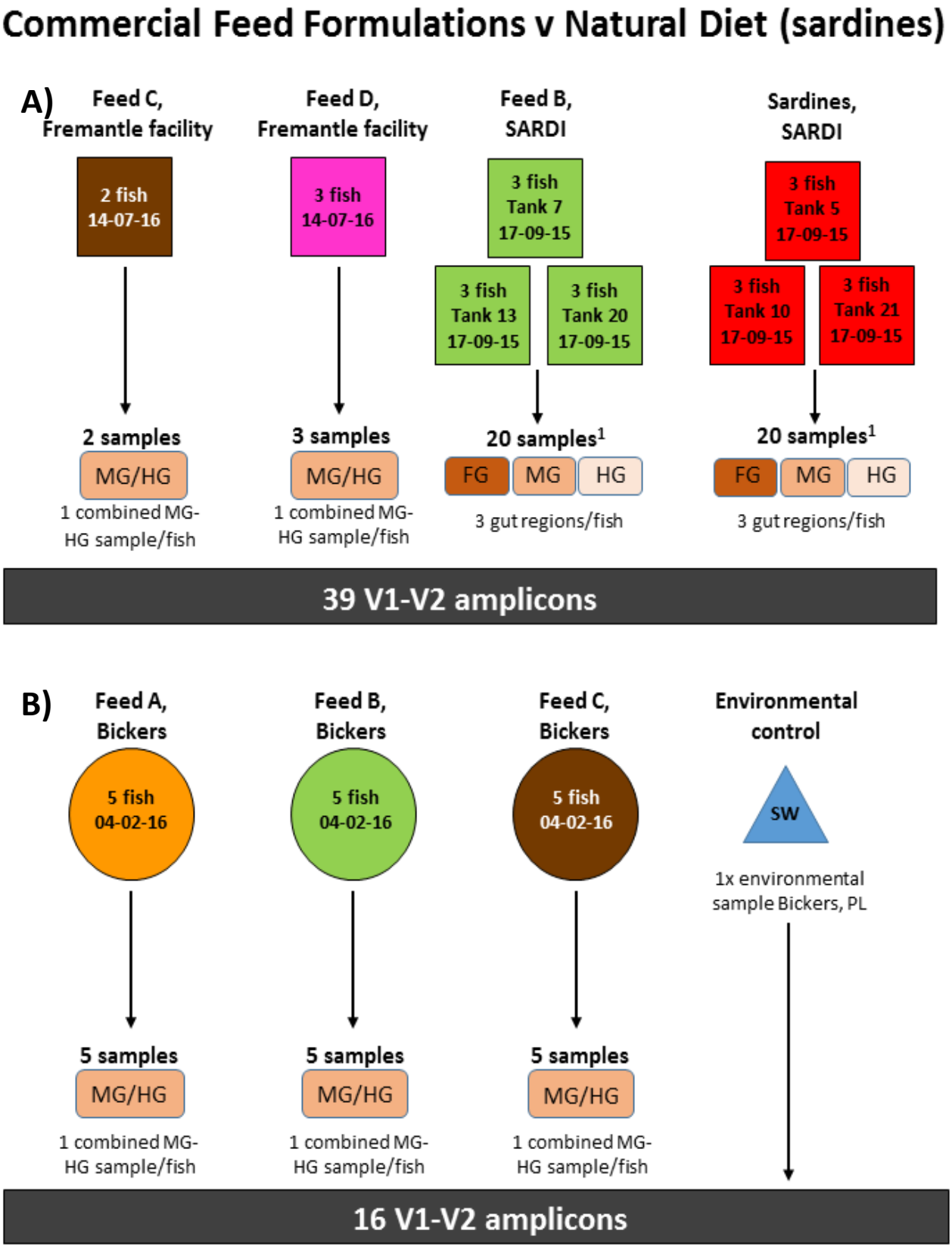


Figure 3.3.1.2.1. Experimental design for commercial feed formulations vs 'natural' (Sardine) diet component in A) tank trials (Fremantle, WA facility and SARDI pool-farm, SA) and B) sea-cages (Bickers, Port Lincoln, SA).
Abbreviations: FG, foregut; HG, hindgut; MG, midgut; SW, seawater.
[1] 20 samples were collected (rather than 27) as the FG was not sampled from all fish from each tank

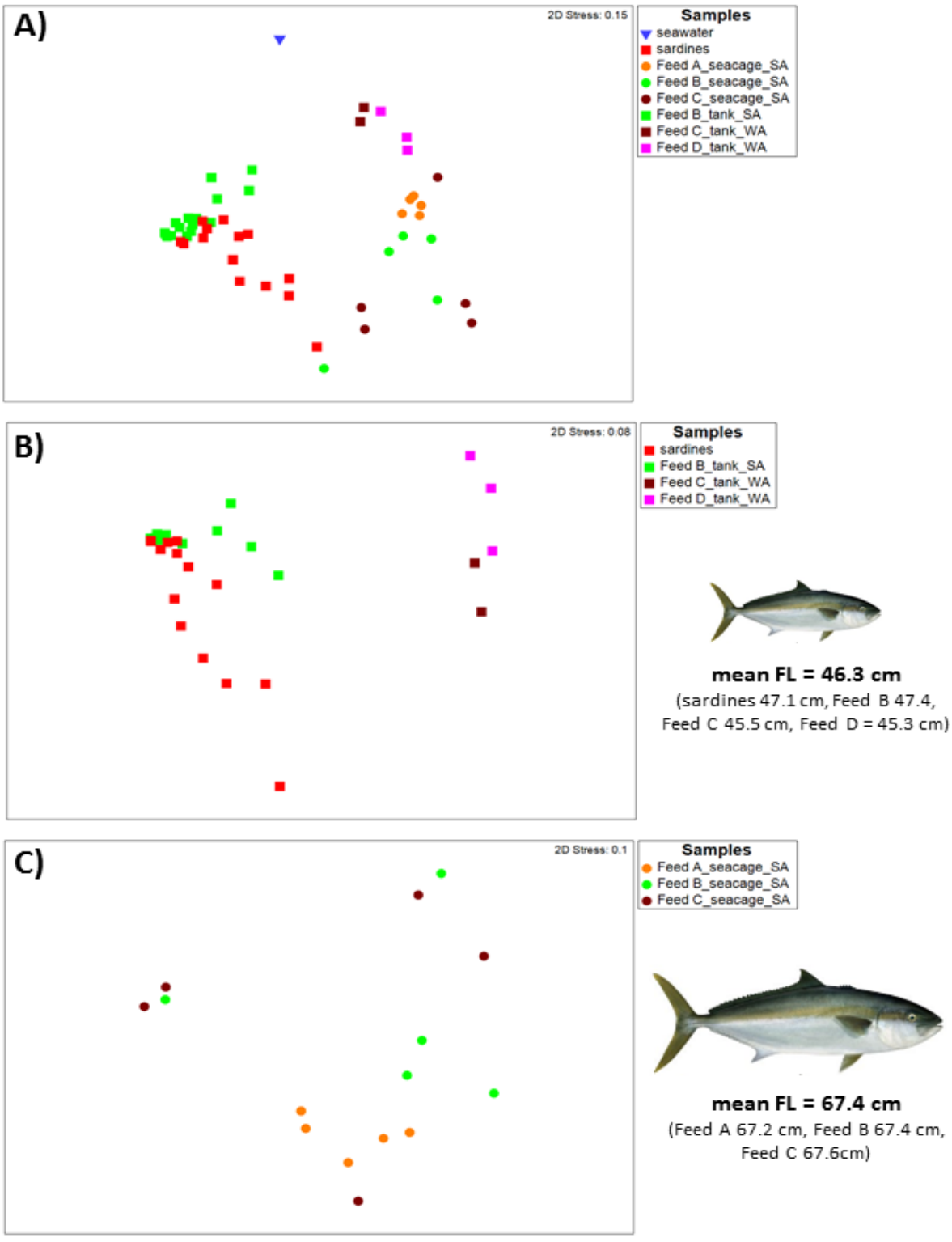


Figure 3.3.1.2.2. Difference between the global community structure of A) all 55 formulated feed and 'natural' (Sardine) diet samples (tank trials and sea-cages), B) tank trial samples only with mean fish length given and C) sea-cage samples only with mean fish length given as analysed by non-metric multidimensional scaling (nMDS).[1]

Abbreviations: FL, fork length; SA, South Australia (SARDI pool-farm facility); WA, Western Australia (Fremantle facility).

[1] Gut scrapings from combined mid- and hindgut sample of 2x Feed C WA, 3x Feed D WA, 5x Feed A sea-cage, 5x Feed B sea-cage and 5x Feed C sea-cage YTK along with gut scrapings from the 3x gut regions (fore-, mid- and hindgut) of 16x Feed B SA and 18x Sardine fed SA YTK. One environmental water sample collected from the Bickers sea-cage site

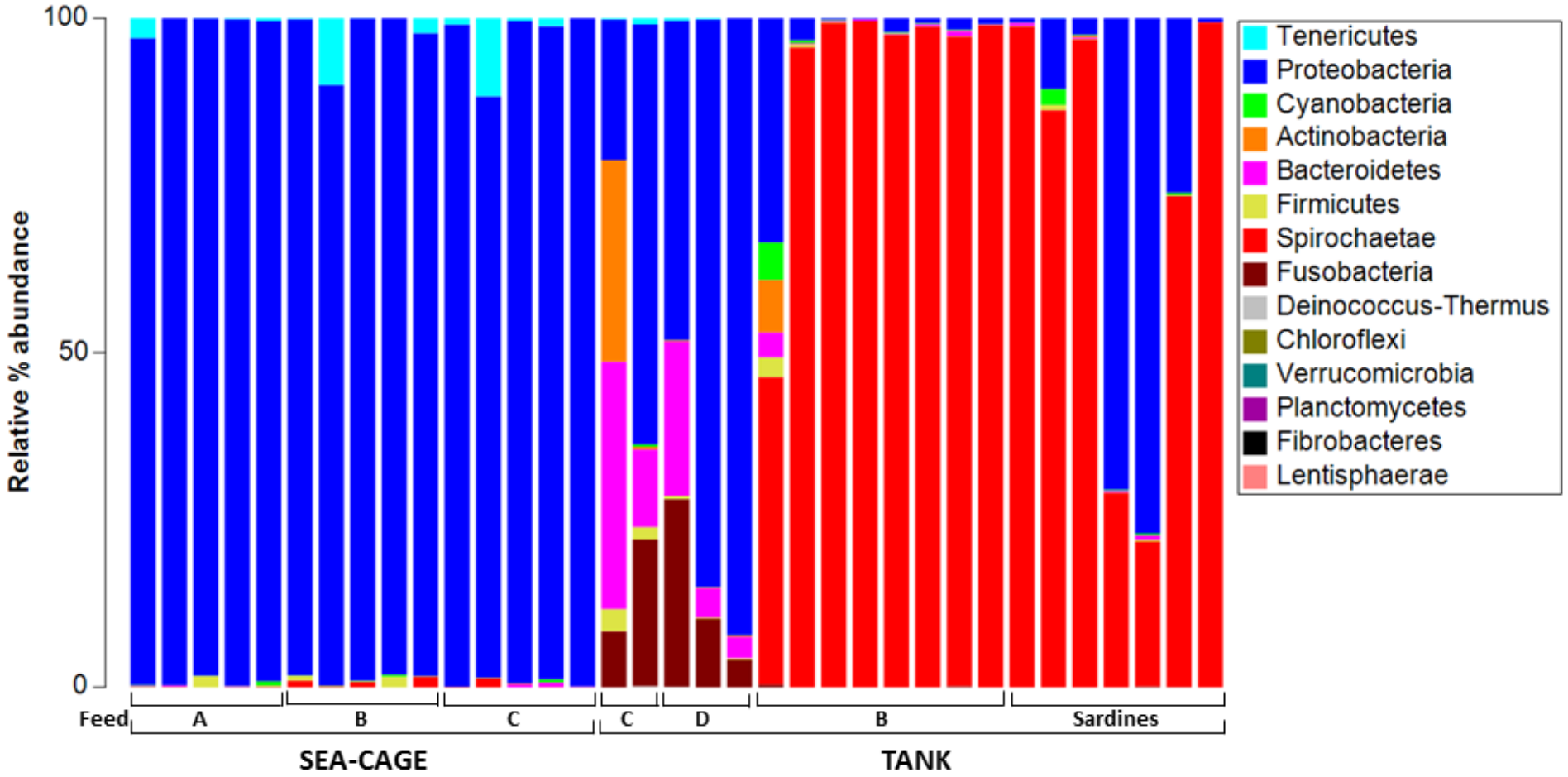


Figure 3.3.1.2.3. Relative percent abundance of bacterial phyla associated with gut scrapings from YTK fed different feeds from the sea-cage and tank trials.[1]

[1] Proprietary commercial feed formulation Feeds A, B and C sea-cage samples collected from Bickers, Port Lincoln, SA (combined MH scraping); proprietary commercial feed formulations Feed C and D tank trial samples collected from Fremantle facility, WA (combined MH scraping); proprietary commercial feed formulation Feed B and Sardine tank trial samples collected from SARDI pool-farm facility, SA (HG scraping only).

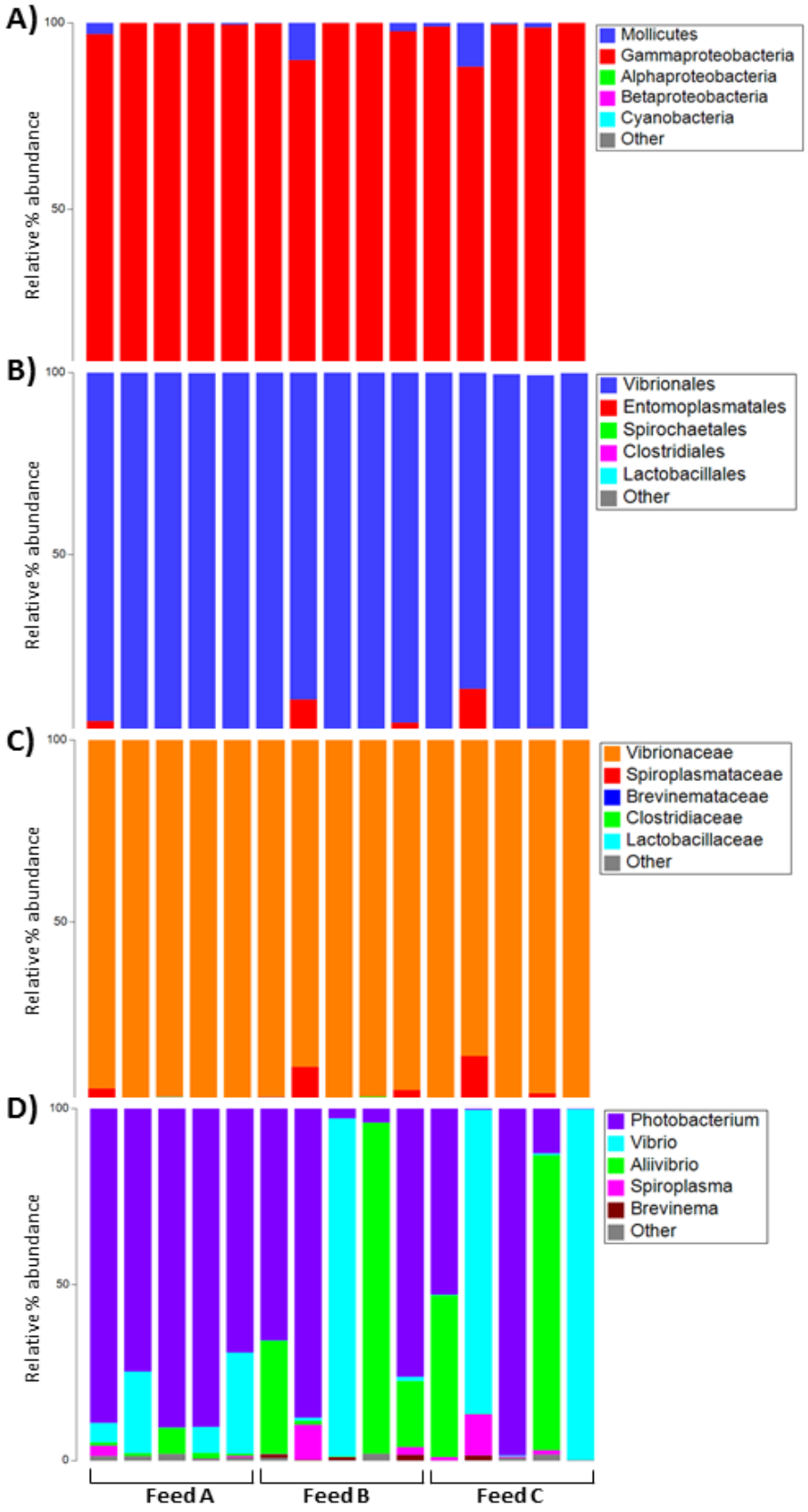


Figure 3.3.1.2.4. Relative percent abundance of bacterial taxa at the A) class, B) order, C) family and D) genus levels associated with gut scrapings from YTK from the three Bickers (Port Lincoln, SA) sea-cage (propritary commercial feed formaultions Feeds A, B or C).

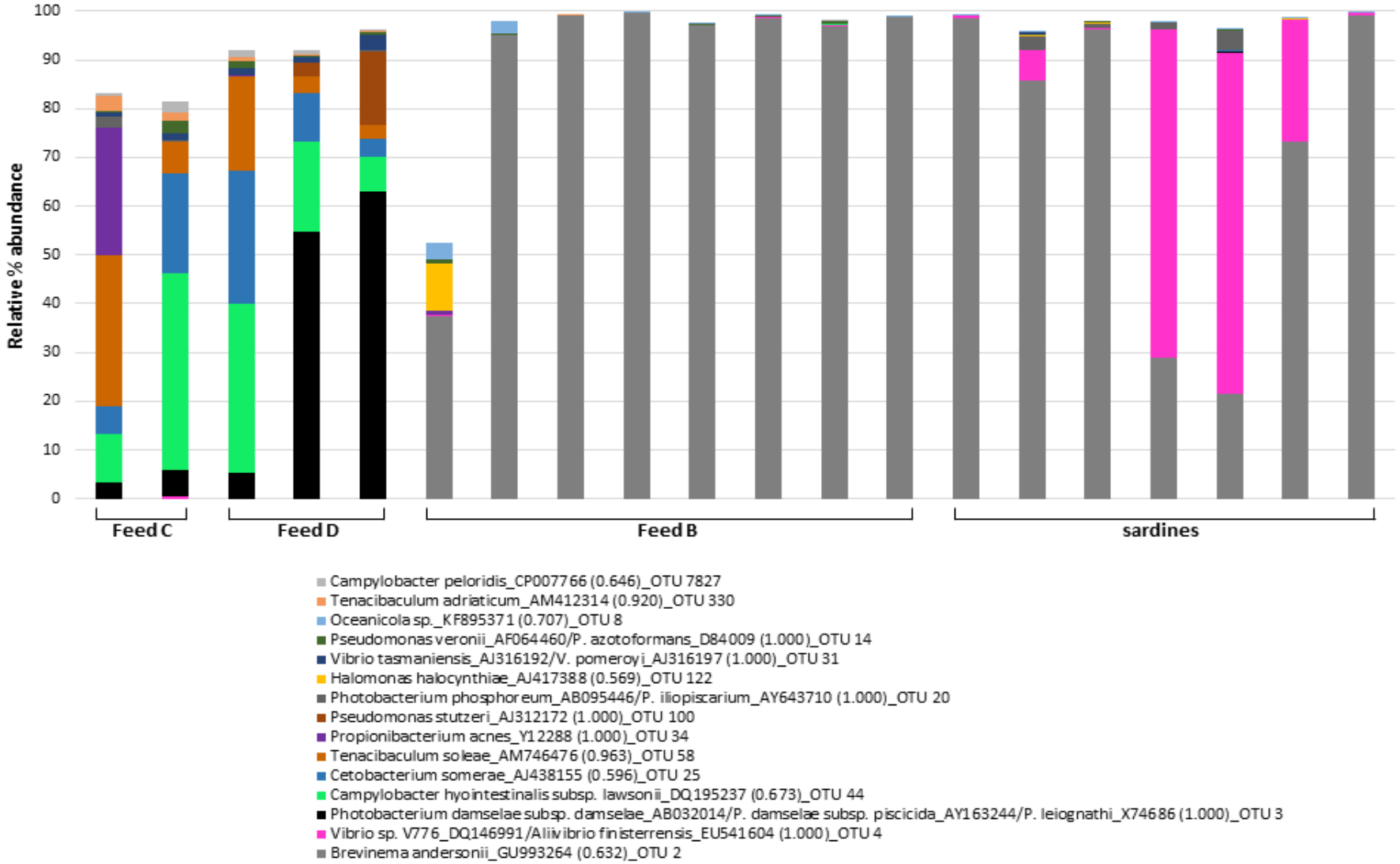


Figure 3.3.1.2.5. Relative percent abundance of the 15 most abundant bacterial OTUs in the tank trial samples representing WA proprietary commercial feed formulations Feed C and Feed D and SARDI pool-farm facility proprietary commercial feed formulation Feed B and 'natural' (Sardine) diets.

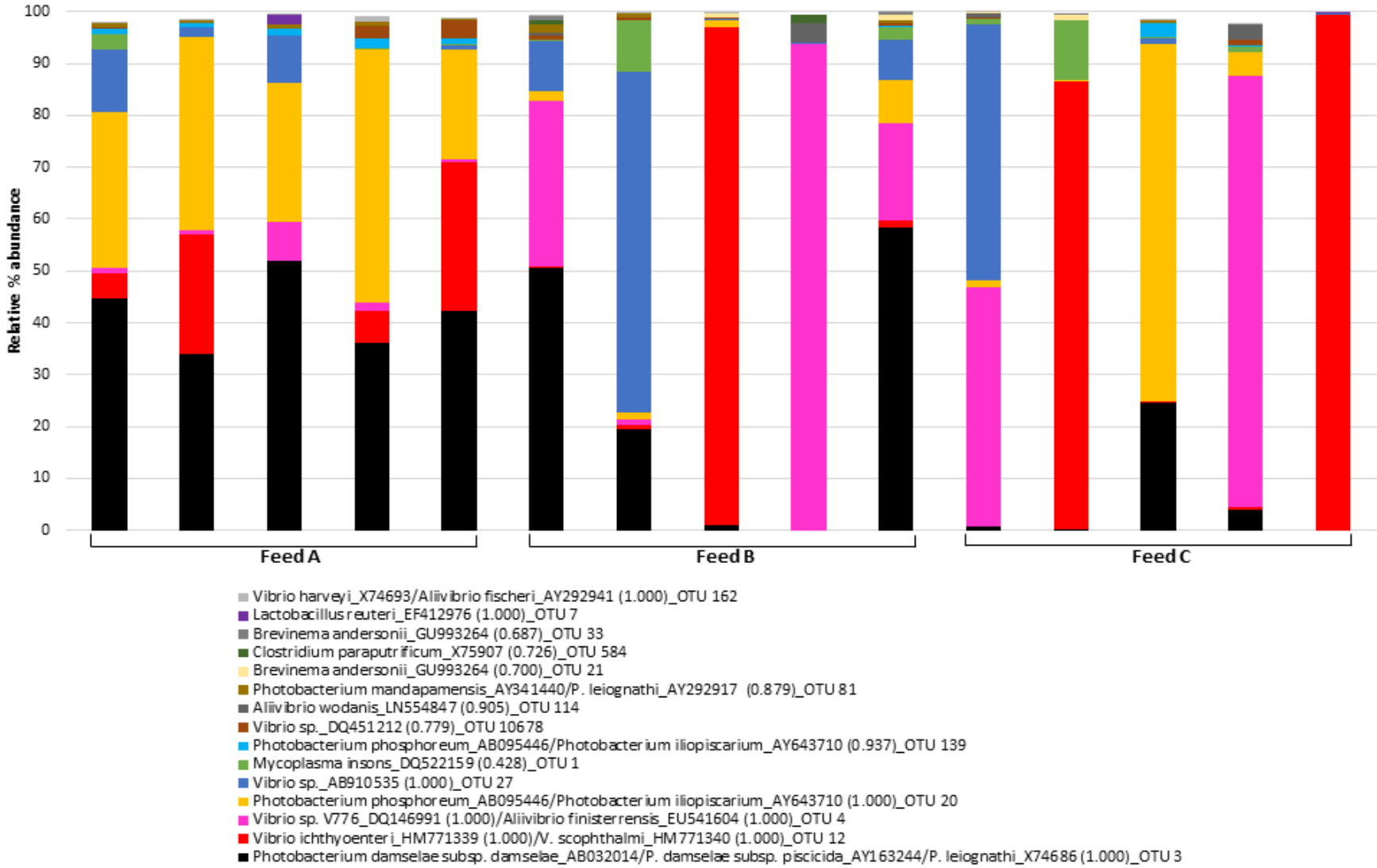


Figure 3.3.1.2.6. Relative percent abundance of the 15 most abundant bacterial OTUs in the Bickers (Port Lincoln, SA) sea-cage samples representing proprietary commerical feed formualtions Feed A, Feed B and Feed C.

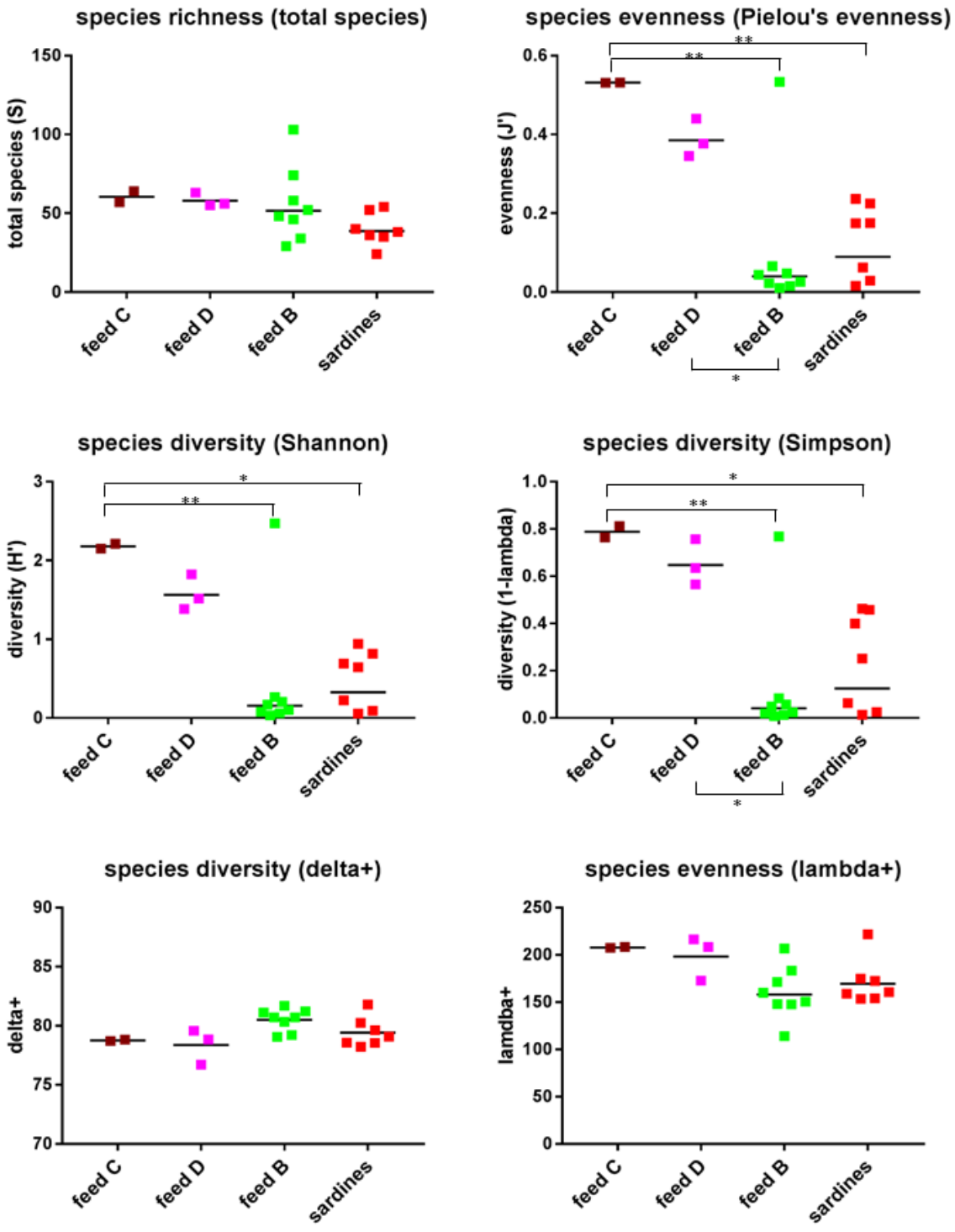


Figure 3.3.1.2.7. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for tank trial samples.[1]

[1] Mean values are plotted for each of the groups of interest (proprietary commercial feed formulation Feed C vs Feed D used in WA vs proprietary commercial feed formulation Feed B vs 'natural' (Sardine) diet used in SA).

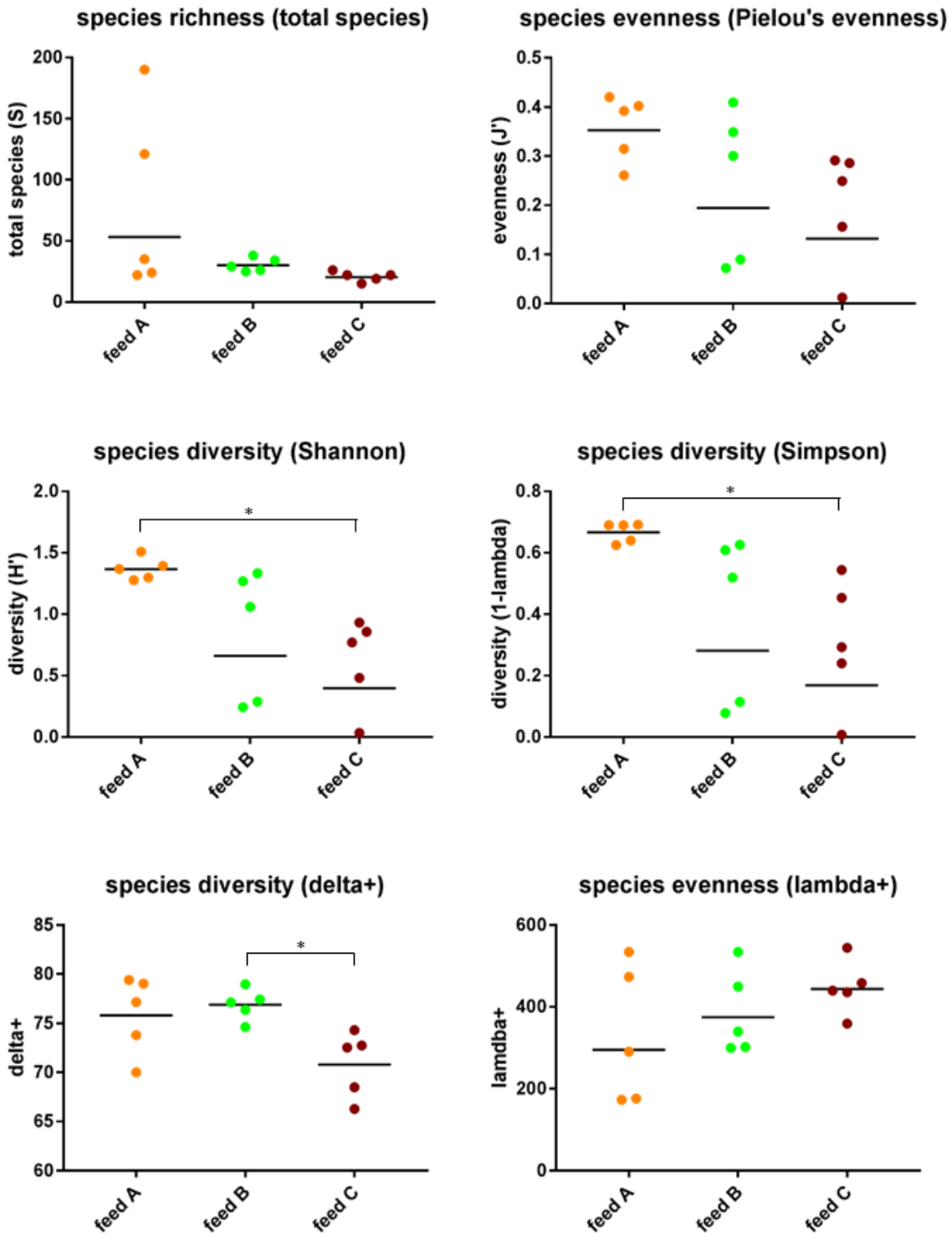


Figure 3.3.1.2.8. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for Bickers sea-cage samples.[1]

[1] Mean values are plotted for each of the groups of interest (proprietary commercial feed formulations Feed A, B and C).

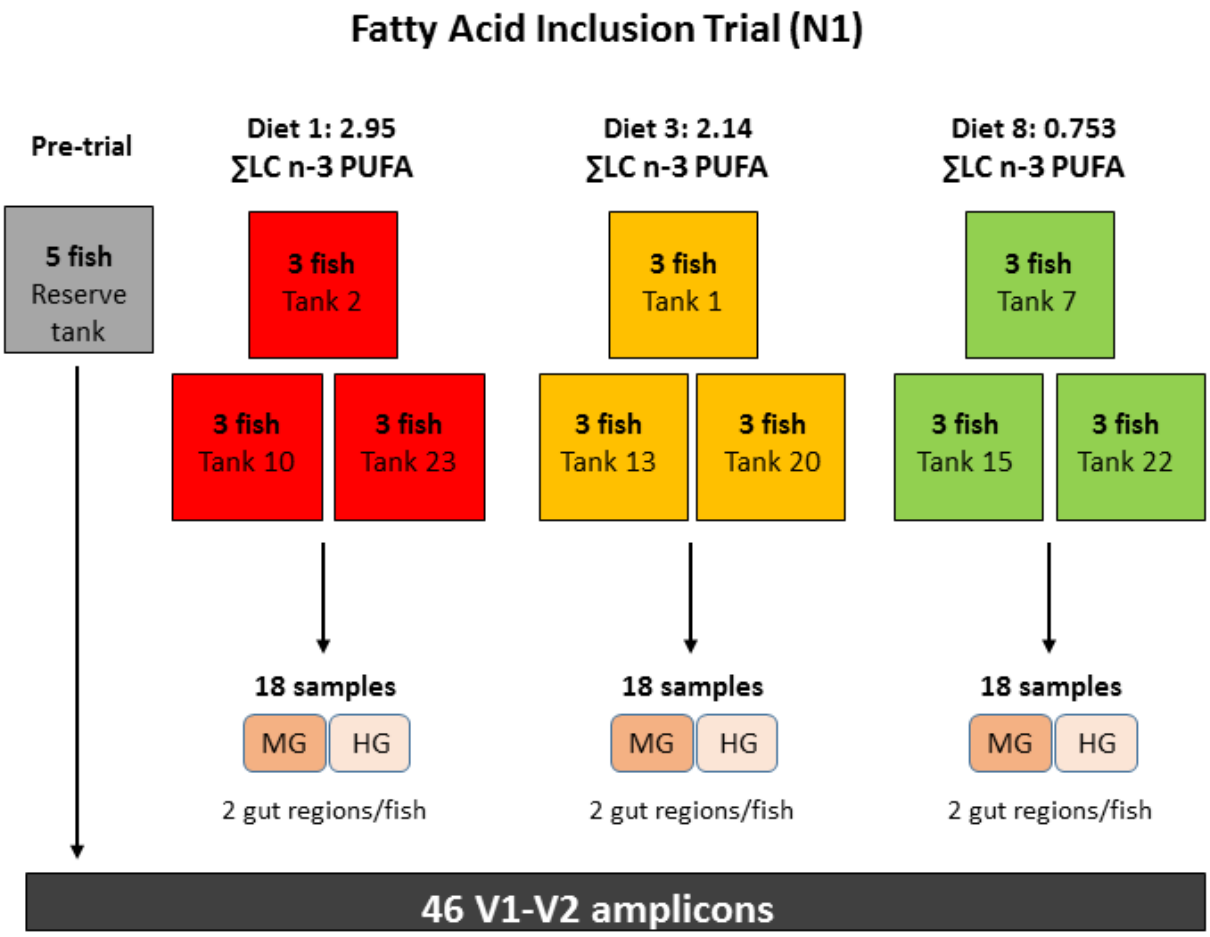


Figure 3.3.1.2.9. Experimental design for fatty acid inclusion trial component.
Abbreviations: HG, hindgut; LC n-3 PUFA, long chain omega-3 polyunsaturated fatty acids; MG, midgut.

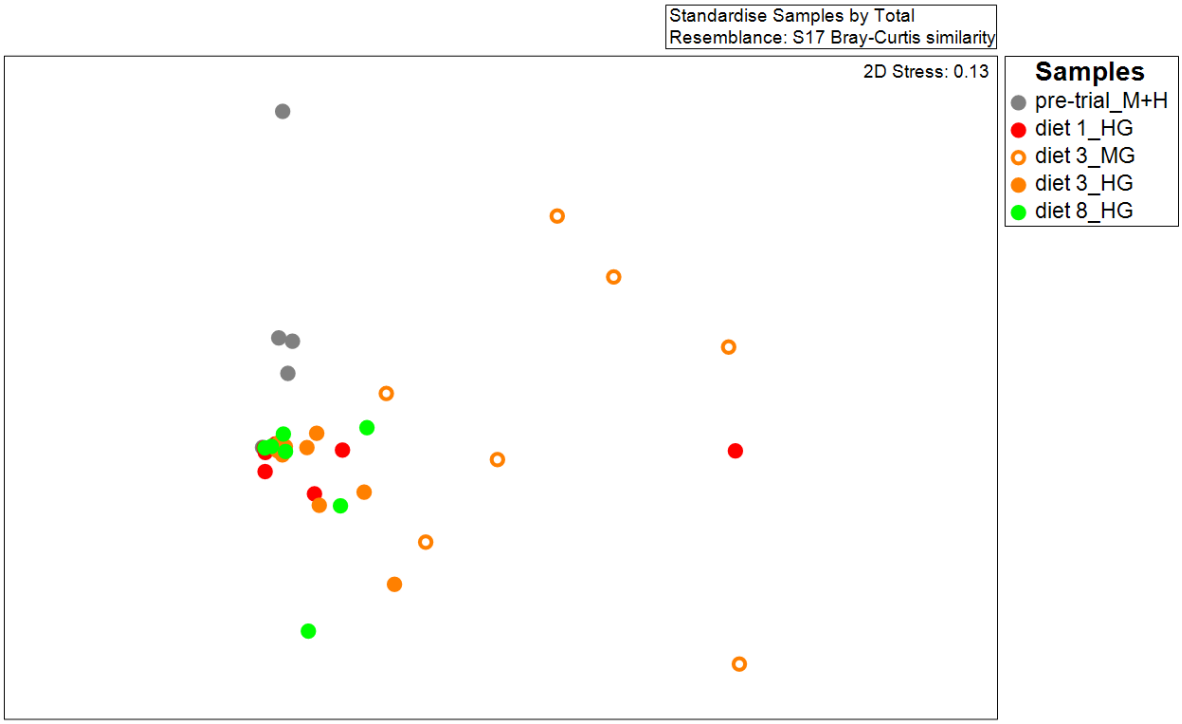


Figure 3.3.1.2.10. Difference between the global community structure of the pre-trial and three fatty acid inclusion diets as analysed by non-metric multidimensional scaling (nMDS).[1]

Abbreviations: HG, hindgut; MG, midgut.

[1] Combined mid- and hindgut scraping from 5x pre-trial (control) fish, HG scraping only from 8x Diet 1 (2.95 ΣLC n-3 PUFA), 9x Diet 3 (2.14 ΣLC n-3 PUFA) and 7x Diet 8 (0.753 ΣLC n-3 PUFA) YTK and MG scraping from 7x Diet 3 (2.14 ΣLC n-3 PUFA) YTK.

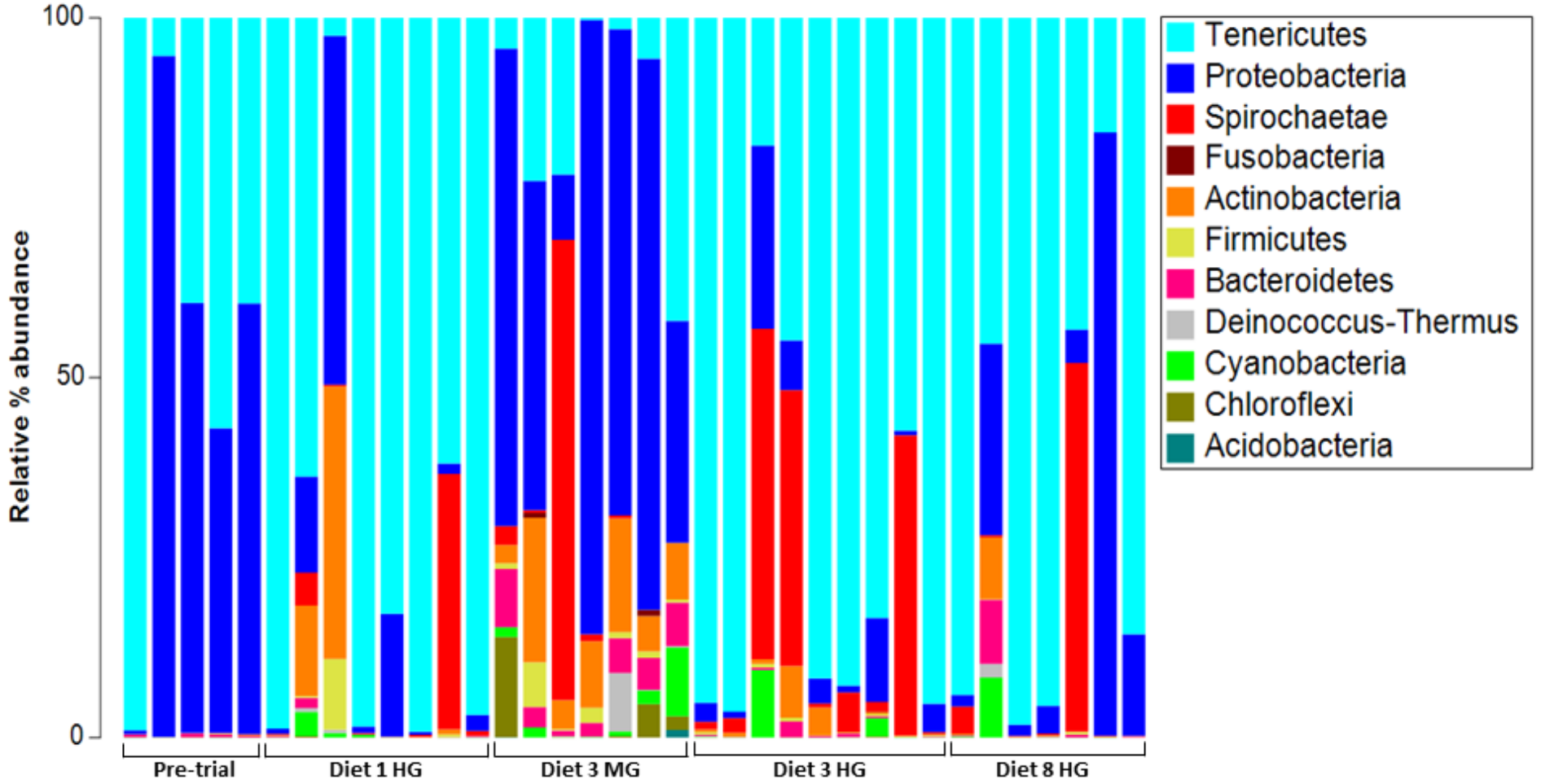


Figure 3.3.1.2.1.11. Relative percent abundance of bacterial phyla associated with gut scrapings from YTK from the fatty acid inclusion trial (including pre-trial samples, Diet 1 HG [2.95 ΣLC n-3 PUFA], Diet 3 MG [2.14 ΣLC n-3 PUFA), Diet 3 HG [2.14 ΣLC n-3 PUFA], Diet 8 HG [0.753 ΣLC n-3 PUFA]).
Abbreviations: HG, hindgut; MG, midgut.

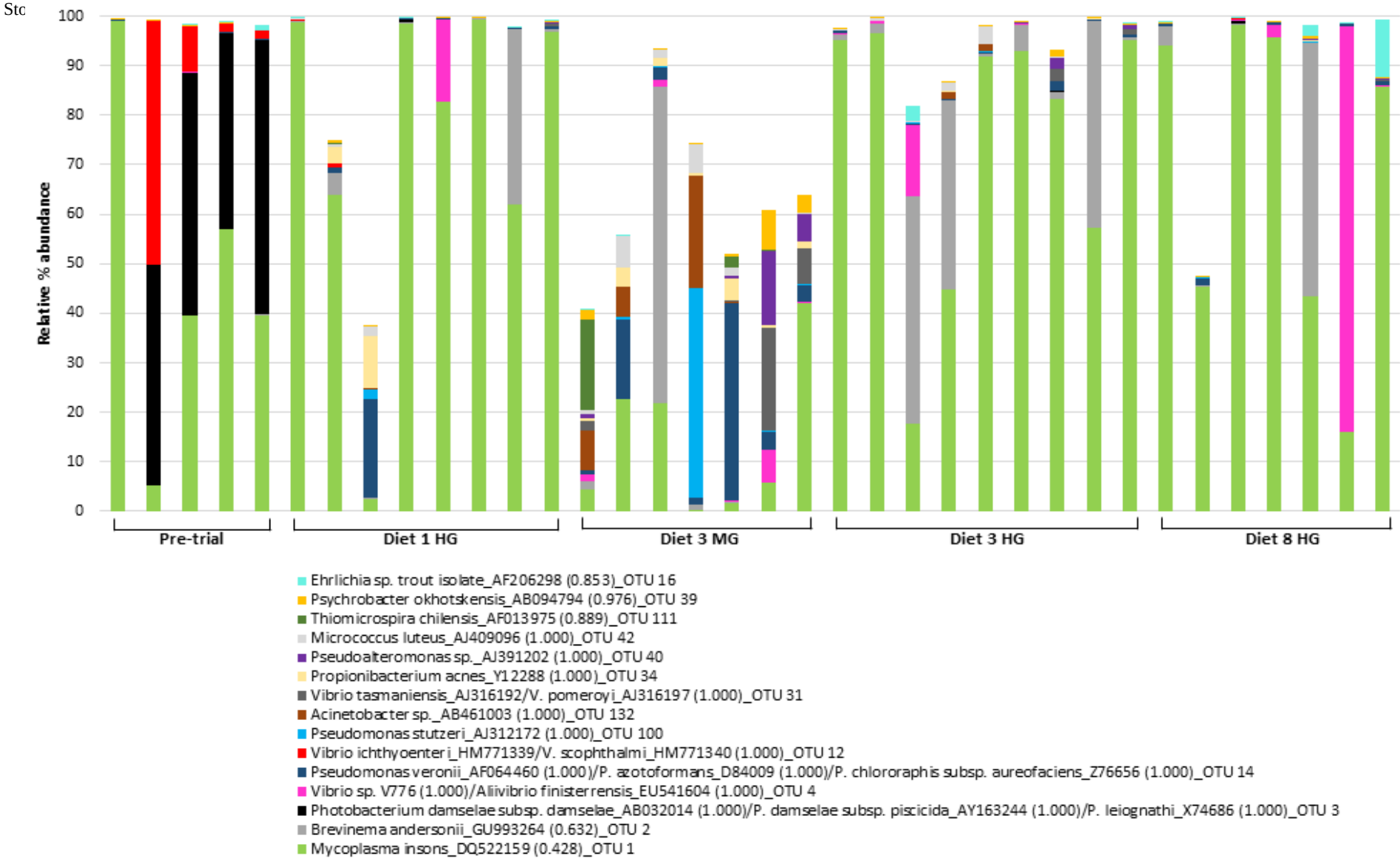


Figure 3.3.1.2.12. Relative percent abundance of the 15 most abundant bacterial OTUs in the fatty acid inclusion trial samples (pre-trial, Diet 1 HG [2.95 ΣLC n-3 PUFA], Diet 3 MG [2.14 ΣLC n-3 PUFA), Diet 3 HG [2.14 ΣLC n-3 PUFA], Diet 8 HG [0.753 ΣLC n-3 PUFA]).
Abbreviations: HG, hindgut; MG, midgut.

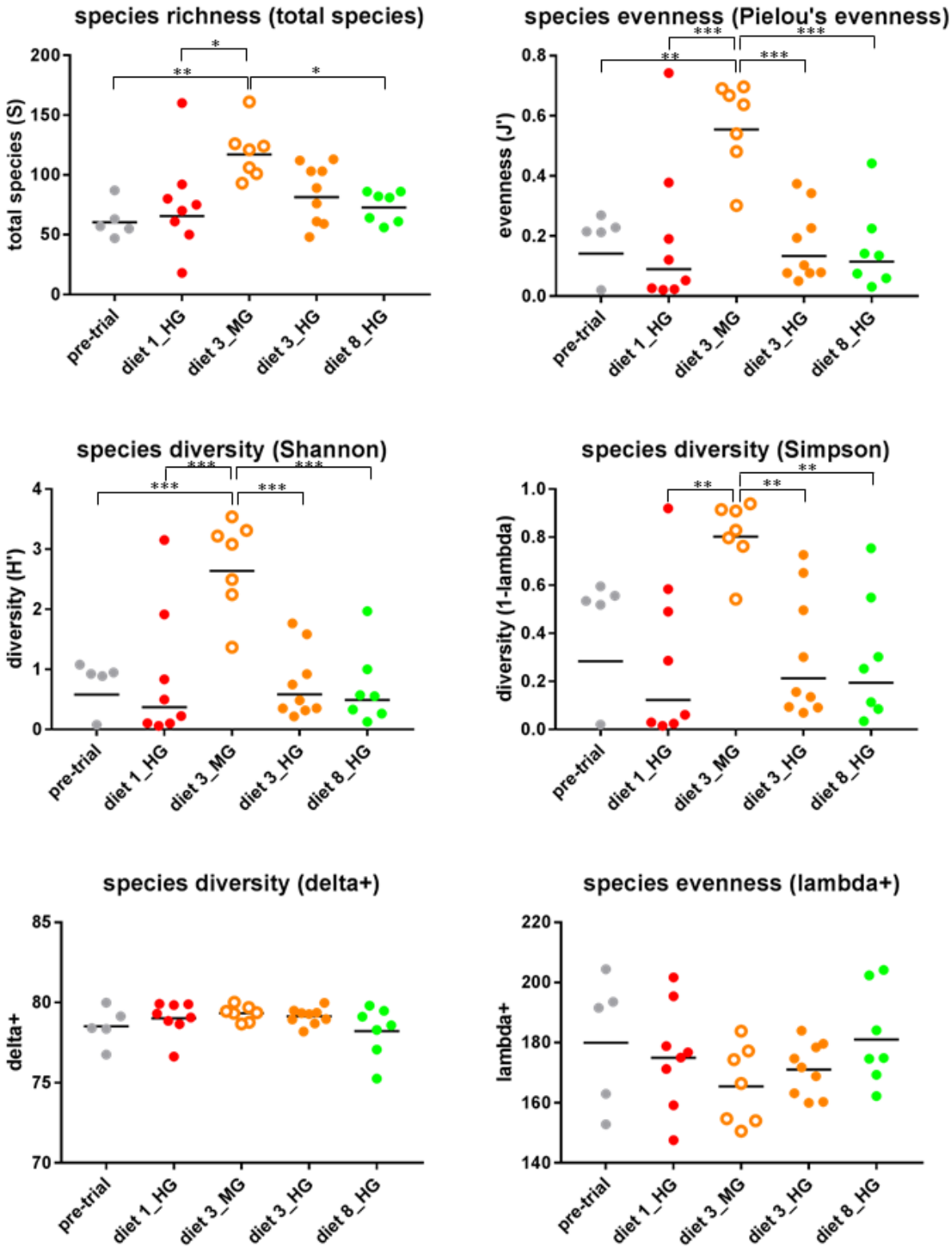


Figure 3.3.1.2.13. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for fatty acid inclusion trial samples.[1]

[1] Mean values are plotted for each of the groups of interest (pre-trial vs Diet 1 HG [2.95 ΣLC n-3 PUFA] vs Diet 3 MG [2.14 ΣLC n-3 PUFA] vs Diet 3 HG [2.14 ΣLC n-3 PUFA] vs Diet 8 HG [0.753 ΣLC n-3 PUFA]).

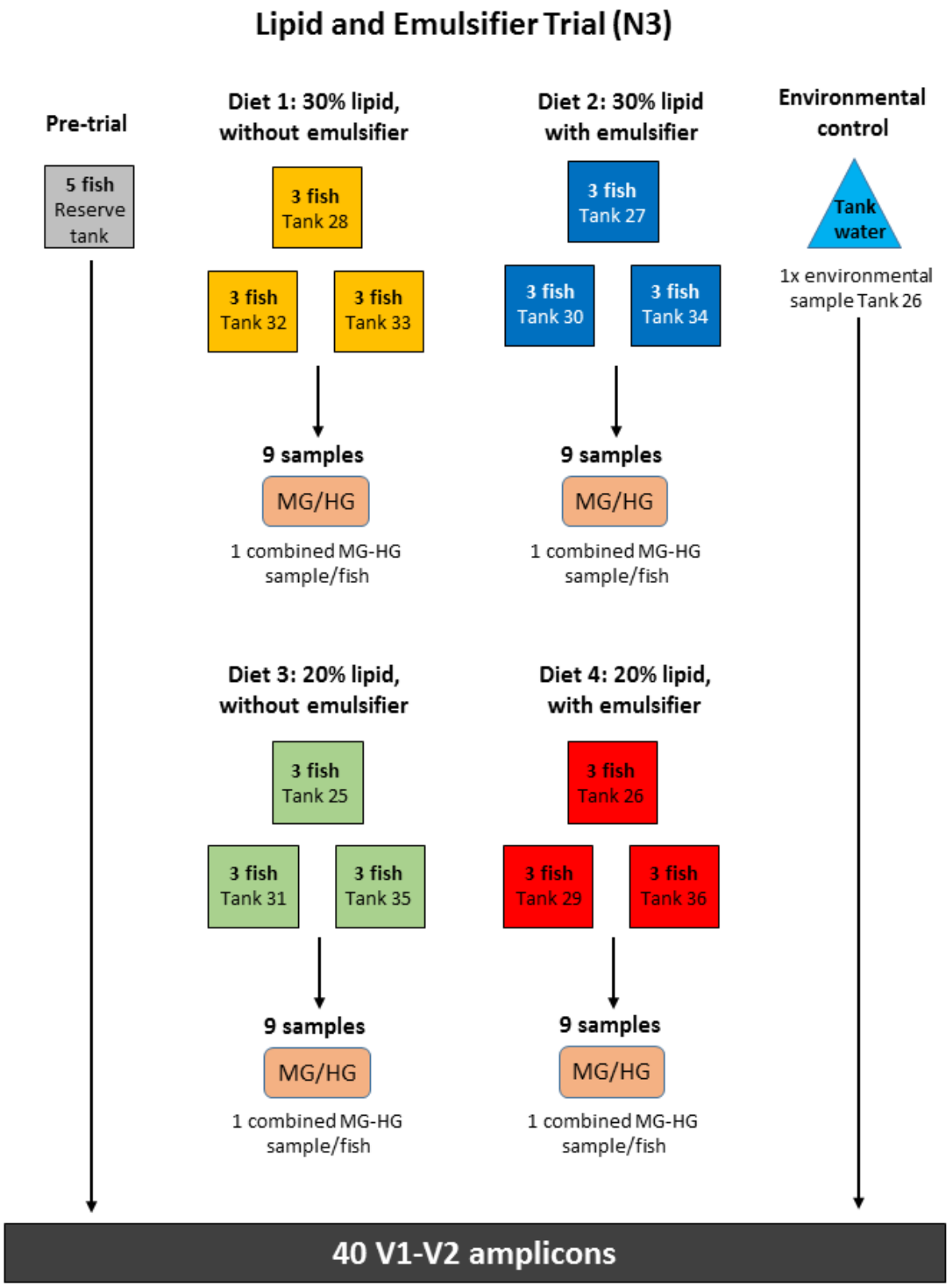


Figure 3.3.1.2.14. Experimental design for the lipid inclusion (with and without emulsifiers) trial component.
Abbreviations: HG, hindgut; MG, midgut.

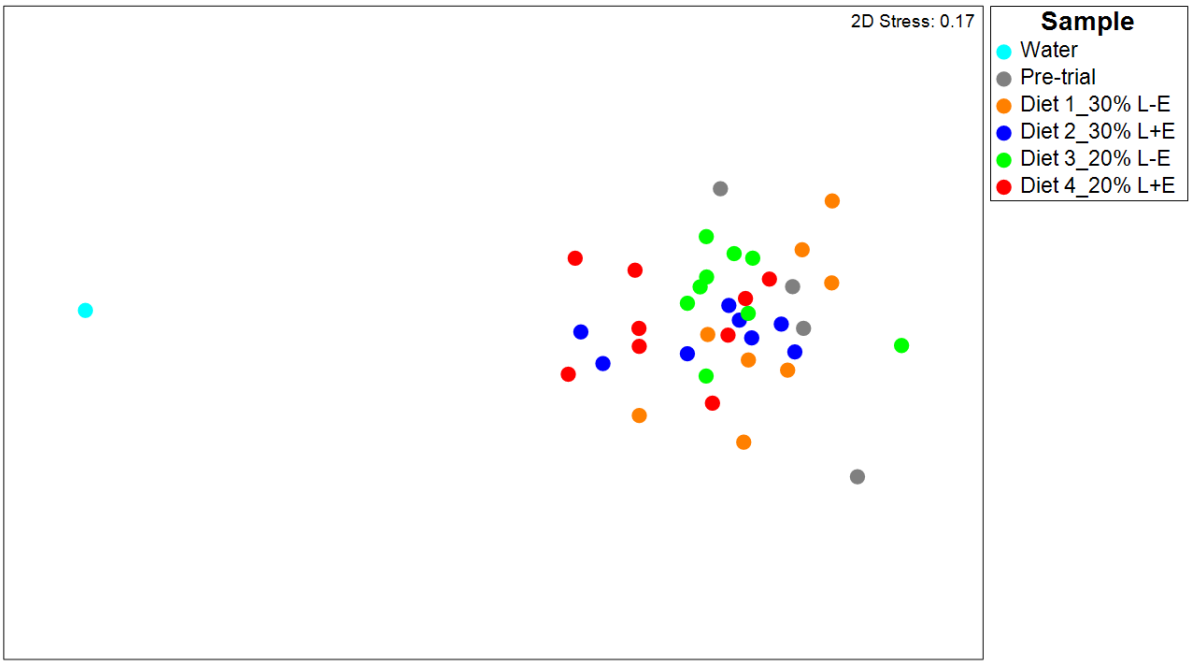


Figure 3.3.1.2.15. Difference between the global community structure of the seawater, pre-trial and four lipid/emulsifier diets as analysed by non-metric multidimensional scaling (nMDS).[1]

[1] Combined mid- and hindgut scraping from 4x pre-trial, 8x Diet 1_30% L -E, 8x Diet 2_30% L +E, 9x Diet 3_20% L –E and 9x Diet 4_20% L +E YTK.

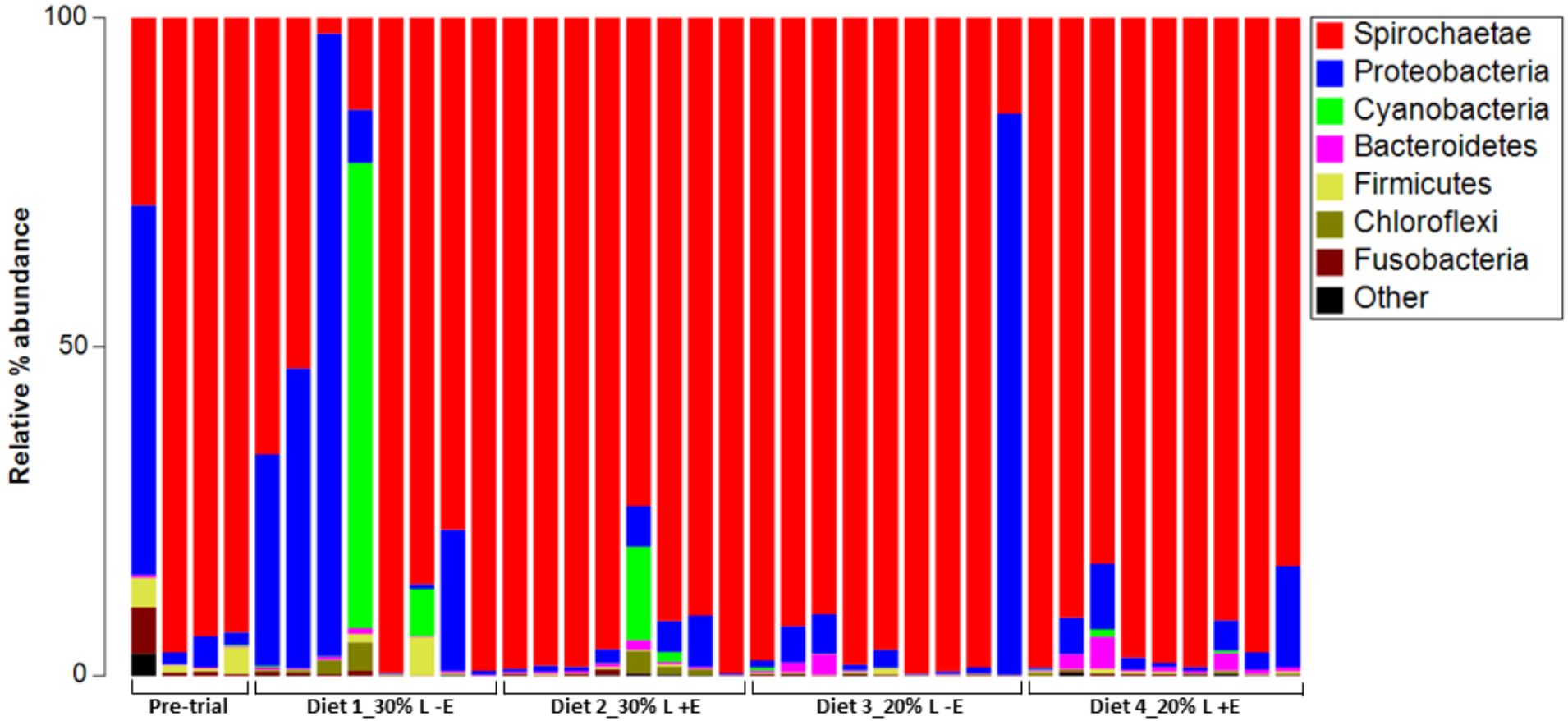


Figure 3.3.1.2.16. Relative percent abundance of bacterial phyla associated with gut scrapings from YTK from the lipid and emulsifier trial (including pre-trial samples, Diet 1_30% L -E, Diet 2_30% L +E, Diet 3_20% L –E, Diet 4_20% L +E).

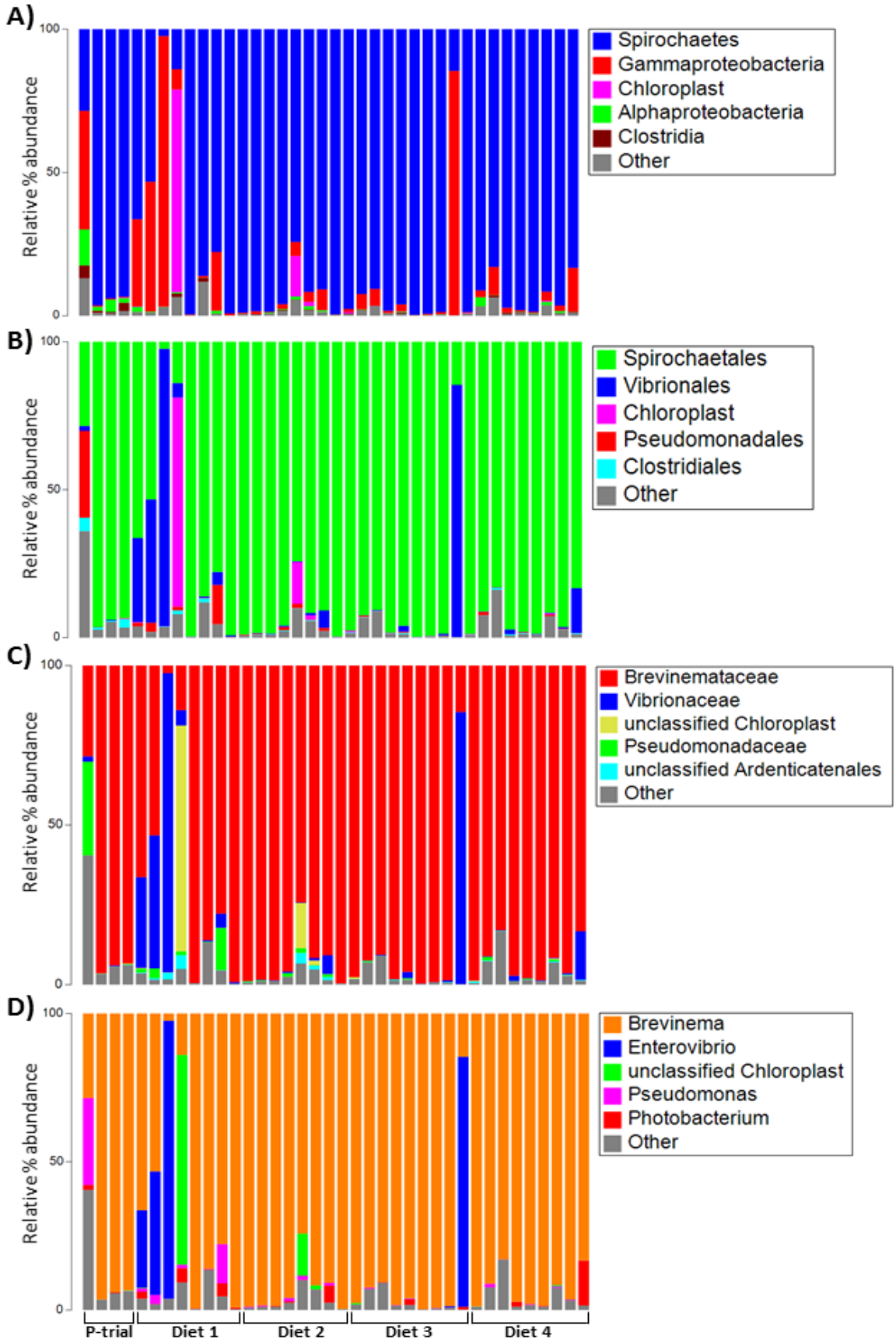


Figure 3.3.1.2.17. Relative percent abundance of bacteria taxa at the A) class, B) order, C) family and D) genus levels associated with gut scrapings from YTK from the pre-trial and four lipid and emulsifier diets.[1]

[1] Diet 1 = 30% L –E; Diet 2 = 30% L +E; Diet 3 = 20% L –E; Diet 4 = 20% L +E.

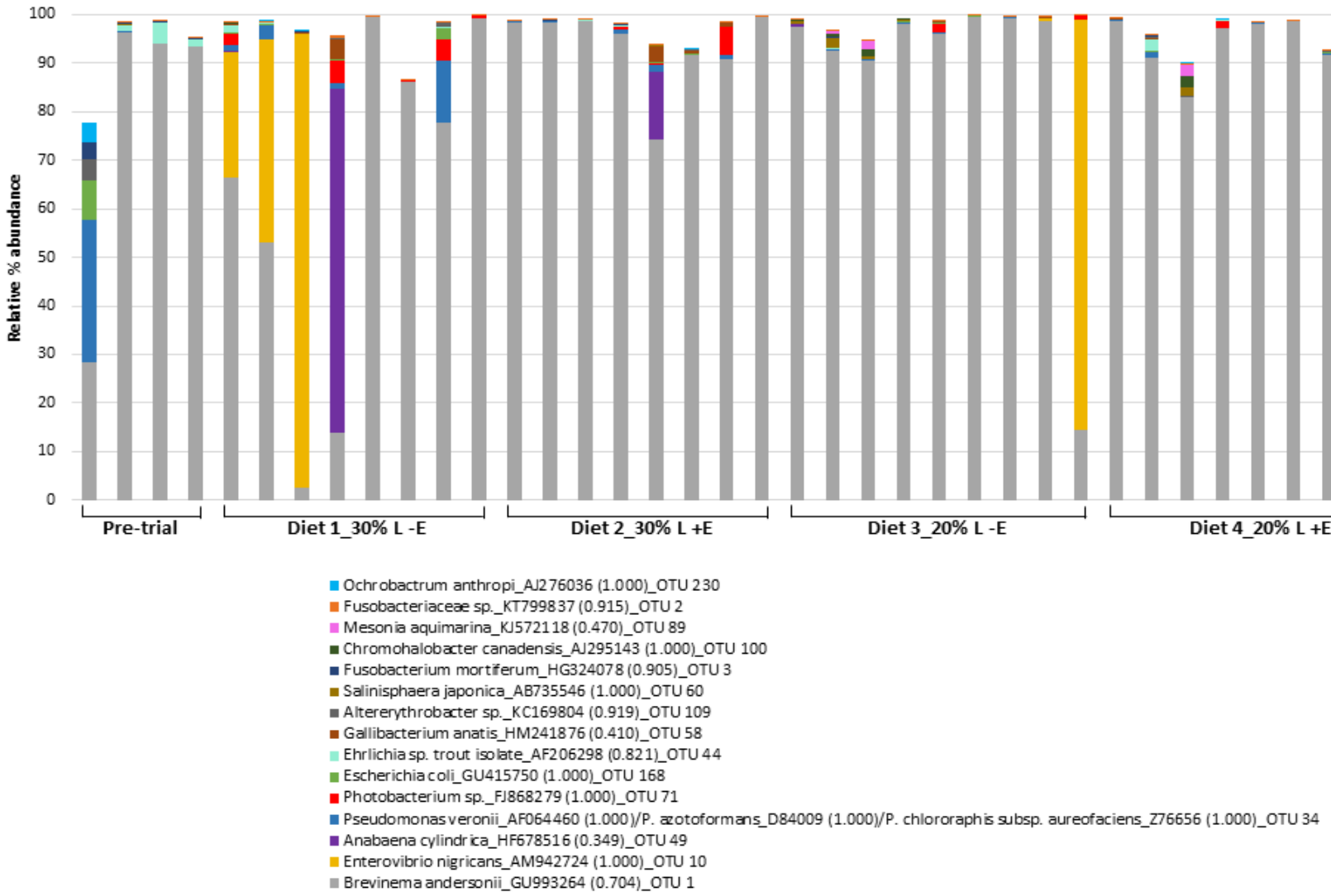


Figure 3.3.1.2.18. Relative percent abundance of the 15 most abundant bacterial OTUs in the lipid and emulsifier samples (pre-trial, Diet 1 30% L -E, Diet 2 30% L +E, Diet 3 20% L -E, Diet 4 20% L +E).

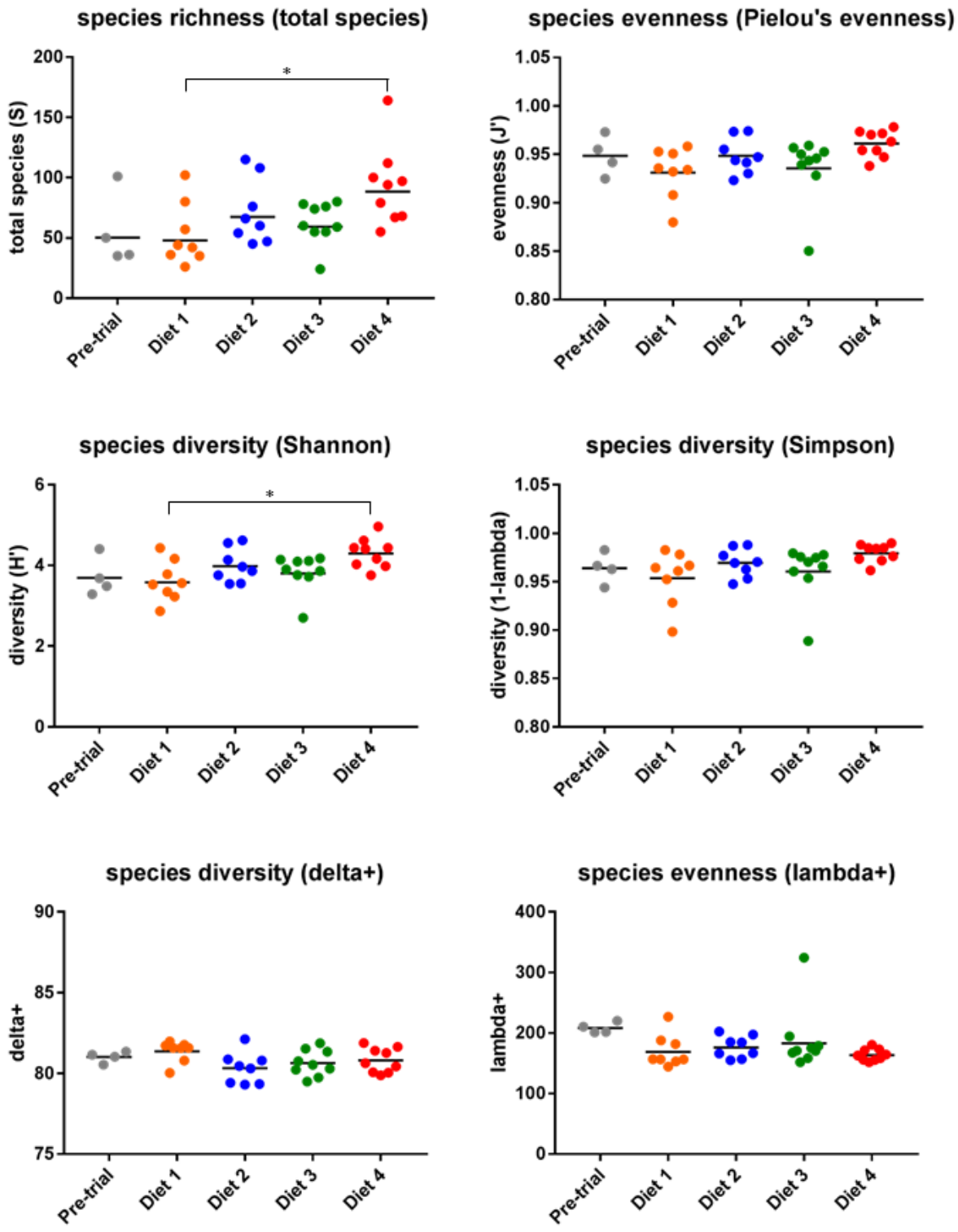


Figure 3.3.1.2.19. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for lipid and emulsifier trial samples.[1,2]

[1] Mean values are plotted for each of the groups of interest (pre-trial vs Diet 1 vs Diet 2 vs Diet 3 vs Diet 4).

[2] Diet 1 = 30% L –E; Diet 2 = 30% L +E; Diet 3 = 20% L –E; Diet 4 = 20% L +E.

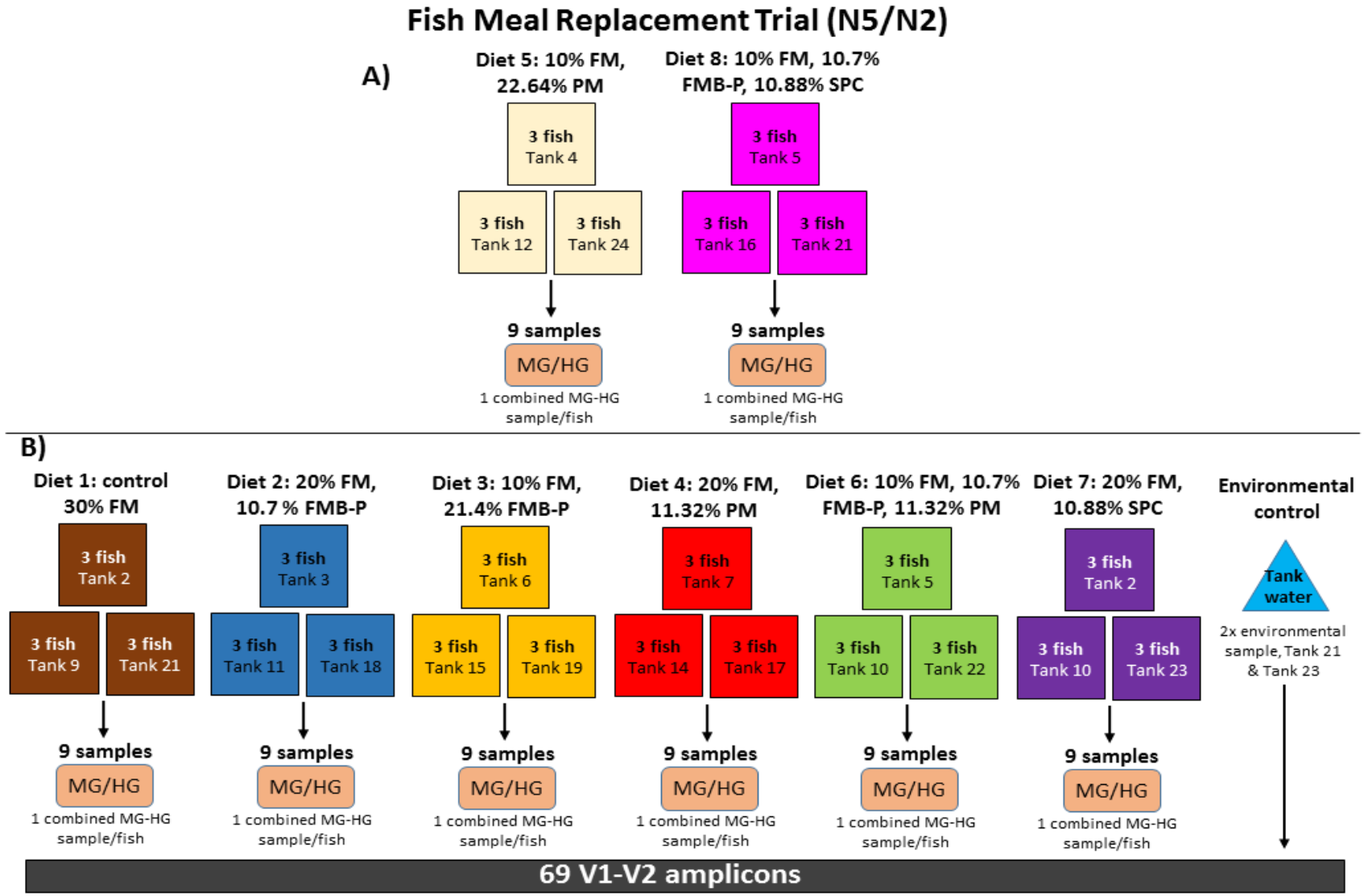


Figure 3.3.1.2.20. Experimental design for the fish meal replacement trial at A) summer water temperatures and B) winter water temperatures.
Abbreviations: FM, fish meal; FMB-P, fish meal by-product protein; HG, hindgut; MG, midgut; PM, poultry meal; SPC, soy protein concentrate.

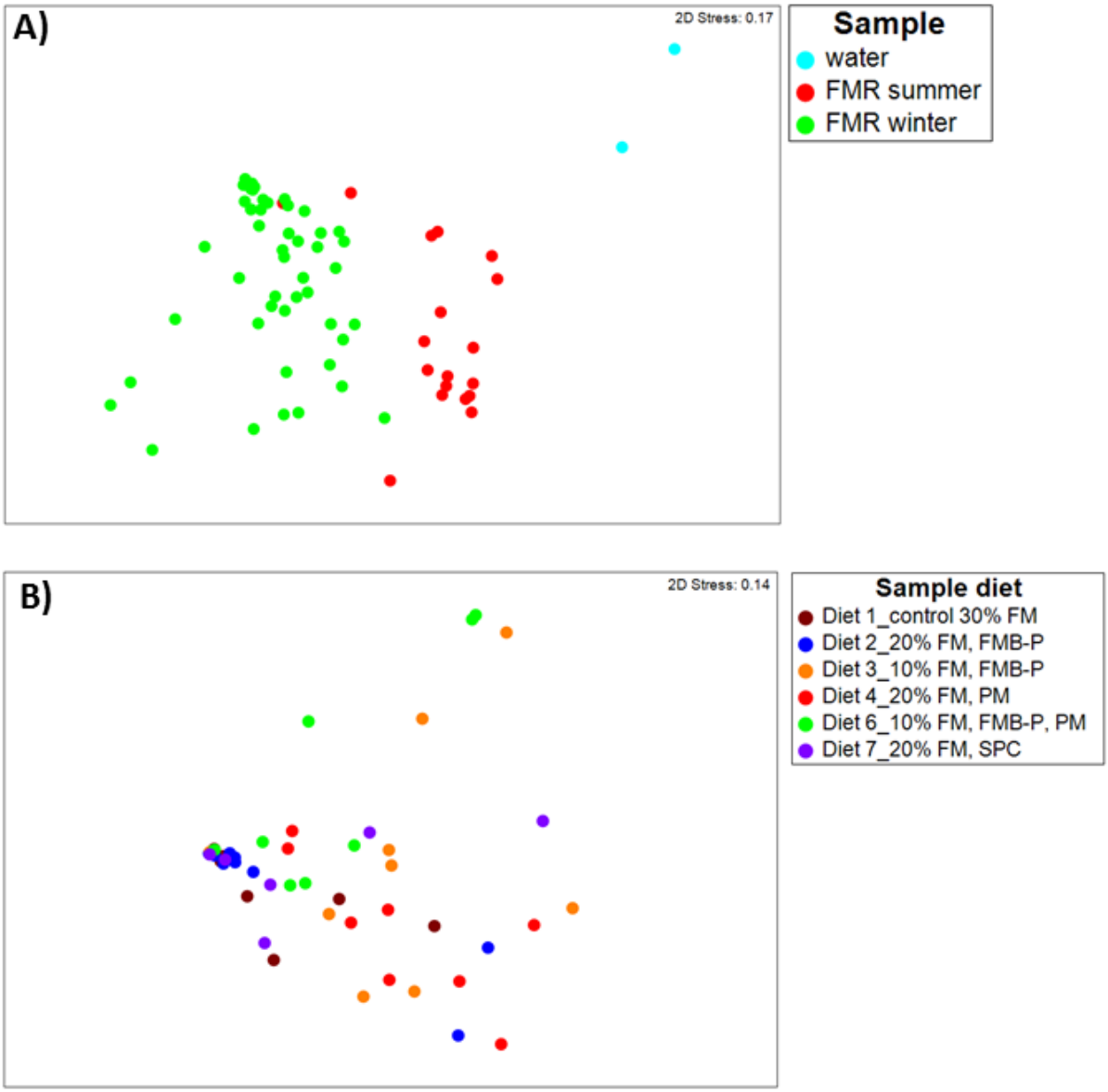


Figure 3.3.1.2.21. Difference between the global community structure of A) all 67 samples from the fish meal replacement trial and B) winter extension (green circles in A) samples only as analysed by non-metric multidimensional scaling (nMDS).[1]

Abbreviations: FM, fish meal; FMB-P, fish meal by-product protein; FMR, fish meal replacement; PM, poultry meal; SPC, soy protein concentrate

[1] Combined mid- and hindgut scraping from 7 Diet 1 (control), 9x Diet 2 (20% FM + 10.7% FMB-P), 9x Diet 3 (10% FM + 21.4% FMB-P), 8x Diet 4 (20% FM +11.32% PM), 9x Diet 5 (10% FM + 22.64% PM), 8x Diet 6 (10% FM, 10.7% FMB-P + 11.32% PM), 6x Diet 7 (20% FM + 10.88% SPC) and 9x Diet 8 (10% FM, 10.7% FMB-P + 10.88% SPC) YTK, along with seawater samples collected from two tanks post-trial.

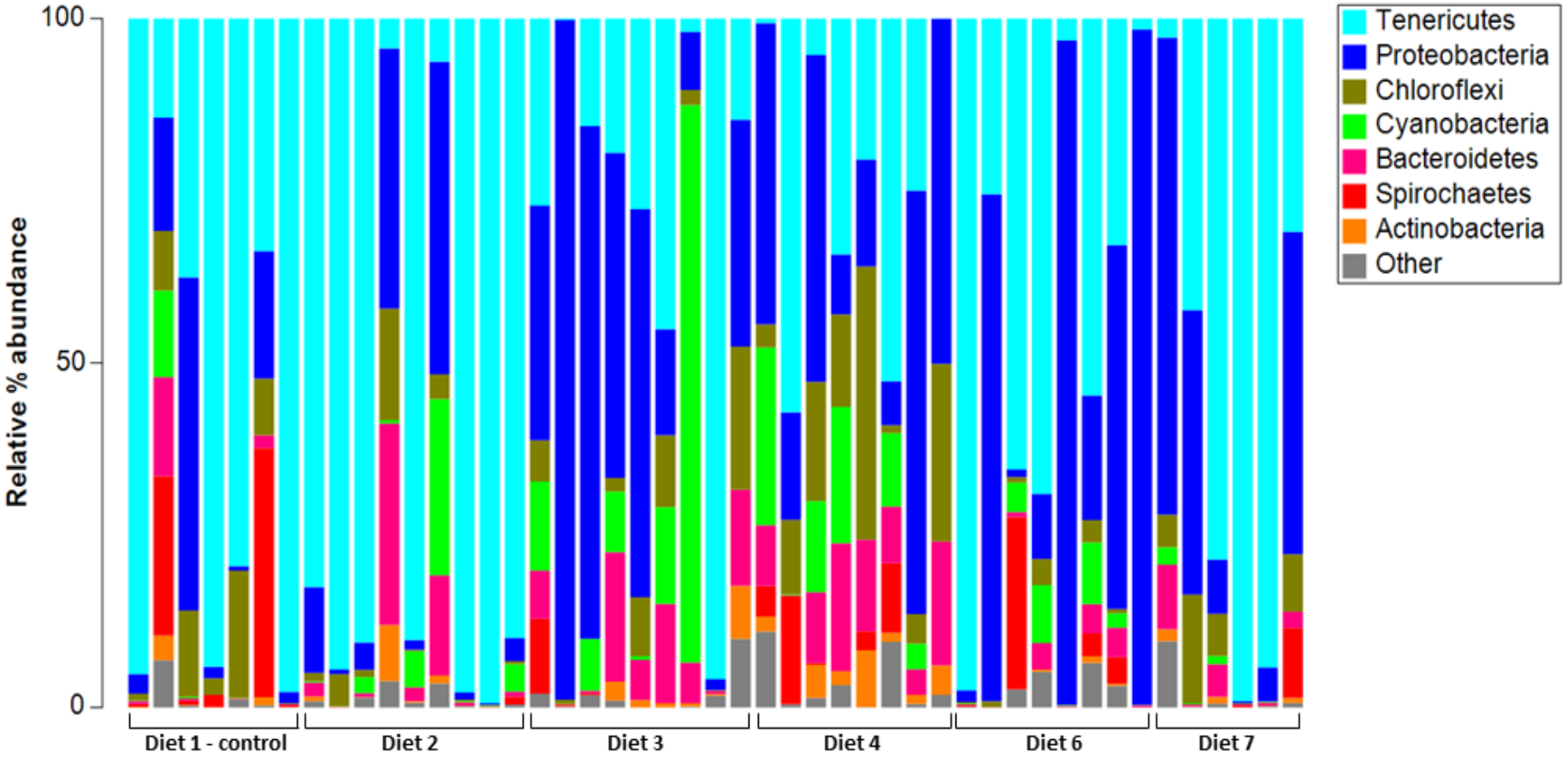


Figure 3.3.1.2.22. Relative percent abundance of bacterial phyla associated with gut scrapings from YTK from the six fish meal replacement winter diets (including Diet 1 [control], Diet 2 [20% FM + 10.7% FMB-P], Diet 3 [10% FM + 21.4% FMB-P], Diet 4 [20% FM +11.32% PM], Diet 6 [10% FM, 10.7% FMB-P + 11.32% PM], Diet 7 [20% FM + 10.88% SPC]).

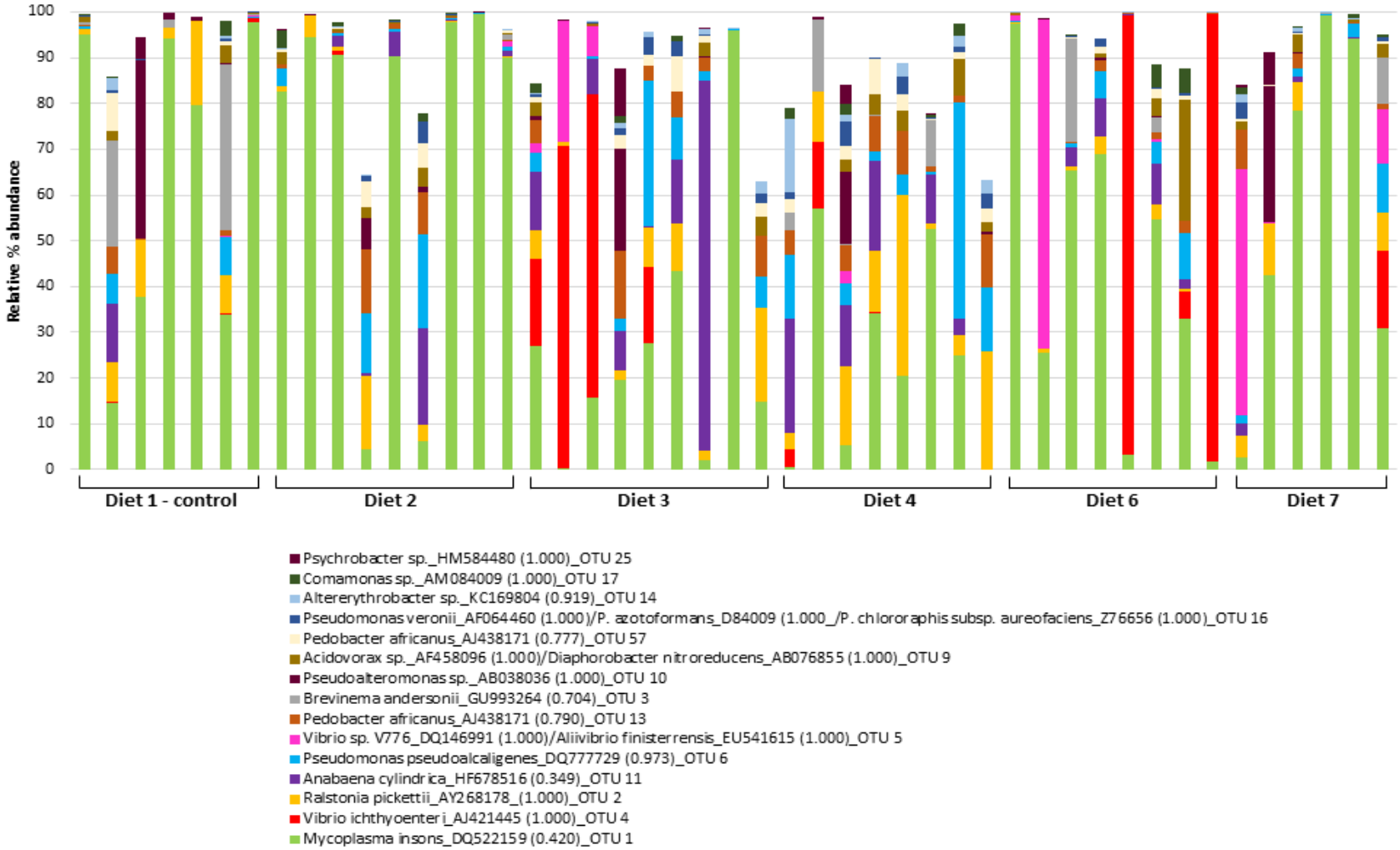


Figure 3.3.1.2.23. Relative percent abundance of the 15 most abundant bacterial OTUs in the fish meal replacement winter trial (Diet 1 [control], Diet 2 [20% FM + 10.7% FMB-P], Diet 3 [10% FM + 21.4% FMB-P], Diet 4 [20% FM +11.32% PM], Diet 6 [10% FM, 10.7% FMB-P + 11.32% PM], Diet 7 [20% FM + 10.88% SPC]).

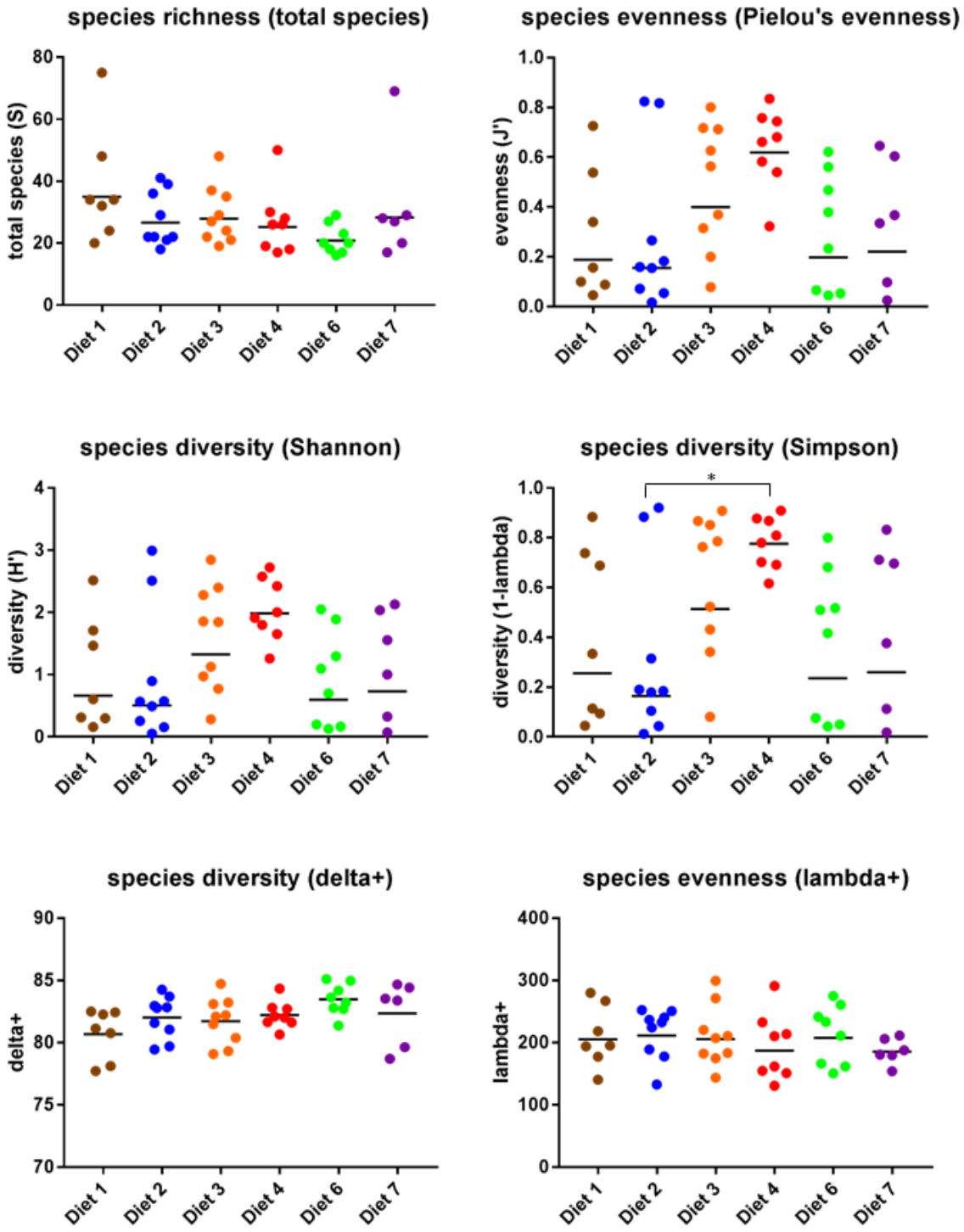
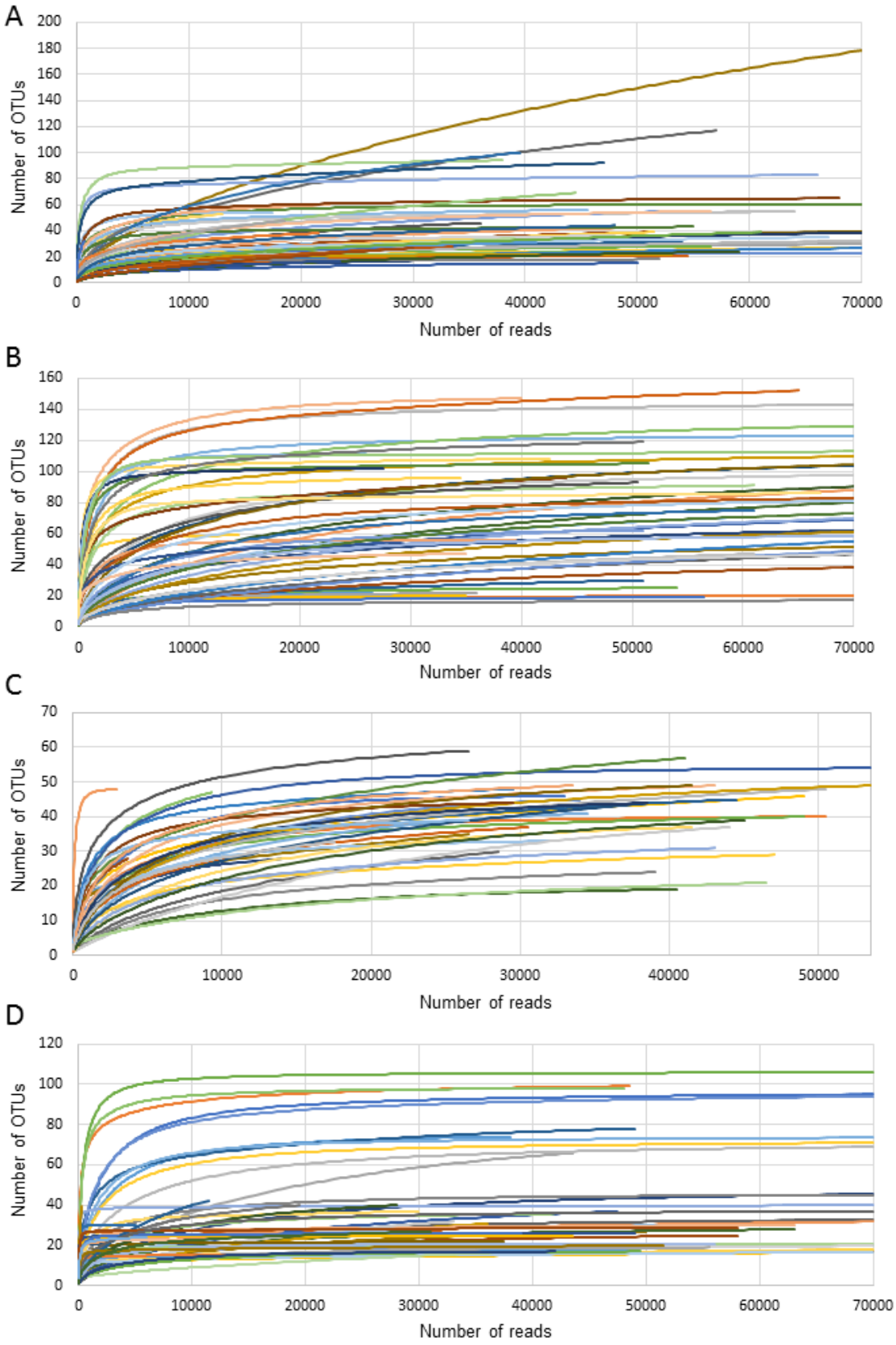


Figure 3.3.1.2.24. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for fish meal replacement winter trial samples.[1]

[1] Mean values are plotted for each of the groups of interest (Diet 1 [control] vs Diet 2 [20% FM + 10.7% FMB-P] vs Diet 3 [10% FM + 21.4% FMB-P] vs Diet 4 [20% FM +11.32% PM] vs Diet 6 [10% FM, 10.7% FMB-P + 11.32% PM] vs Diet 7 [20% FM + 10.88% SPC]).

Appendix 1. Rarefaction curves portraying the number of resolved OTUs against sequencing depth of each sample from component A) Commercial feed formulations vs 'natural' (Sardine) diets, B) Fatty acid inclusion trial, C) Lipid and emulsifier trial and D) Fish meal replacement trial.



3.3.1.3. Manuscript - Assessing the impacts of changing health status on the microbiome of Yellowtail Kingfish (Seriola lalandi).

Sarah R. Catalano [a], Andrew P.A. Oxley [a]

[a] South Australian Research and Development Institute, South Australia Aquatic Sciences Centre, West Beach, SA 5024, Australia.

This manuscript may be referenced as: Catalano, S.R. and Oxley, A.P.A. (2019). 3.3.1.3. Manuscript – Assessing the impacts of changing health status on the microbiome of Yellowtail Kingfish (*Seriola lalandi*) (Outputs 4b and 4d). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.622-705.

Abstract

For assessing the impacts of the gut microbiome on health in Yellowtail Kingfish (*Seriola lalandi*; YTK), analysis of the bacterial communities from samples obtained from a number of common, though poorly resolved, conditions arising in farmed fish were investigated. Specifically, fish exhibiting signs of an enteritis-like condition as well as coccidiosis infection were sampled as part of two separate investigations, with sampling conducted over consecutive years for the more commonly occurring enteritis-like condition. In analysing global patterns of bacterial diversity, samples from these conditions clustered independently from one another, indicating that the contributing features and/or resultant changes in the microbiome are not the same between these two conditions. While the disease agent of coccidiosis is known (*Eimeria* protozoa/*Goussia* parasites), the causative agent responsible for enteritis remains unclear. Nonetheless, even though the disease agents appear to be different, both coccidiosis and enteritis disease cohorts in comparison with healthy controls caused, albeit varied, shifts in the gut microbiome, with both conditions contributing to reduced species richness, diversity and evenness. In both cases, the occurrence of one or more dominant bacterial taxa were also observed. A general trend of a reduction in the numbers of mucous-secreting cells, as well as decreases in villi length and a thinner submucosa, muscle layer and serosa was observed in the hindgut histology slides for fish with the enteritis-like condition, along with enrichment of one specific bacterial taxa with closest similarity to *Photobacterium* sp. (OTU 10950). As an additional observation from this work, when examining the bacterial communities associated with the outer body surfaces (skin and gills) for fish exhibiting the enteritis-like condition, broad changes in the microbiome were also observed. This highlights that gut-associated conditions may induce body-wide microbiome changes, with the occurrence of particular taxa occurring only in disease fish, representing an interesting prospect for the development of novel biomarkers of changing health in these animals on-farm, and may support the implementation of improved and timelier management/intervention strategies that may assist in minimising stock losses. For the fish displaying signs of coccidiosis infection, two *Bacillus* species were notably decreased in abundance in comparison to the healthy controls. With this group of organisms generally considered to be favourable in the host, where they may assist in digestion, nutrient acquisition and competition with pathogens, their loss may represent potentially depleted or diminished microbiome functionality in the gut of these fish. Attempts to restore the occurrence of these taxa and diversify the gut (e.g. through probiotic use or dietary manipulation) should be explored as a potential and alternative strategy to conventional (antimicrobial) therapies for improving health outcomes in these fish.

Introduction

Disease outbreaks pose a significant constraint on the production of fish and shellfish, with the aquatic environment containing a suite of obligate and opportunistic bacterial pathogens that can influence the economic development of this sector (Schulze et al., 2006, Wang et al., 2010). Fish in aquaculture settings are particularly susceptible to disease, as they are often stocked at high densities and fed formulated diets rather than natural feeds which, while used in an effort to reduce production costs (Nakada, 2008), may increase stress and place undue physiological burden on the animal. Under increased levels of physiological stress, opportunistic pathogens that normally occur within the host may proliferate, displacing endogenous species to become dominant features that may led to infection, particularly in animals that have a weakened immune system. As a result, therapeutic treatments are often needed to control for and/or eliminate the pathogen/s, thereby further increasing production costs (Nakada, 2008; Boutin et al., 2013; Sylvain et al., 2016). For infections that are poorly characterised or are of unknown aetiology, this is particularly challenging and may prevent the implementation of effective therapeutic intervention/management strategies. Hence, the capacity to readily identify the responsible pathogen/s and the conditions leading to their emergence, is important for developing and adopting appropriate monitoring and intervention/management strategies for maintaining fish health and productivity in aquaculture. Central to this is an improved understanding of the role the gut microbiome (and select constituents) play in the health and disease of Yellowtail Kingfish (*Seriola lalandi*: YTK).

As in humans and other animals, the gut microbiome of fish is thought to be critical for supporting and maintaining normal healthy immune, digestive and nervous system functioning, and represents an important tool that can be used to monitor changes in health status (Ghanbari et al., 2015; Colston and Jackson, 2016). However, under physiological stress, these systems may become compromised, whereby a loss or change in bacterial community diversity can lead to immune dysfunction, susceptibility to toxins and reduced efficiency to acquire nutrients, thereby increasing the risk of disease (Xia et al., 2014; Parris et al., 2016). In contrast, under normal conditions, a healthy gut microbiome comprises diverse bacterial assemblages that aid in regulating and supporting gut homeostasis, for example through the supply of exogenous nutrients and extracellular enzymes, fatty acids and vitamins (Austin, 2006; Dehler et al., 2017). Gut microbes can also protect the host by depriving invading pathogens of nutrients and secreting a range of antimicrobial substances (Nayak, 2010). A balanced microbiome is therefore key to maintaining overall fish health (Austin, 2006).

Microbiome research in fish, especially those of importance to aquaculture, has been developed over the past few years, largely due to the revolution and reduced cost of next generation sequencing (NGS) technologies (Leonard et al., 2014; Llewellyn et al., 2014). Collectively, these technologies paired with the availability of 16S rRNA databases (i.e. RDP, SILVA and Greengenes) provide a powerful tool to count, classify and describe microbial communities, including uncultivable members, that occur in a system or under a given treatment or condition (Llewellyn et al., 2014; Federici et al., 2015). For conditions where no clear aetiological agent has been identified, or where the mechanisms behind or driving infection are unknown, such tools offer a tantalising new approach to understanding disease processes in aquaculture. Specifically, in examining the community as a whole, rather than using conventional targeted assays which seek to identify/exclude a specific pathogen, such procedures provide an opportunity to identify new biomarkers of changing health status. For specific diseases occurring in YTK such as enteritis, where symptomatic features required for diagnosis frequently appear at the later (chronic) stages of infection when it is often too late for therapeutic intervention (Sheppard, 2005; Bansemer et al., 2015), such an approach is especially enticing and may assist in identifying biomarkers that, when adapted for use using more rapid molecular assays (e.g. quantitative [q]PCR), can be used for early detection. In doing so, a platform for supporting the implementation of improved and timelier management/intervention strategies may be developed, and may assist in minimising stock losses from such diseases. As a further benefit, in analysing the microbiome during health and disease, potentially novel species that may be important for supporting normal, healthy functioning may also be identified and may represent organisms that could be used as novel probiotics (or enriched for using prebiotics) for inhibiting pathogens, or boosting the host's immune response or metabolic processes to promote and sustain health and performance (Merrifield et al., 2010; Cruz et al.,

2012; Gomez et al., 2013; Ghanbari et al., 2015; Hoseinifar et al., 2015; Dittman et al., 2017). This may aid in improved resilience of fish to disease, and currently represents a more attractive alternative to the use of conventional (antimicrobial) therapeutics within the industry, which may otherwise promote antibiotic resistance in pathogens (Dittman et al. 2017; Miranda et al. 2018) and/or a reduction of microbial gut diversity with the loss of commensal, beneficial taxa (Egerton et al., 2018).

In this manuscript, we investigate the gut, skin and gill microbiome (bacterial assemblages) of YTK in health and disease, with a specific focus on conditions of the gastrointestinal system that may arise within this species, including an enteritis (or enteritis-like) condition that typically arises in suboptimal temperatures in fish fed diets containing plant proteins (Bansemer et al. 2015; Sheppard 2004) and coccidiosis, which is primarily caused by species of *Eimeria* protozoa and *Goussia* parasites and has been recorded from other teleost fish, such as Whiting-pout (*Trisopterus luscus*) (Gestal and Azevedo, 2006). These investigations were undertaken as part of two separate studies, with sampling conducted over two consecutive years for the more commonly occurring enteritis-like condition. Comparisons between the conditions and with underlying pathology and potential drivers (i.e. diet, environment, fish size and year class) were also investigated. The data generated in this manuscript thus serves as a resource for improving our understanding of these conditions in farmed YTK and may aid in the development of improved monitoring programs that enable optimal health management strategies on-farm.

Aim

The aim of this experimental work is to assess the impacts of changing health status on the microbiome (bacterial assemblages) of YTK and to identify the involvement of microbiome changes (or select bacterial constituents) in specific diseases of the gastrointestinal system (namely enteritis and coccidiosis); as observed from comparisons of the gut, skin and/or gills from healthy and diseased fish, and in comparing variations between disease events, individual (enteritis/coccidiosis) conditions, changes in underlying pathology and potential drivers (namely location/site, fish size, year class and feed type).

A. Healthy vs Disease 2016 - Enteritis

A. Methods

Experimental design

A total of 36 fish were sampled for this experiment in March 2016, and comprised 12 healthy fish from a control sea-cage displaying no signs of infection (Port Lincoln, South Australia [SA], Bickers site, cage 15-002B) and 24 fish from a second sea-cage in the same region (Port Lincoln, SA, Northern Site, cage 15-004A). In this second sea-cage, fish were classified into two groups (with 12 samples taken per group). This included a 'healthy intermediate' group, where no or minor external symptomatic features were observed, and a 'disease' group, where obvious external symptomatic features were apparent. Categorisation of the health groups was performed by Clean Seas veterinarian Dr Matt Landos as confirmed earlier by necropsy and histopathological assessment (data not shown). Fish from both sea-cages were of the same year class (2015) and fed the same diet from manufacturer A (9 mm pellet diameter). A seawater sample was taken from both the healthy control and disease sea-cage locations and processed in parallel to control for the influence the environment may have on the structure and composition of the gut bacterial community (Figure 3.3.1.3.1).

Fish sampling – microbiomic analyses and histopathology

Each fish was euthanised in AQUIS solution, weighed (g) and measured (fork length, cm). The body cavity was opened and the entire GI tract removed and placed onto a clean surface. The GI tract was

cut into three sections with a sterile scalpel, representing the fore-, mid- and hindgut regions. For the microbiome analysis, a new sterile scalpel was used to open each region to expose the inner surfaces, then a scraping was taken with a sterile glass slide to collect the gut contents/mucosa. The scraping was immediately placed in a 50 mL falcon tube containing stabilising buffer (RNAlater™, Ambion), labelled and stored at 4 °C for 1-2 days before being stored for up to a month at -20 °C prior to RNA extraction. Gloves, aluminium foil and scalpel blades were discarded and forceps were cleaned with ethanol after sampling each fish to avoid cross contamination.

For histopathological analysis, a 1 cm^2 longitudinally opened biopsy section from each gut region from each fish were collected for histological analysis prior to taking the gut scraping sample. Biopsy samples were placed into standard histopathology cassettes and fixed immediately in 10% seawater formalin for > 48 h and stored at room temperature prior to analysis. Histological samples collected from the three gut regions of the three 'wild' and seven wild charter fish (refer to Manuscript 3.1.1.1) were assessed here with the health and disease microbiome samples for comparative analysis. A number of gastrointestinal morphological parameters were evaluated from each tissue section from Hematoxylin and Eosin (H E) and Periodic acid-Schiff Alcain Blue (PAS AB pH 2.5) stained slides and included: number of mucous cells per 100 µm (mean values derived from a minimum of three measurements per slide); length of villi (mean values derived from a minimum of three measurements per slide and from the longest villi that could be found in a given section); thickness of submucosa (mean values derived from a minimum of two measurements per slide); thickness of muscle and serosa (single measurements were taken per slide due to the general consistency of the layers); and number of melanomacrophage centres (MMCs) in the submucosa (total counts given for each section, with relative rather than absolute values denoted as some tissue sections were larger than others). Histological slides were prepared by Dr Rebecca Forder and Cheryl Day (School of Animal and Veterinary Science, University of Adelaide, Roseworthy Campus), with histopathological evaluations and measurements conducted by Dr Fran Stephens (Veterinarian and Diagnostic Consultant, Morangup, WA).

RNA extraction for gut microbiome samples

RNA was extracted on ice from stabilised samples according to the methods detailed in Szafranska et al. (2014). In brief, the stabilising buffer was removed from each sample and 1 mL of cold (4 °C) RLT buffer supplemented with 1% β-mercaptoethanol was added and transferred to lysing matrix B tubes (MP Biomedicals). Samples were disrupted via bead-beating using the FastPrep-24™ 5G instrument (MP Biomedicals) at an intensity of 5.5 for 45 s, placed on ice for 3 min then disrupted a second time as described above prior to centrifugation at 14,000 × g for 10 min at 4 °C. The supernatant was transferred to 1.5 mL RNase-free Biopur centrifuge tubes (Eppendorf) and the RNeasy minikit was used to extract the RNA according to manufacturer's instructions. RNA was eluted in 30 µL of RNase free water, passed through the spin column twice to concentrate each sample and quantified using a NanoDrop 2000 spectrophotometer. To remove any source of potential contaminating gDNA, a routine DNase treatment was performed for all samples using the Turbo DNA-free™ kit (Life Technologies) following the manufacturer's instructions. All samples were precipitated with ethanol using standard procedures, reconstituted in 30 µL of RNase free water and the RNA re-quantified using NanoDrop. Samples were stored at -80 °C prior to use in down-stream procedures.

DNA extraction for environmental samples

One litre of seawater was collected in a sterile Schott bottle from each of the sea-cage locations (Bickers site healthy vs Northern Site disease, Port Lincoln, SA). Each bottle was labelled with the site location and stored at 4 °C prior to filtration and DNA extraction. Each seawater sample was filtered onto separate sterile 0.22 µM filters prior to DNA extraction using the FastDNA™ Spin Kit for Soil (MP Biomedicals) following the manufacturer's instructions. In brief, the filter paper was placed in a lysing matrix E tube with sodium phosphate and MT buffer and cells were lysed via bead-beating using the FastPrep-24™ 5G instrument (MP Biomedicals) at an intensity of 5.5 for 45 s. Samples were

subsequently centrifuged for 10 min at 14,000 × *g* and the supernatant transferred to 1.5 mL DNA LoBind tubes (Eppendorf). Following the addition of a protein precipitation solution, the samples were mixed and centrifuged to pellet the precipitation before the supernatant was transferred to a clean 15 mL centrifuge tube supplemented with Binding Matrix solution. The DNA was captured on SPIN filter tubes and washed, re-eluted in 100 µL of DES and quantified using a NanoDrop 2000 spectrophotometer followed by precipitation with ethanol using standard procedures. The pelleted DNA was reconstituted in 30 µL of RNase free water, re-quantified using the NanoDrop and stored at 4 °C prior to use in down-stream procedures.

cDNA synthesis, PCR amplification, AMPure bead-purification and Picogreen quantification

For the gut scraping samples only, the RNA extracts were converted to cDNA to assess for the active (and likely resident) bacterial community members using the Superscript™ III First Strand Synthesis System (Life Technologies) following the manufacturer's instructions and stored at -20 °C prior to PCR amplification. The V1-V2 hypervariable region of the 16S rRNA gene was amplified for all samples (DNA and cDNA samples) as described by Camarinha-Silva et al. (2014)); though included an initial pre-enrichment of the V1-V2 target region by conducting a 20 cycle PCR reaction with primers 27F and 338R as described by Chaves-Moreno et al. (2015). However, in a first 20 cycle PCR reaction the 16S rDNA target was enriched using the previously described 27F and 338R primers (Chaves-Moreno et al., 2015). Specifically, 2 µL of cDNA and 5 µL of each environmental DNA extract was used as template in the first round of PCR, with 1 µL aliquots from this reaction used as template in a second 15 cycle PCR reaction to append sample specific barcodes and reverse adapter sequences complementary to the Illumina platform specific adaptors. One microlitre of the second round PCR reaction mixture were subsequently used as a template in a third 10 cycle PCR to append the Illumina multiplexing sequencing and index primers. PCR amplicons were visualised via agarose gel electrophoresis and products of the expected size (~438 bp) were purified using Agencourt AMPure XP beads (Beckman Coulter). Samples were quantified in duplicate using the Quant-iT™ Picogreen® dsDNA kit (Life Technologies) following the manufacturer's instructions. Approximately 100 samples were pooled for each library in equimolar ratios and sequenced on the MiSeq platform (Illumina, San Diego, CA) using 250 nt paired-end sequencing chemistry through the Australian Genome Research Facility (AGRF). As a sequencing control, amplicons generated from a single bacterial species (*Lactobacillus reuteri*) were included within each Illumina index within each of the libraries. The final list of samples that generated good-quality microbiomic libraries for this component of work are presented in Table 3.3.1.3.1.

Bioinformatics analysis

In total, 3,348,468 million sequence reads were derived from 89 samples (of the 110 that were collected). Twenty one samples failed to amplify enough material to produce good-quality NGS libraries (majority being foregut samples), which was accounted for in the experimental approach by allowing for ample replication of fish (and sampling of multiple gut regions). Sequence reads were paired using PEAR (version 0.9.5) (Zhang et al., 2014), where primers were identified and removed. Paired-end reads were quality filtered, with removal of low-quality reads, full-length duplicate sequences (after being counted) and singleton sequences using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010), USEARCH (version 8.0.1623) (Edgar, 2010; Edgar et al., 2011) and UPARSE software (Edgar, 2013). Reads were mapped to Operational Taxonomic Units (OTUs) using a minimum identity of 97%, with putative chimeras removed using the RDP-gold database as a reference (Cole et al., 2014). These OTUs were further filtered as conducted previously (Zhang et al., 2016) where only those that contributed to > 0.01% of the host-associated dataset (gut samples only) or > 0.01% of the environmental (seawater) samples were included (see Table 3.3.1.3.2 for a summary of OTUs remaining post-filtering). Rarefaction curves were used to inspect (retrospectively) sampling depth for each gut sample (Appendix 1A). Filtered OTUs were taxonomically interrogated using the SeqMatch function of the RDP database (Wang et al., 2007) as

well as SILVA (Quast et al., 2013), whereby lineages based on the SILVA taxonomy and best hits from RDP were assigned to each OTU alongside the corresponding RDP sequence similarity value (SeqMatch, S_ab score). The S_ab score represents the number of unique 7-base oligomers shared between an OTU and a known sequence contained in the RDP database divided by the lowest number of unique oligos in either of the two sequences. A S_ab score of 1.000 represents an identical match to the nearest database sequence, with values closer to 1.000 providing greater confidence in the identification of the OTU sequence.

Statistical analysis

In order to explore for patterns across the global bacterial communities, the data matrix comprising the percent standardised abundances of OTUs was used to construct a sample-similarity matrix using the Bray-Curtis algorithm (Bray and Curtis, 1957), where samples were then ordinated using non-metric multidimensional scaling (nMDS) with 50 random restarts (Clarke et al., 2001). Significant differences between *a priori* pre-defined groups of samples (e.g. environmental water samples vs gut scraping samples) were evaluated using permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations, allowing for type III (partial) sums of squares, fixed effects sum to zero for mixed terms, and exact *P* values generated using unrestricted permutation of raw data (Anderson, 2001). Groups of samples were considered significantly different if $P < 0.05$. Pairwise tests in PERMANOVA were used to determine which *a priori* pre-defined categories (e.g. healthy vs healthy intermediate vs disease) were significantly different. The multivariate analyses, relative percent abundance of bacterial phyla and rarefaction curves were performed and calculated using PRIMER (v.7.0.11), PRIMER-E, Plymouth Marine Laboratory, UK (Clarke et al., 2001).

Conventional measures of species diversity, richness and evenness were calculated using algorithms for total OTUs (S), Pielou's evenness (J'), Shannon diversity (H') and Simpson (1-λ), while taxonomic diversity was calculated using algorithms for taxonomic distinctness: average taxonomic distinctness (avTD - delta+) and variation in taxonomic distinctness (varTD - lambda+) using PRIMER (v.7.0.11) (Clarke et al., 2001). These univariate indicators of diversity (S, J', H', 1-λ, avTD, varTD), along with the mean values for each histological parameter (mucous cells, length of villi, thickness of submucosa, thickness of muscle and serosa, melanomacrophage centres in submucosa), were compared between *a priori* groups of samples (e.g. healthy vs healthy intermediate vs disease) using one-way ANOVA and plotted in Prism v. 7.01 (Graphpad Software Inc.). Variables were considered to be significantly different if $P < 0.05$, for which a Tukey's post-hoc multiple comparisons test was then performed (Prism v. 7.01).

For further presentation of the data, relative abundance plots of the top 15 most abundant gut OTUs was constructed in Excel. To obtain the identification of the closest cultured species for each of the most abundant OTUs, the corresponding sequence was blasted against the RDP isolate database only. A similarity (S_ab) score in parenthesis is presented for each OTU in the top 15 OTUs plot.

A. Results

Global community structure

As observed in the baseline data (see Manuscript 3.3.1.1), there was a clear separation and significant difference (PERMANOVA, $P = 0.0004$, table not shown) between the global bacterial community structure of the two environmental (seawater) samples and all gut samples collected from the healthy, healthy intermediate and disease fish (Figure 3.3.1.3.2). For the three health status groups, the disease samples clustered together with overlap by the healthy intermediate samples, which also spread outwards towards the healthy samples. The healthy samples had minimal overlap with the disease group and were more widely spread compared to the healthy intermediate samples. These trends were confirmed by PERMANOVA, with a significant difference from pairwise comparisons between healthy vs healthy intermediate ($P = 0.0001$) and healthy vs disease ($P = 0.0001$), but not between healthy intermediate vs disease ($P = 0.3107$) (Table 3.3.1.3.3). Though three gut regions were sampled (i.e. the

fore-, mid- and hindgut), there was no significant difference between these regions (PERMANOVA, $P = 0.2979$, table not shown) or when gut regions were crossed with the three health classes (PERMANOVA, $P = 0.9199$, table not shown).

Bacterial phyla

Differences in the phyla composition of the healthy, healthy intermediate and disease fish were observed. In particular, the healthy fish were mainly dominated by phyla *Proteobacteria*, with dominance by *Tenericutes* seen for some samples and minor representation from *Actinobacteria*, *Bacteroidetes* and *Spirochaetae* in a few samples (Figure 3.3.1.3.3). Healthy intermediate fish were predominantly dominated by the phyla *Tenericutes* with representation by *Spirochaetae* and *Proteobacteria* in some samples (Figure 3.3.1.3.3). The disease fish were almost exclusively dominated by phyla *Tenericutes*, with some samples having decreased representation from *Bacteroidetes*, *Actinobacteria* and *Cyanobacteria*, and increased representation from *Spirochaetae* (Figure 3.3.1.3.3).

Top 15 OTUs

Disease samples were dominated by a single OTU that was most closely associated with an unclassified *Mollicutes* (phylum *Tenericutes*) species, namely *Mycoplasma insons* (OTU 1, similarity [S_ab] score 0.420) (Figure 3.3.1.3.4). In some individuals, the relative abundance of this organism reached almost 100%, with a median relative abundance of 93% among the disease samples. There was also minor representation from taxa with closest sequence similarity to *Aliivibrio* sp. (OTU 4, S_ab score 0.948) in the disease samples (Figure 3.3.1.3.4).

High relative abundance of *M. insons* (OTU 1, S_ab score 0.420) was recorded in most healthy intermediate samples with a reduced median abundance (83%) to that observed for the disease samples. There was also representation in some healthy intermediate samples by taxa with closest sequence similarity to *Aliivibrio* sp. (OTU 4, S_ab score 0.948), *Brevinema andersonii* (OTU 22, S_ab score 0.734; and OTU 13, S_ab score 0.741), and *Photobacterium damselae* subsp. *damselae*/*P. leognathi* (OTU 2, S_ab score 1.000) (Figure 3.3.1.3.4).

M. insons (OTU 1, S_ab score 0.420) was recorded in the healthy fish, though at a much lower abundance (median 11%) than that observed for either the healthy intermediate or disease fish. Samples from the healthy fish also appeared to have greater heterogeneity (or inter-individual variation), with variable dominance between samples observed for various taxa which had closest sequence similarity to *Aliivibrio* sp. (OTU 4, S_ab score 0.948), *P. damselae* subsp. *damselae*/*P. leognathi* (OTU 2, S_ab score 1.000), *P. phosphoreum/iliopiscarium* (OTU 17, S_ab score 1.000), *Vibrio* sp. (OTU 16, S_ab score 1.000), *B. andersonii* (OTU 13, S_ab score 0.741) and *Ehrlichia* sp. trout isolate (OTU 12, S_ab score 0.821) (Figure 3.3.1.3.4).

Diversity indices

Between the healthy and disease samples, there was a significant decrease in species evenness ($P < 0.0001$) and diversity (Shannon's diversity $P = 0.0011$ and Simpson's diversity $P = 0.0014$) in the disease fish compared to the healthy fish, although species richness was significantly greater in the disease cohort ($P = 0.0019$) (Figure 3.3.1.3.5, Table 3.3.1.3.4). The same trend was also observed between the healthy and healthy intermediate samples, with a significant decrease in species evenness ($P = 0.0007$) and diversity (Shannon's diversity $P = 0.0115$ and Simpson's diversity $P = 0.0309$) but significant increase in species richness ($P = 0.0374$) in the healthy fish compared to the healthy intermediate fish (Figure 3.3.1.3.5, Table 3.3.1.3.4). No clear patterns or significant differences were observed between the healthy intermediate and disease samples for all diversity indices (Figure 3.3.1.3.5, Table 3.3.1.3.4).

Histological parameters

For the foregut tissue samples, the thickness of the submucosa was the only parameter that was significantly different between health groups, with a greater thickness recorded in the healthy intermediate fish compared to the healthy ($P = 0.0123$) and disease fish ($P = 0.0021$) (Figure 3.3.1.3.6, Table 3.3.1.3.5). When including samples collected as part of the wild charter dataset (refer to Manuscript 3.3.1.1), both the number of mucous cells and thickness of muscle and serosa were significantly different between the wild charter fish and certain farmed YTK health groups. In particular, a greater number of mucous cells was recorded in the farmed healthy ($P = 0.0007$) and disease fish ($P = 0.0234$) compared to the wild charter fish, and the muscle and serosa was thicker in the wild charter fish compared to the farmed healthy ($P < 0.0001$), healthy intermediate ($P < 0.0001$) and disease ($P < 0.0001$) fish (Figure 3.3.1.3.6, Table 3.3.1.3.5). Length of villi and number of MMCs in the submucosa was not significantly different between the wild fish and farmed health groups, or between samples from the three health groups.

For the midgut tissue samples, both the length of villi and thickness of submucosa were significantly different between certain health groups, with a greater villi length in healthy and healthy intermediate fish compared to disease fish ($P = 0.0050$ and $P = 0.0258$ respectively) and thicker submucosa in healthy intermediate fish compared to healthy and disease fish ($P = 0.0118$ and $P = 0.0006$ respectively) (Figure 3.3.1.3.7, Table 3.3.1.3.6). Similar to the foregut tissue, the muscle and serosa was significantly thicker in the wild charter fish compared to the farmed healthy ($P = 0.0072$), healthy intermediate ($P = 0.0058$) and disease ($P < 0.0001$) fish, and the number of mucous cells was significantly greater in the farmed healthy ($P = 0.0011$), healthy intermediate ($P = 0.0034$) and disease fish ($P = 0.0085$) compared to the wild charter fish (Figure 3.3.1.3.7, Table 3.3.1.3.6). While not significant in the foregut tissue, the length of villi was significantly greater in the farmed healthy fish compared to the 'wild' ($P = 0.0004$) and wild charter fish ($P = 0.0284$), as well as between the healthy intermediate and 'wild' fish ($P = 0.0013$) in the midgut tissue (Figure 3.3.1.3.7, Table 3.3.1.3.6). As observed in the foregut tissue samples, the number of MMCs in the submucosa was not significantly different between the wild fish and health groups, as well as within the three health groups.

Similar to the midgut tissue samples, the length of villi was significantly different between certain health groups in the hindgut tissue, with a greater villi length in the healthy and healthy intermediate fish compared to disease fish ($P < 0.0001$ for both) (Figure 3.3.1.3.8, Table 3.3.1.3.7). The muscle and serosa was significantly thicker in healthy compared to disease ($P = 0.0124$) fish. Additionally, in the hindgut tissue, and consistent with the foregut and midgut finding, the muscle and serosa was significantly thicker in the wild charter fish compared to the farmed healthy ($P < 0.0001$), healthy intermediate ($P < 0.0001$) and disease fish (< 0.0001) (Figure 3.3.1.3.8, Table 3.3.1.3.7). A significantly greater number of mucous cells was recorded in the healthy compared to wild charter fish ($P = 0.0340$) and the villi length was significantly less in the disease fish compared to the wild charter fish ($P < 0.0001$), but greater in the healthy and healthy intermediate fish compared to the wild fish ($P = 0.0250$ and $P = 0.0437$ respectively) (Figure 3.3.1.3.8, Table 3.3.1.3.7). As observed in the foregut and midgut tissue, the number of MMCs in the submucosa was not significantly different between the wild fish and health groups, as well as within the three health groups (i.e. healthy, healthy intermediate and disease).

B. Gastrointestinal Health (Enteritis vs Coccidiosis)

B. Methods

Experimental design

To discern whether the microbiome varies between different gut conditions (namely enteritis and coccidiosis), 12 additional fish from a sea-cage comprising individuals displaying signs of a coccidiosis infection (as determined earlier by Clean Seas health staff) were collected on the 18th July 2016 from sea-cage AB16-8 at site AB3-AB in Arno Bay, SA (Figure 3.3.1.3.9); and were compared alongside the 36 samples collected earlier for the health vs disease (enteritis) component (Figure 3.3.1.3.1; and

detailed in section A. Methods). Of the 12 fish that were collected for this work, six were classed as 'healthy' (where no visible external symptomatic signs of infection or weight loss were observed), and six as 'disease' (where obvious external symptomatic features were apparent, including notable loss of weight and body condition). All fish from the coccidiosis and enteritis groups had been fed the same diet from manufacturer A, except the pellet diamter was smaller (6 mm compared to 9 mm) for the coccidiosis cohort. A seawater sample was also taken and processed in parallel from the Arno Bay sea-cage site to control for the influence the environment may have on the structure and composition of the gut bacterial community. The initial gut enteritis dataset was merged with the coccidiosis samples and analysed as a single dataset here to allow for a deeper insight into the general properties of disease and its influence on the microbiome in YTK.

Fish sampling - microbiomics

Refer to section A. Methods. No histological samples were collected for the coccidiosis investigations.

RNA extraction for gut microbiome samples

Refer to section A. Methods.

DNA extraction for environmental sample

Refer to section A. Methods.

cDNA synthesis, PCR amplification, AMPure bead-purification and Picogreen quantification

Refer to section A. Methods. The final list of samples that generated good-quality microbiomic libraries for the components of this work are presented in Table 3.3.1.3.8.

Bioinformatics analysis

In total, 4,663,108 million sequence reads were derived from 122 samples (of the 183 that were collected). Sixty one samples failed to amplify enough material to produce good-quality NGS libraries, which is accounted for in the experimental approach by allowing for ample replication of fish. Table 3.3.1.3.9 provides a summary of the OTUs remaining post-filtering. Rarefaction curves were used to inspect (retrospectively) sampling depth (Appendix 1B). For detailed methods on the quality filtering and mapping reads, refer to section A. Methods.

Statistical analysis

Refer to section A. Methods. In addition, the Similarity Percentage (SIMPER) routine was applied in PRIMER (v.7.0.11) where there was a significant difference and moderate variation to seek for those OTUs that contribute mostly to the observed difference between *a priori* pre-defined categories (e.g. for coccidiosis 'healthy' vs coccidiosis 'disease' fish). The univariate indicators of diversity (S, J', H', 1-λ, avTD, varTD) were compared between *a priori* groups of samples (e.g. coccidiosis 'healthy' vs coccidiosis 'disease' fish) using the unpaired Welch's t-test (not assuming equal variance) and plotted in Prism v. 7.01 (Graphpad Software Inc.).

B. Results

Global community structure

The seawater sample collected in this component of the work clustered with those from the earlier health vs disease (enteritis) investigations in the nMDS and, as previously observed, were significantly different to the gut samples, irrespective of health grouping (PERMANOVA, $P = 0.0001$, table not shown) (Figure 3.3.1.3.10). The global community structure of the two disease conditions were significantly different ($P = 0.0001$) (Table 3.3.1.3.10), with clustering of the coccidiosis disease samples on the left of the plot and enteritis disease on the right of the plot (Figure 3.3.1.3.10).

Both conditions included samples of healthy individuals, however these healthy fish did not group together and were significantly different ($P = 0.0001$) (Table 3.3.1.3.10). This reiterates the importance of appropriate age- and size- specific controls as presented in the baseline dataset (Manuscript 3.3.1.1), as the enteritis fish ranged from 2-4 kg whereas the coccidiosis fish ranged from 200-600 g, hence a universal 'healthy control' could not be applied for both groups.

For coccidiosis only, healthy and disease samples separated out in the nMDS plot (Figure 3.3.1.3.10), with a significant difference confirmed by PERMANOVA pairwise comparison ($P = 0.0002$) (Table 3.3.1.3.10). Like that observed earlier for the enteritis samples, there was no significant difference between the individual gut regions ($P = 0.6223$) or between the coccidiosis health groups crossed with the gut regions ($P = 0.4967$) (Table 3.3.1.3.11), therefore gut region was removed as a factor in all remaining analyses.

Bacterial phyla

For the coccidiosis samples, both healthy and disease individuals were primarily characterised by the two phyla *Proteobacteria* and *Cyanobacteria*, with minor representation from *Fusobacteria*. While *Firmicutes* were present in all coccidiosis healthy fish, this phyla was absent in some of the coccidiosis disease samples, with greater dominance by *Proteobacteria* when *Firmicutes* were absent (Figure 3.3.1.3.11).

Between the disease samples, the bacterial phyla profile of coccidiosis compared to enteritis fish were markedly different. In particular, the dominant phyla in the enteritis group (*Tenericutes*) was not observed (or occurred in very low abundance) from the coccidiosis disease samples (Figure 3.3.1.3.11) which, as stated above, comprised higher abundances of *Proteobacteria*, *Cyanobacteria*, *Fusobacteria* and *Firmicutes* (Figure 3.3.1.3.11).

Top 20 OTUs and greatest taxa contributors

For the coccidiosis samples, there was displacement of the microbiome between the healthy and disease groups with a greater contribution by four key taxa in the disease fish compared to the healthy fish. The four key bacterial taxa enriched in the disease fish had closest sequence similarity to *Vibrio* sp. V776/*Aliivibrio finisterrensis* (OTU 4, similarity [S_ab] score 1.000), *Anabaena cylindrica* (OTU 15, S_ab score 0.376), *V. tasmaniensis*/*V. pomeroyi* (OTU 31, S_ab score 1.000) and *Brevinema andersonii* (OTU 2, S_ab score 0.632) (Figure 3.3.1.3.12, Table 3.3.1.3.12). Coccidiosis disease samples were also characterised by a loss in *Synechococcus* species (OTU 29, S_ab score 0.987; and OTU 23, S_ab score 1.000), *Pseudoalteromonas* sp. (OTU 40, S_ab score 1.000), *Campylobacter hyointestinalis* subsp. *lawsonii* (OTU 44, S_ab score 0.673), *Pseudomonas veronii*/*P. azotoformans*/*P. chlororaphis* subsp. *aureofaciens* (OTU 14, S_ab score 1.000), *Cetobacterium somerae* (OTU 25, S_ab score 0.596) and *Bacillus*/*Geobacillus* species (OTU 45, S_ab score 1.000; OTU 83, S_ab score 1.000; and OTU 234, S_ab score 0.977) (Table 3.3.1.3.12).

As also noted in the bacterial phyla plot (Figure 3.3.1.3.11), the specific taxa profiles between the coccidiosis and enteritis disease samples were distinctly different (Figure 3.3.1.3.12). The single,

dominant OTU observed from the enteritis disease samples (with closest sequence similarity to *Mycoplasma insons*; OTU 1, S_ab score 0.428), was not observed in the coccidiosis disease samples which instead, comprised the four main taxa highlighted above (Figure 3.3.1.3.12).

Diversity indices

For the coccidiosis investigation, a trend of reduced species richness and significantly lower species diversity (Shannon's diversity $P = 0.0032$ and Simpson's diversity $P = 0.0365$) and evenness ($P = 0.0032$) was observed for the disease compared to the healthy samples (Figure 3.3.1.3.13, Table 3.3.1.3.13). This was similarly observed for the enteritis disease samples, though with increased (rather than reduced) species richness (Figure 3.3.1.3.13, Tables 3.3.1.3.4 and 3.3.1.3.13).

C. Healthy vs Disease extension (2016 and 2017 enteritis samples)

C. Methods

Experimental design

To discern whether the microbiome of fish varies over re-occuring disease events, or between variables such as location/site, fish size, year class and feed type, samples from a second enteritis-like event were collected in 2017. Specifically, 20 fish were sampled in February 2017 from two sea-cages (17-4 and 17-8) in Arno Bay, SA (10 fish per cage), with a further 40 fish sampled in April 2017 from the same two sea-cages (20 fish per cage) (Figure 3.3.1.3.14 A, Table 3.3.1.3.14). Note that while cage 17-4 remained in Arno Bay for both sampling events, cage 17-8 was moved from Arno Bay to the Northern site (Port Lincoln, SA) without prior knowledge, and was subsequently sampled at this new location in April (Figure 3.3.1.3.14 A, Table 3.3.1.3.14). In February, fish were being fed a 4mm pellet diet (manufactured by feed company B), whereas in April they had moved from the 4mm to 6mm pellets and then to 9mm pellets (manufactured by feed company B), at which time they were sampled (Figure 3.3.1.3.14 A). Fish at the first time point (before feed change) were reported to be of ideal health, whereas at the second time point (after feed change), some fish appeared to exhibit signs of an enteritis-like disease (as reported by Clean Seas field personnel Jay Dent and Declan Sambrook, and Clean Seas veterinarian Dr James Fensham), resulting in fish being classified into 'healthy' and 'disease' cohorts (Figure 3.3.1.3.14 A, Table 3.3.1.3.14). In parallel, samples were also taken from a second location at Port Lincoln, SA in February 2017 where signs of an enteritis-like disease had also been recorded. From this location, a total of 78 fish were sampled from two sites (Site 1, Point Boston; and Site 2, Bickers), with 40 fish sampled from two sea-cages at Site 1 (20 fish per cage from 17-2 and 17-3), and 38 fish from two sea-cages at Site 2 (18 fish 16-4GS, and 20 from 16-3A) (Figure 3.3.1.3.14 B). Fish were again classed into two cohorts ('healthy' or 'disease') based on observational assessment by Clean Seas veterinarian Dr James Fensham, with identical numbers of each health group sampled per cage (Figure 3.3.1.3.14 B, Table 3.3.1.3.14). Fish in cage 17-2 at Point Boston and 16-4GS at Bickers were both fed a 9mm pellet diet (manufactured by feed company B), whereas fish in cage 17-3 at Point Boston were fed a 6mm pellet diet (manufactured by feed company B) and fish in cage 16-3A at Bickers a 9mm pellet diet (manufactured by feed company C) (Figure 3.3.1.3.14 B).

As stated above, the criteria for classing fish into the two health states (i.e. 'healthy' and 'disease') was determined by Clean Seas veterinarian Dr James Fensham. As part of this process, fish were excluded from inclusion if there were any obvious physical abnormalities that could impede on fish performance, including: eye lesions; jaw, spinal, opercular and/or fin deformity or damage; and physical trauma. 'Healthy' fish were of a live weight and fork length approximately equivalent to or greater than the cage population average, with an optimum body condition and no external symptomatic signs of poor health (e.g. a darkened body colour). 'Diseased' fish were of a live weight and fork length approximately below the respective cage population, with poor body condition (e.g. sunken head, drawn in abdomen and/or concave dorsal musculature) and clearly discernable external signs of poor health (e.g. diarrhoea, red vent, darkened body colour/appearance, slow swimming).

For comparing samples between the 2016 and from 2017 investigations, only hindgut samples from healthy and disease fish were considered, as no 'healthy intermediate' or foregut and midgut samples were collected in 2017. Additional samples from the skin and gills were collected though, to verify the differences that were discerned earlier between healthy and disease fish in the outer body surfaces as part of the student extension work conducted on this project (see Section 4).

Fish sampling – microbiomics and histology

For microbiome sampling, fish were collected from sea-cages using a dip net (disinfected between each fish capture with ethanol) and the skin and gills promptly swabbed using sterile FLOQSwabs (Copan Flock Technologies). For the gills, samples were collected from between the gill filaments, and for the skin, posterior from the pectoral fin above and below the lateral line. Swabs were immediately placed in 200 µl of RNAlater™ (Ambion) and stored at 4 °C for 1-2 days before being stored for up to a month at -20 °C prior to RNA extraction. Each fish was then euthanised in AQUIS solution, weighed (g) and measured (fork length, cm). The body cavity was opened and the entire GI tract removed. The hindgut was then separated from the fore- and midgut using a sterile scalpel blade and placed on a clean surface. Using a clean pair of forceps and sterile scalpel, an incision was made along the length of the hindgut to expose the inner surface, and then a single scraping was taken with a sterile glass slide to collect the gut contents/mucosa. Scrapings were immediately placed in 50 mL falcon tubes containing stabilising buffer (RNAlater™, Ambion), labelled and stored at 4 °C for 1-2 days before being stored for up to a month at -20°C prior to RNA extraction. Gloves, aluminium foil and scalpel blades were discarded and forceps were cleaned with ethanol after sampling each fish to avoid cross contamination.

For histopathological analysis, hindgut tissue from three healthy and three disease fish at each sea-cage was collected as described in section A. Methods. Comparative histological analyses were conducted alongside the 2016 samples (as detailed in section A. Methods), and those obtained from the three 'wild' and seven wild charter fish sampled in Manuscript 3.3.1.1.

RNA extraction for gut microbiome samples

Refer to section A. Methods. However, for extracting RNA from the swabs of the skin and gills, the whole tip of the swab was transferred from the stabilisation buffer (RNAlater™, Ambion) into the lysing matrix B tube (MP Biomedicals) containing 1 mL of cold (4 °C) RLT buffer supplemented with β-mercaptoethanol before disruption via bead-beating.

cDNA synthesis, PCR amplification, AMPure bead-purification and Picogreen quantification

Refer to section A. Methods. The final list of samples that generated good-quality microbiomic libraries for the *components* of this work are presented in Table 3.3.1.3.14.

Bioinformatics analysis

The gut scraping raw dataset contained ~12 million raw reads. This was derived from a total of 218 samples (of the 257 that were collected). Thirty nine samples failed to amplify enough material to produce good-quality NGS libraries. This was accounted for in the experimental approach by allowing for ample replication of fish. For the gill and skin swab samples, the raw dataset contained ~13 and ~12 million raw reads respectively. This was derived from a total of 177 gill and 177 skin swab samples, with no samples failing to amplify. Table 3.3.1.3.15 provides a summary of OTUs remaining post-filtering. Rarefaction curves were used to inspect (retrospectively) sampling depth for the gut, skin and gill samples (Appendix 1C-1E). Due to the low numbers of sequence reads and occurrences as outliers in the non-metric multidimensional scaling (nMDS) plot, three samples were removed from the gut

dataset (see Table 3.3.1.3.14, Appendix 1C). For detailed methods on quality filtering and mapping reads, refer to section A. Methods.

Statistical analysis

Refer to section A. Methods. In addition, the Similarity Percentage (SIMPER) routine was applied in PRIMER (v.7.0.11) where there was a significant difference and moderate variation to seek for those OTUs that contribute mostly to the observed differences between *a priori* pre-defined categories.

C. Results

To delineate variations in the gut microbiome (bacterial assemblages) between sampling periods or due to other factors (e.g. site/environment, fish size, feed type/pellet size, year class/run), a series of different comparisons were made. Firstly, the 2016 and 2017 gut samples were analysed as a combined dataset to determine if there was a significant difference in the microbiome structure of healthy vs disease fish with time (irrespective of other factors such as feed type, site, fish size and year class). Secondly, the 2017 before and after feed change gut samples from healthy fish from two sea-cages at Arno Bay/Northern Site were then analysed to assess likely changes occurring with increases in size/age and the possible need for appropriate controls, where the before fish were much smaller compared to the after fish which were sampled ~8 weeks later. As a third component, to further delineate factors contributing to microbiome changes, the gut samples were split into three discreet data subsets to explore for the influence of site (environment), fish size, feed type (pellet size), feed manufacturer (A, B or C) or year class/run. These included a 'temporal scale', 'same feed manufacturer and feed type', and a 'same feed manufacturer, site and year class' dataset (Table 3.3.1.3.16). The first data subset ('temporal scale') comprised gut samples from fish fed the same feed type (9 mm pellet diameter) and which were of the same size (healthy ~3.5 kg, disease ~1.6 kg), though with variations in the site of collection (for the 'disease' fish), year class and feed manufacturer. The second data subset ('same feed manufacturer and feed type') comprised gut samples from fish fed feed from the same manufacturer (B) and feed type (9 mm pellet diameter), though year class, site and fish size were variable. The third data subset ('same feed manufacturer, site and year class') comprised gut samples from fish fed feed from the same manufacturer (B) and which were from the same site (Point Boston) and year class (2017), though feed type (pellet size) and fish size were variable (Table 3.3.1.3.16). As additional components, common taxa occurring in all disease fish (irrespective of other factors) were noted as well as microbiome (bacterial assemblage) differences between healthy and disease samples of the skin and gills (between the 2016 and 2017 datasets).

Global community structure – combined gut dataset

In the 3D nMDS plot of the combined 2016 and 2017 gut datasets, a pattern of clustering of the healthy samples to the right of the plot and disease samples to the left of the plot was observed (Figure 3.3.1.3.15). This was confirmed by PERMANOVA ($P = 0.0002$, table not shown), highlighting that on a global microbiome (bacterial) community structure level, the healthy fish are significantly different to the diseased fish, irrespective of other factors such as diet, site, size and year class.

Global community structure – assessing age- and size appropriate controls

Previous findings for the gastrointestinal health component, whereby coccidiosis samples were compared with enteritis samples, showed that healthy individuals did not cluster together in the nMDS plot, highlighting the need for age- and size- appropriate controls as the coccidiosis fish were much smaller than the enteritis fish (see section B. Results, Figure 3.3.1.3.10). This observation was also validated here for the 2017 Arno Bay/Northern Site healthy samples from two sea-cages (17-4 and 17-

8). There was a significant difference in the global bacterial community between the healthy before and after feed change fish (sampled ~8 weeks apart) collected from sea-cage 17-4 (P = 0.0003, Table 3.3.1.3.17), as well as from 17-8 (P = 0.0004, Table 3.3.1.3.17), but not between healthy before 17-4 fish and healthy after 17-8 fish (P = 0.1403, Table 3.3.1.3.17) (Figure 3.3.1.3.16). This is correlated to fish size rather than feed type, as a significant difference in the global bacterial community structure was seen when feed shifted from a 4 mm to a 9 mm diameter pellet and fish size was different (e.g. 200 g to 800 g for 17-8 and 600 g to 1400 g for 17-4), but not when feed shifted from a 4 mm to 9 mm diameter pellet and fish size was similar (e.g. 600 g to 800 g, before 17-4 and after 17-8) (Figure 3.3.1.3.16 A and B).

Global community structure, SIMPER analysis and histological parameters – gut data subset 1

For data subset 1 ('temporal scale'), the feed type (9mm pellet) and fish size (healthy ~3.5 kg, disease ~1.6 kg) were constant, while the year and site of disease samples (Northern Site 2015 vs Bickers 2016), year class (one year apart) and feed manufacturer (A, B or C) were variable. In analysing the global bacterial community structure from these samples, no significant differences were observed between the healthy samples from the three sea-cages (Bi 15-002B, Bi 16-4GS and Bi 16-3A) (Figure 3.3.1.3.17, Table 3.3.1.3.18), although the submucosa was significantly thicker in the Bi 16-4GS fish compared to the other two sea-cages (Figure 3.3.1.3.18). However, for the disease samples, the global community structure of fish in sea-cage NS 15-004A compared to Bi 16-3A was significantly different (Figure 3.3.1.3.17, Table 3.3.1.3.18). This was due to a greater contribution of OTUs most closely related to *Mycoplasma insons* (OTU 1, similarity [S_ab] score 0.428) and *Brevinema andersonii* (OTU 25, S_ab score 0.700) in the 15-004A disease fish, compared to OTUs most closely related to *Vibrio* sp. V776/*Aliivibrio finisterrensis* (OTU 4, S_ab score 1.000), *Photobacterium* sp. (OTU 10950, S_ab score 0.875) and *Pseudoalteromonas atlantica* (OTU 37, S_ab score 1.000) in the 16-3A disease fish (Table 3.3.1.3.19 A). No significant differences in the histological parameters were recorded for these two groups (Figure 3.3.1.3.18).

Between the healthy and disease samples at each sea-cage locality, the global bacterial community structure of healthy vs disease fish in sea-cage 16-3A and in sea-cage 15-002B/15-004A were significantly different (Figure 3.3.1.3.17, Table 3.3.1.3.18, with the latter already presented in part A as these corresponded to the associated 2016 gut enteritis samples). For sea-cage 16-3A, a greater contribution and increased abundance of OTUs most closely related to *Vibrio* sp. V776/*A. finisterrensis* (OTU 4, S_ab score 1.000), *Photobacterium* sp. (OTU 10950, S_ab score 0.875), *Pseudoalteromonas atlantica* (OTU 37, S_ab score 1.000) and *Synechococcus* sp. (OTU 32, S_ab score 1.000) was observed in the disease compared to healthy samples (Table 3.3.1.3.19 B). The occurrence of OTU 1 (*M. insons*, S_ab score 0.428) was also observed in the healthy individuals in this sea-cage, but was decreased in abundance in the disease samples (Table 3.3.1.3.19 B). Three additional taxa, with closest sequence similarity to *Photobacterium phosphoreum*/*P. iliopiscarium* (OTU 28, S_ab score 1.000) and *Anabaena cylindrica* (OTU 16, S_ab score 0.377; and OTU 12360, S_ab score 0.350) were also decreased in abundance in the disease compared to healthy samples (Table 3.3.1.3.19 B). For the histological parameters measured in the hindgut tissue sections collected from these fish, there was no significant difference between the healthy and disease fish from this sea-cage (Figure 3.3.1.3.18).

For healthy sea-cage 15-002B and disease sea-cage 15-002A, the greatest contribution to the observed difference in the disease compared to healthy individuals was due to the increased abundance of OTU 1 (*M. insons*, S_ab score 0.428) (Table 3.3.1.3.19 C). Four additional taxa, with closest sequence similarity to *Vibrio* sp. V776/*Aliivibrio finisterrensis* (OTU 4, S_ab score 1.000), *Photobacterium damselae* subsp. *damselae*/*P. leiognathi* (OTU 6, S_ab score 1.000), *Brevinema andersonii* (OTU 25, S_ab score 0.700) and *P. phosphoreum*/*P. iliopiscarium* (OTU 28, S_ab score 1.000) were also decreased in abundance in the disease compared to healthy samples (Table 3.3.1.3.19 C). In evaluating the histology sections collected from these samples, a significantly shorter villi length was recorded in the disease compared to healthy fish (Figure 3.3.1.3.18). In general, while not significantly different, a reduction in the number of mucous cells and decrease in villi length, and thickness of the submucosa and muscle and serosa was observed in the hindgut tissue sections from disease compared to healthy

samples (Figure 3.3.1.3.18). Samples from the wild charter fish also displayed a pattern of longer villi and thicker submucosa, muscle and serosa compared to those from the disease fish (Figure 3.3.1.3.18).

Global community structure, SIMPER analysis and histological parameters – gut data subset 2

For data subset 2 ('same feed manufacturer and feed type'), the feed manufacturer (B) and feed type (9mm pellet) were constant, while the year class (2016 or 2017), site (Bickers, Arno Bay, Point Boston or Northern Site) and fish size (healthy 0.8-3.5 kg, disease 0.4-1.7 kg) were variable. For the healthy samples, there was a significant difference in the global bacterial community structure between healthy fish across the four sea-cage sites (Figure 3.3.1.3.19, Table 3.3.1.3.20). However, there was no significant difference in the histological parameters measured in the hindgut tissue sections collected from these fish, with the exception of those from sea-cage Bi 16-4GS which had a significantly thicker submucosa compared to those from sea-cage NS 17-8 (Figure 3.3.1.3.20). Like the healthy fish, there was also a significant difference in the global bacterial community structure between disease fish across the sea-cage sites, with the exception of those from cage AB17-4 compared to cage PB 17-2 (Figure 3.3.1.3.19, Table 3.3.1.3.20). No significant differences between disease samples were apparent for the histological parameters measured from these fish (Figure 3.3.1.3.20).

In comparing between the healthy and disease samples at each sea-cage locality, the global bacterial community structures were not significantly different, with the exception of those from cage AB 17-4 (Figure 3.3.1.3.19, Table 3.3.1.3.20). For this sea-cage, a greater contribution and abundance of an OTU most closely related to *Vibrio harveyi*/*Aliivibrio fischeri* (OTU 10, S_ab score 1.000) was observed in the disease compared to healthy samples (Table 3.3.1.3.21). Alongside this was a decrease in abundance of three OTUs with closest sequence similarity to *Photobacterium* sp. (OTU 10950, S_ab score 0.875), *P. damselae* subsp. *damselae*/*P. leiognathi* (OTU 6, S_ab score 1.000) and *Neorickettsia helminthoeca* (OTU 26, S_ab score 0.533) in the disease samples (Table 3.3.1.3.21). Note though that while OTU 10950 (*Photobacterium* sp., S_ab score 0.875) was of lower abundance in the disease compared to healthy samples in this sea-cage (AB 17-4), when compared to the healthy before samples, its average abundance did increase from 0.01 to 10.20% (Table 3.3.1.3.21). There were no significant differences in the histological parameters measured in the hindgut tissue sections collected from the healthy vs disease fish samples in AB 17-4, though the samples from healthy fish in cage Bi 16-4GS had a significantly thicker submucosa compared to those from disease fish cage (Figure 3.3.1.3.20).

As similarly observed for samples from data subset 1, while not significantly different, a reduction in the number of mucous cells and decrease in villi length, and thickness of the submucosa and muscle and serosa was observed in the hindgut tissue sections from disease compared to healthy samples (Figure 3.3.1.3.20). Samples from the wild charter fish also displayed a pattern of longer villi and thicker submucosa, muscle and serosa compared to those from the disease fish (Figure 3.3.1.3.20).

Global community structure, SIMPER analysis and histological parameters – gut data subset 3

For data subset 3 ('same feed manufacturer, site and year class'), the feed manufacturer (B), site (Point Boston) and year class (2017) were constant, while the feed type (6 mm or 9 mm pellet diameter) and fish size (healthy 0.8-1.2 kg, disease 0.5-0.6 kg) were variable. For the two healthy samples from sea-cages 17-2 and 17-3 and the two disease samples from sea-cages 17-2 and 17-3, there was no significant difference in the global bacterial community structure (Figure 3.3.1.3.21, Table 3.3.1.3.22) or any of the histological parameters measured from these fish (Figure 3.3.1.3.22).

In comparing healthy and disease samples from each of the two sea-cage locations, the global bacterial community structure of healthy vs disease fish at sea-cage 17-2 was not significantly different, though in the histology samples a greater villi length was recorded in heathy compared to disease fish (Figure 3.3.1.3.22). In contrast, for sea-cage 17-3, the global bacterial community structure was significantly different (Figure 3.3.1.3.21, Table 3.3.1.3.22), though the histological parameters were the same (Figure 3.3.1.3.22). For sea-cage 17-3, a greater contribution and increased abundance of OTUs most closely related to *Vibrio harveyi*/*Aliivibrio fischeri* (OTU 10, S_ab score 1.000) and *Photobacterium* sp. (OTU

10950, S_ab score 0.875) was observed in the disease compared to healthy samples (Table 3.3.1.3.23). Alongside this was a decreased in abundance of two OTUs with closest sequence similarity to *Vibrio* sp. V776/*A. finisterrensis* (OTU 4, S_ab score 1.000) and *M. insons* (OTU 1, S_ab score 0.428) in the disease samples (Table 3.3.1.3.23).

As similarly observed for samples from data subsets 1 and 2, while not significantly different, a reduction in the number of mucous cells and decrease in villi length, and thickness of the submucosa and muscle and serosa was observed in the hindgut tissue sections from disease compared to healthy samples (Figure 3.3.1.3.22). Samples from the wild charter fish also displayed a pattern of longer villi and thicker submucosa, muscle and serosa compared to those from the disease fish (Figure 3.3.1.3.22).

Common taxa occurring in disease fish (irrespective of other factors)

In assessing for common taxa occurring among disease samples in general, a single OTU with closest taxonomic similarity to *Photobacterium* sp. (OTU 10950, S_ab score 0.875) was observed to be one of the top 5 contributors in all disease samples across the sites (i.e. Northern Site, Arno Bay, Point Boston or Bickers) (Figure 3.3.1.3.23). This organism was also found in samples from healthy individuals at all sites and sea-cages, however, occurred at a much lower abundance compared to those from disease fish (Table 3.3.1.3.24).

Global community structure – skin and gills of healthy and disease fish

For the skin and gill datasets (2016 samples combined with 2017 extension samples) there was a significant difference between healthy and disease samples (PERMANOVA $P = 0.0038$ skin; and $P = 0.0287$ gill, table not shown) with some level of clustering evident in the nMDS plots (Figure 3.3.1.3.24). Therefore as observed for the gut, on a global community structural level, the skin and gill microbiome (bacterial assemblages) of healthy fish are significantly different to those from diseased fish and is consistent with the findings reported for the student extension component on this work; who also reported broad differences between the skin and gill communities, and the enrichment of select taxa belonging to the *α-proteobacteria*, *γ-proteobacteria* and *Actinobacteria* at early (acute) stages of disease (see Section 4).

Discussion

This work aimed to assess the impacts of changing health status on the microbiome (bacterial assemblages) of YTK and to identify the involvement of microbiome changes (or select bacterial constituents) in enteritis and coccidiosis. Samples were collected on-farm from fish displaying symptoms of an enteritis-like condition and coccidiosis infection. While coccidiosis samples were collected from a single sea-cage site at one time point, samples for the more commonly occurring enteritis-like condition were collected over two consecutive years at multiple sites with fish fed different diet formulations to further explore the potential drivers (i.e. diet, environment, fish size and year class) which may contribute to this condition. Gut scraping samples were collected from disease individuals alongside healthy controls for both disease conditions, with skin and gill swabs and hindgut histology sections also collected and analysed for the enteritis cohort. Compositional comparisons were evaluated in relation to those of the surrounding seawater to determine if environmentally-independent gut community assemblages are selected for in the gut. Overall, the global bacterial community composition between the environmental samples (surrounding seawater) and the gut samples was markedly different, validating that YTK are able to regulate and maintain their own environmentally-independent bacterial communities in the gut (as also reported in Manuscripts 3.3.1.1 and 3.3.1.2).

Enteritis disease in YTK

On a global bacterial community level, the gut microbiome of healthy and disease enteritis fish was significantly different, with a significant decrease in species diversity and evenness recorded for the enteritis disease fish compared to the healthy fish. This loss in diversity is characteristic of a dysbiotic state with reduced functionality, as there is no longer the diverse suite of bacteria present that are able to perform all the required tasks, such as breaking down of food compounds, development and training of the immune system, metabolism of therapeutics, resistance to pathogens and biosynthesis of vitamins and amino acids (Alonso and Guarner, 2013; Montalban-Arques et al., 2015; Valdes et al., 2018). General patterns, although not significantly different (small sample size, three healthy and three disease fish sampled per sea-cage), were also observed in the histological parameters between the healthy and disease enteritis fish. In particular, disease fish were characterised by a reduction in the number of mucous cells and decrease in villi length, thickness of submucosa and thickness of muscle and serosa in hindgut prepared slides. The mucus is one of the most important innate defense mechanisms present at mucosal surfaces (Gomez et al., 2013), therefore a reduction in the number of cells that produce this compound, as observed in the disease cohort, can lead to greater susceptibility to pathogenic organisms. Differences in the skin and gill global bacterial community structure was also recorded between healthy and enteritis disease fish, suggesting a body-wide effect in response to underlying gut health changes, as also observed by Legrand et al. (2018). For the 2016 dataset, where healthy intermediate fish were also sampled, there was no significant differences between the healthy intermediate and diseased fish in regards to global bacterial community structure and diversity indices (e.g. total species richness, diversity [Simpson, Shannon, delta+] and evenness [Pielou's, lambda+]), although a decrease in villi length, thickness of the submucosa, muscle and serosa was observed in the disease compared to the healthy intermediate fish from the foregut, midgut and hindgut prepared slides. Histological abnormalities away from the norm are clearly evident in the enteritis disease cohort, further validated by comparisons with the wild charter fish in which number of mucous cells, villi length and submucosa, muscle and serosa thickness were also lower in the disease samples. These findings, particularly for the healthy intermediate group, highlights the progression of the disease condition, as although fish might not be displaying clear symptomatic features of the disease (hence classed as 'healthy intermediate'), their associated gut microbiota (e.g. global level patterns and measures of diversity) and tissue integrity (e.g. reduction in number of mucous producing cells, decrease in villi length, thinner submucosa, muscle and serosa) already presents the diseased phenotype. Whether recovery from this healthy intermediate state is possible is worth investigating, as if therapeutic strategies (e.g. probiotic supplementation) could be applied to revert the gut microbiome back to a healthy state where diversity is subsequently recovered, fish losses from this intermediate group, presenting no visual symptoms but an internal disease phenotype, may be avoided. In addition, for some of the sea-cages sampled as part of the 2017 enteritis extension component, there was no significant difference in the global community structure between healthy and disease fish (e.g Bi 16-4GS, PB 17-2 and NS 17-8). Unlike the 2016 sampling, whereby one healthy control sea-cage was sampled alongside a second, distinct disease sea-cage, which included a mixed cohort of intermediate and disease fish, the 2017 sampling of healthy and disease individuals were taken from the same sea-cage. Therefore, for the three 2017 sea-cages (e.g Bi 16-4GS, PB 17-2 and NS 17-8) where no significant difference in global community structure was recorded, may indicate samples of healthy intermediate and disease fish were collected (rather than healthy and disease fish), with the healthy intermediate fish not displaying any clear signs or symptoms of disease (hence originally classed as healthy), yet presenting with a gut microbiome diseased phenotype, as observed for the 2016 healthy intermediate fish. Furthermore, average abundance of the dominant OTU 10950 (*Photobacterium* sp., similarity [S_ab] score 0.875) in these 2017 'healthy' fish was similar to the abundance recorded in the disease fish (e.g. 8.46 in 'healthy' vs 9.84 in disease for Bi 16-4GS, Table 3.3.1.3.24), whereas when a significant difference was recorded in the global community structure of the 2017 healthy vs disease fish (e.g. as observed for sea-cage Bi 16-3A), average abundance of OTU 10950 was disparate (e.g. 0.35 in healthy vs 17.21 in disease, Table 3.3.1.3.24). This supports the use of the gut microbiome, or development of specific and rapid assays based on these findings, to be used as early-detection markers of changing health status in YTK on-farm.

Differences were also recorded between healthy and disease enteritis fish at the bacterial phyla and taxa level. Healthy samples had greater taxa representation from a range of phyla, whereas a common property in the disease samples was dominance by one or a few bacterial phyla and taxa. Dominance is commonly linked to a dysbiotic state, whereby functionality has been lost (Heiman and Greenway, 2016). Only one taxa, with closest sequence similarity to *Photobacterium* sp. (OTU 10950, S_ab score 0.850), was recorded and enriched in all the enteritis disease samples collected, irrespective of sea-cage site, fish size, feed type, feed manufacturer and year class. *Photobacterium* sp. are known to cause disease in marine fish including *Seriola* sp., with *P. damselae* subsp. *piscicida* recorded as the causative agent of pseudotuberculosis in Japanese Yellowtail (*S. quinqueradiata*) cultures in Japan (Romalde, 2002), although other species, such as *P. leiognathi*, are known to be symbionts of marine fish with no reports of pathology or disease (Bannister and Parker, 1985; Dunlap et al., 2012). More detailed taxonomic information is needed to ascertain whether this *Photobacterium* sp. (OTU 10950) is related to other pathogenic species, and whether it is a causative agent of enteritis disease or an opportunistic pathogen able to proliferate under the disease conditions.

Other taxa were observed as dominant or with a greater contribution to the enteritis disease group, however this varied with year class, site, feed type, feed manufacturer and fish size. This included taxa with greatest sequence similarity to *Mycoplasma insons* (OTU 1, S_ab score 0.420), *Brevinema andersonii* (OTU 25, S_ab score 0.700), *Vibrio* sp. V776/*Aliivibrio finisterrensis* (OTU 4, S_ab score 1.000), *V. harveyi*/*A. fischeri* (OTU 10, S_ab score 1.000), *Pseudoalteromonas atlantica* (OTU 37, S_ab score 1.000) and *Synechococcus* sp. (OTU 32, S_ab score 1.000), although the first three taxa (*M. insons*, *B. andersonii* and *Vibrio* sp. V776/*A. finisterrensis*) were also recorded in some healthy individuals at certain sites. Note again that the classification of either 'healthy' or 'disease' was performed based on visual symptomatic features in the field by Clean Seas veterinarian staff, therefore although classed as 'healthy', certain individuals in the population sampled may still have an underlying disease condition (and rather would be better classed as 'healthy intermediate'). For OTU 1, sequence similarity for the taxa classification to *M. insons* (class *Mollicutes*) was low (S_ab score 0.420), therefore without knowing a definitive identification, it is difficult to assess associated pathogenicity. Nonetheless, it was recorded with high abundance in the enteritis disease fish, suggesting that it is an important potentially opportunistic pathogen that requires further elucidation. Note that other taxa from the *Mollicutes* have been linked to disease in marine organisms, including cytopathic effects in Salmon (Emerson et al., 1979), gill disease in Tench (Kirchhoff et al., 1987) and systematic infection in Crayfish, Crabs and Shrimp (Nunan et al., 2005; Wang et al., 2005; Wang, 2011). Spirochetes, including *B. andersonii* (OTU 25, S_ab score 0.700), are free-living or host-associated bacteria, some of which are pathogenic to animals (Paster and Dewhirst, 2000). Taxa from this genus have also been recorded as predominant organisms in the intestinal tracts of other teleost's, including Rainbow Trout (*Oncorhynchus mykiss)* (Lyons et al., 2016) and Pufferfish (*Takifugu niphobles*) (Shiina et al., 2006), as well as in the intestinal mucosa of three species of Carp (Li et al., 2015). Although *A. finisterrensi* and *A. fischeri* (syn with *V. fischeri*) are not known to be pathogenic (Austin et al., 2005; Ruby et al., 2005; Beaz-Hidalgo et al., 2010), other *Vibrio* species have been reported to induce disease in a range of fish species (Schulze et al., 2006; Vanhove et al., 2015). In particular, *V. anguillarum* is the main causative agent of vibriosis, a deadly haemorrhagic septicaemic disease in fish, bivalves and crustaceans (Kusuda and Kawai, 1998; Frans et al., 2011). It is regarded as one of the most important bacterial pathogen affecting aquaculture worldwide and is responsible for severe economic losses in this industry (Kumar et al., 2008). Additionally, skin lesions, exophthalmia and mortalities recorded in cage-cultured Seabass (*Lates calcarifer*) in the Philippines was attributed to infection with *V. harveyi*, which has been described as an opportunistic pathogen that infects stressed fish (Tendencia, 2002). *Pseudoalteromonas atlantica* has previously been documented from Edible Crabs (*Cancer pagurus*), with extracellular products isolated from this bacteria found to have a potent effect leading to rapid paralysis and mortality in healthy edible crabs (Costa-Ramos and Rowley, 2003). Other *Pseudoalteromonas* species have been identified as virulent pathogens, however, some species are also being explored for their use in probiotic strains as they can synthesise biologically active compounds with antibacterial, algicidal, anti-algal and bacteriolytic properties (Schulze et al., 2006). These key bacterial taxa dominant in the enteritis disease samples may therefore be directly involved in the enteritis disease process, or opportunistic pathogens able to take hold under the disease conditions. Either way, they represent important, previously

unknown markers that could be used to infer shifts towards a dysbiotic state in YTK. Furthermore, alongside enrichment of specific bacterial taxa, there was also down-regulation of others in the enteritis disease samples, including *Photobacterium phosphoreum*/*P. iliopiscarium* (OTU 28, S_ab score 1.000), *Neorickettsia helminthoeca* (OTU 26, S_ab score 0.533) and *Anabaena cylindrica* (OTU 16, S_ab score 0.377; and OTU 12360, S_ab score 0.350). Further elucidation of these four bacterial taxa (in particular the classification of OTU 26, OTU 16 and OTU 12360 with low sequence similarities) would be beneficial in order to explore their potential for inclusion into probiotic supplementations, allowing for gut diversity to be restored and health outcomes to be improved.

Factors influencing the gut microbiome of enteritis disease fish

Although three discreet data subsets were analysed to explore for the potential factors (e.g. site [environment], fish size, feed type [pellet size], feed manufacturer [A, B or C] or year class/run) that influence the enteritis disease process, no consistent pattern was observed for all the factors, although site (environment) and year class (host genetics) appear to be primary drivers of change. Note that other authors have highlighted site (habitat), host phylogeny and trophic level as the most likely factors influencing the gut microbiota of fish (Sullam et al., 2012). In particular for our study, differences in the global bacterial community was observed in disease samples at different sites (e.g. Northern Site vs Bickers in subset 1, Bickers vs Arno Bay vs Northern Site and Bickers vs Point Boston vs Northern Site in subset 2), with no significant difference at the same site (e.g. subset 3, Point Boston). Similarly, differences in the global bacterial community was observed in disease samples of different year classes (e.g. subset 1 and subset 2), with no significant difference for the same year class (e.g. subset 3), although for the former, site was also different and for the latter, site was the same. Diet does not appear to be as an important driver, with differences observed between disease samples on the same feed type (e.g. subset 1 and subset 2, 9 mm pellet), but no difference when on different feed types (e.g. subset 3, 6mm vs 9mm pellet). Collectively this highlights the complexity of the underlying enteritis disease process, as no one individual taxa was the dominant constituent across all the disease samples (although *Photobacterium* sp. [OTU 10950, S_ab score 0.850] was found to be enriched in all the disease samples), and no single factor was universally identified as the sole driver for disease, although site and/or year class appear to play important roles.

Coccidiosis in YTK

When comparing the second disease, coccidiosis, with the 2016 enteritis dataset, the coccidiosis samples were found to cluster independently to the enteritis samples with a significant difference recorded in the global community structure, highlighting that the change in the gut microbiome is not the same between these two disease conditions. Coccidiosis disease samples were dominated by completely different phyla compared to the enteritis disease samples, with the former having a greater level of phyla diversity with representation from *Proteobacteria*, *Cyanobacteria*, *Fusobacteria* and *Firmicutes* while the latter were dominated by *Tenericutes*. Nonetheless, a reduction in species richness and significantly lower species diversity and evenness was observed in the coccidiosis disease samples compared to the healthy samples, indicting similarly to the enteritis disease fish, a dysbiotic state with loss in functionality for the disease samples. Furthermore, coccidiosis samples were characterised by an increase in four specific taxa, *Vibrio* sp. V776/*Aliivibrio finisterrensis* (OTU 4, S_ab score 1.000), *Anabaena cylindrica* (OTU 15, S_ab score 0.376), *V. tasmaniensis*/*V. pomeroyi* (OTU 31, S_ab score 1.000) and *Brevinema andersonii* (OTU 2, S_ab score 0.632). Down-regulation of nine taxa were also observed in the coccidiosis disease samples, including two *Synechococcus* species (OTU 29, S_ab score 0.987 and OTU 23, S_ab score 1.000), *Pseudoalteromonas* sp. (OTU 40, S_ab score 1.000), *Campylobacter hyointestinalis* subsp. *lawsonii* (OTU 44, S_ab score 0.673), *Pseudomonas veronii*/*P. azotoformans*/*P. chlororaphis* subsp. *aureofaciens* (OTU 14, S_ab score 1.000), *Cetobacterium somerae* (OTU 25, S_ab score 0.596) and three *Bacillus*/*Geobacillus* species (OTU 45, S_ab score 1.000; OTU 83, S_ab score 1.000 and OTU 234, S_ab score 0.977). *Bacillus* species are considered to be favourable taxa that aid in digestion, enhance the immune response, compete with potential

pathogens and produce inhibitory compounds (Merrifield et al., 2010; Romero et al., 2014). Some *Bacillus* species, along with lactic acid bacteria (*Carnobacterium* sp., *Lactobacillus* sp., *Lactococcus* sp., *Streptococcus* sp., *Weissella* sp.), *Pseudomonas* sp. (*P. flurescens*) and *Vibrio* sp. (*V. alginolyticus, V. salmonicida*-like) have been applied as probiotics in aquaculture to improve aquatic animal growth, survival and health (Schulze et al., 2006; Pérez et al., 2010). Ultimately this reduction in microbiome diversity and loss of key taxa involved in nutritional and defense mechanisms can lead to an overall loss of function for the fish.

The need for appropriate age and size class controls

Age and size class appropriate controls were once again highlighted as important considerations when analyzing the gut microbiome of YTK. This was validated in three separate instances with the healthy vs disease dataset: 1) healthy before samples from two sea-cages with a different global microbiome structure when size classes were different (e.g. 200 g vs 800 g and 600 g vs 1400 g), but not when size classes were similar (600 g vs 800 g). Additionally for these two similar size classes (600 g vs 800 g), although feed type was different (pellet size 4mm vs 9mm), a similar microbiome structure was observed, suggesting fish size rather than pellet size (diet) as an important driver of changes to the gut microbiome; 2) healthy fish in both the enteritis and coccidiosis datasets characterised by a significantly different microbiome composition, with the coccidiosis fish being much smaller than enteritis fish; and 3) no significant difference in the global community structure of healthy fish from the same site (Bickers, three sea-cages 15-002B, 16-4GS and 16-3A) and fish size (~3.5 kg) even though they were of different year classes (2015 vs 2016), sampled through time (15-002B sampled in 2016, 16-4GS and 16-3A sampled in 2017) and on feeds from three distinct manufacturers (A, B or C).

Conclusion and Recommendation

The general properties of coccidiosis and enteritis and its influence on the microbiome were established, including that a shift in the global community structure occurs and is associated with a significant reduction in species richness, diversity and evenness and is accompanied by the dominance of one or more select taxa. Diminished microbiomes are likely to be associated with a loss of overall functionality which may have consequences to the health and fitness of the animal. A general trend of loss of mucous cells, decrease in villi length and thinner submucosa, muscle and serosa was observed in fish with enteritis, along with a change in epithelial and gill microbiota with changing gut health status. This suggests that underlying diseases of the gut are likely to cause body-wide microbiome changes in the outer surfaces, the consequences of which are unknown, though may led to weakened barrier functions that may make the fish more susceptible to secondary infections. As a loss of tissue integrity from skin histology samples was also noted in the student extension component of this work (see Section 4), such a scenario seems likely and the pathophysiology of the condition should be investigated. These disease conditions appear to be complex and multifactorial, as no single factor was observed to account for the changes in the microbiome in fish with enteritis, with age/size class- appropriate controls required for further studies. As part of the student extension components of this work (see Section 4), the utility of more advanced omics-based procedures that have the capacity to delineate the underlying functional genes expressed under these conditions from both the microbiome and the host are likely to improve our understanding of these complex conditions, which may include the relevance of other non-bacterial taxa and if host immune deficiency. Further characterisation and elucidation of the involvement of specific taxa universally identified across the disease samples (namely an unidentified *Photobacterium* species) is recommended, as this was the only organism that was found to be enriched in all samples irrespective of site, fish size, year class or feed type, and hence may be specific to the disease condition or represent an opportunistic pathogen. The occurrence of other prevalent and dominant taxa from samples in the 2016 evaluations of gut enteritis also require further investigation. This includes the relevance of *Mycoplasma*, which is related to other known pathogenic bacteria. No significant differences were observed between gut regions in the 2016 healthy vs disease and coccidiosis samples, therefore future work should be directed at taking a single hindgut scraping, which would allow for

more samples to be processed at the same cost. This would increase the capacity to sample across multiple sea-cages, seasons and sites to provide a greater overview of farm-wide changes in health status. Using the new catalogue of bacterial taxa across different health conditions generated here, early-detection biomarkers of changing health status for coccidiosis and enteritis infections could be established using more targeted, rapid and cost-effective tools (e.g. q-PCR). With notable changes occurring in the skin of diseased fish (see Section 4) occurring in early stages of disease where fish are asymptomatic, such tools could be implemented non-invasively by taking swabs of the skin, and could be implemented as part of routine health surveys for the early detection of disease. This would facilitate better health assessment and aid in implementing timelier intervention/management strategies. In addition, further efforts should be directed to the involvement and replenishment of organisms that may be of benefit to the host (e.g. *Bacillus* species) that were otherwise diminished in the microbiomes of YTK with coccidiosis. Development of strategies which promote broader microbial diversity in the gut of fish are particularly warranted as they are most likely to improve the robustness of the fish to potentially opportunistic pathogens. The results outlined in this manuscript serve as a resource for further improving our understanding of disease in farmed YTK.

Findings

This component of work found marked differences in the global community structure, taxonomic composition and species richness, evenness and diversity between healthy and diseased YTK. Fish with an underlying enteritis-like condition presented a different gut microbiome composition to those displaying symptoms of coccidiosis, but both diseases were characterised by dominance of one or more bacterial taxa, a reduction in species richness, and significant decrease in diversity and evenness. These are features indicative of a dysbiotic state whereby functionality is lost. A general trend of loss of mucous cells, decrease in villi length and thinner submucosa, muscle and serosa was observed for the enteritis-like fish, along with a change in the epithelial and gill microbiota with changing gut health status. This was supported in the findings from the student extenstion component of this work (Section 4), which revealed that these changes are most notable in the early stages of disease where the host is asymptomatic and is accompanied by a loss of barrier integrity as associated with a decrease in skin thickness and the number of mucous cells. One taxon, with closest similarity to *Photobacterium* sp. (OTU 10950, S_ab score 0.875), was found to be enriched in all enteritis samples, irrespective of other factors (i.e. diet, site/environment, fish size and year class), highlighting that it may be specific to the disease condition or an opportunistic pathogen that is able to proliferate under these conditions. Other taxa with greatest relative contribution to the enteritis-like condition or coccidiosis infection, were reported, along with potential favourable organisms (including *Bacillus* species) which were notably decreased in abundance in the coccidiosis disease samples. Although multiple factors were explored to investigate their influence on the gut microbiome of healthy and diseased enteritis fish, site (environment) and/or year class (host genetics) appear to be the key drivers.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit program, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds. The authors would also like to acknowledge the support of the South Australian Research and Development Institute (SARDI) and

provision of the Molecular Laboratory facilities at West Beach, South Australia. Thanks to Dr Trent D'Antignana of Nutrisea, Dr Jay Dent and Dr Erin Bubner of Clean Seas along with Clean Seas health team staff for coordinating and assisting with sample collections on-farm. Thanks to Dr Rebecca Forder, Cheryl Day and Dr Fran Stephens for histological slide preparation and evaluations, and Dr Melissa Wos-Oxley of Flinders University for supporting and guiding the statistical analyses.

References

Alonso, V.R., Guarner, F., 2013. Linking the gut microbiota to human health. British Journal of Nutrition 109, S21-S26.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology 26, 32-46.

Austin, B., 2004. The bacterial microflora of fish, revised. The Scientific World Journal 6, 931-945.

Austin, B., Austin, D., Sutherland, R., Thompson, F., Swings, J., 2005. Pathogenicity of vibrios to rainbow trout (*Oncorhynchus mykiss*, Walbaum) and *Artemia* nauplii. Environmental Microbiology 7 (9), 1488-95.

Bannister, J.V., Parker, M.W., 1985. The presence of a copper/zinc superoxide dismutase in the bacterium *Photobacterium leiognathi*: A likely case of gene transfer from eukaryotes to prokaryotes. Proceedings of the National Academy of Science USA 82, 149-152.

Bansemer, M.S., Forder, R.E.A., Howarth, G.S., Suitor, G.M., Bowyer, J., Stone, D.A.J., 2015. The effect of dietary soybean meal and soy protein concentrate on the intestinal mucus layer and development of subacute enteritis in Yellowtail Kingfish (*Seriola lalandi*) at suboptimal water temperature. Aquaculture Nutrition 21, 300-310.

Beaz-Hidalgo, R., Doce, A., Balboa, S., Barja, J.L., Romalde, J.L., 2010. *Aliivibrio finisterrensis* sp. nov., isolated from Manila clam, *Ruditapes philippinarum* and emended description of the genus *Aliivibrio*. International Journal of Systematic and Evolutionary Microbiology 60, 223-228.

Boutin, S., Bernatchez, L., Audet, C., Derôme, N., 2013. Network analysis highlights complex interactions between pathogen, host and commensal microbiota. PLoS ONE 8(12), e84772.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. Ecological monographs 27(4), 325-349.

Camarinha-Silva, A., Jáuregui, R., Chaves-Moreno, D., Oxley, A.P.A., Schaumburg, F., Becker, K., Wos-Oxley, M.L., Pieper, D.H., 2014. Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. Environmental Microbiology 16(9), 2939-2952.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7(5), 335-336.

Chaves-Moreno, D., Plumeier, I., Kahl, S., Krismer, B., Peschel, A., Oxley, A.P.A., Jauregui, R., Pieper, D.H., 2015. The microbial community structure of the cotton rat nose. Environmental Microbiology Reports 7(6), 929-935.

Clarke, K.R., Warwick, R.M.R.M., Laboratory, P.M., 2001. Change in marine communities: an approach to statistical analysis and interpretation: Plymouth, U.K.: PRIMER-E Ltd.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research* 42 (Database issue), D633-D642.

Colston, T.J., Jackson, C.R., 2016. Microbiome evolution along divergent branches of the vertebrate tree of life: what is known and unknown. Molecular Ecology 25, 3776-800.

Costa-Ramos, C., Rowley, A.F., 2004. Effect of extracellular products of *Pseudoalteromonas atlantica* on the edible crab *Cancer pagurus*. Applied and Environmental Microbiology 70(2), 729-735.

Cruz, P.M., Ibanez, A.L., Hermosillo, O.A.M., Saad, H.C.R., 2012. Use of probiotics in aquaculture. ISRN Microbiology, 1-13.

Dehler, C.E., Secombes, C.J., Martin, S.A.M., 2017. Environmental and physiological factors shape the gut microbiota of Atlantic salmon parr (*Salmo salar* L.). Aquaculture 467, 149-157.

Dittmann, K.K., Rasmusen, B.B., Castex, M., Gram, L., Bentzon-Tilla, M., 2017. The aquaculture microbiome at the centre of business creation. Microbial Biotechnology 10, 1279-1282.

Dunlap, P.V., Gould, A.L., Wittenrich, M.L., Nakamura, M., 2012. Symbiosis initiation in the bacterially luminous sea urchin cardinalfish *Siphamia versicolor*. Journal of Fish Biology 81, 1340-1356.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19), 2460-2461.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nature Methods 10, 996-998.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27(16), 2194-2200.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., Ross, R.P., 2018. The gut microbiota of marine fish. Frontiers in Microbiology 9, 1-17.

Emerson, M., Nicholson, B.L., Bayer, R., 1979. Effects of *Acholeplasma laidlawii* and an unidentified mycoplasma on selected fish cultures and the replication of fish viruses. Journal of Fish Diseases 2(3), 227-238.

Federici, E., Rossi, R., Fidati, L., Paracucchi, R., Scargetta, S., Montalbani, E., Franzetti, A., La Porta, G., Fagotti, A., Simoncelli, S., Cenci, G., Di Rosa, I., 2015. Characterization of the skin microbiota in Italian stream frogs (*Rana italica*) infected and uninfected by a cutaneous parasitic disease. Microbes and Environment 30, 262-269.

Frans, I., Michiels, C.W., Bossier, P., Willems, K.A., Lievens, B., Rediers, H., 2011. *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. Journal of Fish Disease 34, 643-661.

Gestal, C., Azevedo, C., 2006. Ultrastructural aspects of hepatic coccidiosis caused by *Goussia lusca* n. sp. (Apicomplexa: Coccidia) infecting *Trisopterus luscus* (Gadidae) from the NE Atlantic Ocean. Diseases of Aquatic Organisms 71, 25-31.

Ghanbari, M., Kneifel, W., Domig, K.J., 2015. A new view of the fish gut microbiome: Advances from next-generation sequencing. Aquaculture 448, 464-475.

Gomez, D., Sunyer, J.O., Salinas, I., 2013. The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. Fish Shellfish Immunology 35(6), 1729-1739.

Heiman, M.L., Greenway, F.L., 2016. A healthy gastrointestinal microbiome is dependent on dietary diversity. Molecular Metabolism 5, 317-20.

Hoseinifar, S.H., Esteban, M.A., Cuesta, A., Sun, Y., 2015. Prebiotics and fish immune responses: a review of current knowledge and future perspectives. Reviews in Fisheries Science and Aquaculture 23, 315-328.

Kirchhoff, H., Beyene, P., Fischer, M., Flossdorf, J., Heitmann, J., Khattab, B., Lopatta, D., Rosengarten, R., Seidel, G., Yousef, C., 1987. *Mycoplasma mobile* sp. nov., a new species from fish. International Journal of Systematic Bacteriology 37(3), 192-197.

Kusuda, R., Kawai, K., 1998. Bacterial diseases of cultured marine fish in Japan. Fish Pathology 33(4), 221-227.

Kumar, S.R., Ahmed, V.P.I., Sudhakaran, R., Babu, V.S., Hameed, A.S.S. (2008). Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in Asian sea bass (*Lates calcarifer*) to protect from *Vibrio (Listonella*) *anguillarum.* Fish and Shellfish Immunology 25, 47-56.

Legrand, T.P.R.A., Catalano, S.R., Wos-Oxley, M.L., Stephens, F., Landos, M., Bansemer, M.S., Stone, D.A.J., Qin, J.G., Oxley, A.P.A., 2018. The inner workings of the outer surface: skin and gill microbiota as indicators of changing gut health in Yellowtail Kingfish. Frontiers in Microbiology 8, 2664.

Leonard, A.B., Carlson, J.M., Bishoff, D.E., Sendelbach, S.I., Yung, S.B., Ramzanali, S., Manage, A.B.W., Hyde, E.R., Petrosino, J.F., Primm, T.P., 2014. The skin microbiome of *Gambusia affinis* is defined and selective. Advances in Microbiology 4, 335-343.

Li, T., Long, M., Gatesoupé, Zhang, Q., Li, A., Gong, X., 2015. Comparative analysis of the intestinal bacterial communities in different species of carp by pyrosequencing. Microbiology of Aquatic Systems 69, 25-36.

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H., Derome, N., 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Frontiers in Microbiology 5, 1-17.

Lyons, P.P., Turnbull, J.F., Dawson, K.A., Crumlish, M., 2016. Phylogenetic and functional characterization of the distal intestinal microbiome of rainbow trout *Oncorhynchus mykiss* from both farm and aquarium settings. Journal of Applied Microbiology 122, 347-363.

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bøgwald, J., Castex, M., Ringø, E., 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture 302, 1-18.

Miranda, C.D., Godoy, F.A., Lee, M.R., 2018. Current status of the use of antibiotics and the antimicrobial resistance in the Chilean Salmon farms. Frontiers in Microbiology 9, 1284.

Montalban-Arques, A., De Schryver, P., Bossier, P., Gorkiewicz, G., Mulero, V., Gatlin, D.M., Galindo-Villegas, J., 2015. Selective manipulation of the gut microbiota improves immune status in vertebrates. Frontiers in Immunology 6, 1-14.

Nakada, M., 2008. Capture-based aquaculture of yellowtail. In A. Lovatelli, P.F. holthus (eds). Capture-based aquaculture. Global overview. FAO Fisheries Technical Paper. No. 508. Rome, FAO, pp. 199-215.

Nayak, S.K., 2010. Role of gastrointestinal microbiota in fish. Aquaculture Research 41, 1553-1573.

Nunan, L.M., Lightner, D.V., Oduori, M.A., Gasparich, G.E., 2005. *Spiroplasma penaei* sp. nov., associated with mortalities in *Penaeus vannamei*, Pacific white shrimp. International Journal of Systematic and Evolutionary Microbiology 55(6), 2317-2322.

Parris, D.J., Brooker, R.M., Morgan, M.A., Dixson, D.L., Stewart, F.J., 2016. Whole gut microbiome composition of damselfish and cardinalfish before and after reef settlement. PeerJ 4, e2412.

Paster, B.J., Dewhirst, F.E., 2000. Phylogenetic foundation of spirochetes. Journal of Molecular Microbiology and Biotechnology 2, 341-44.

Pérez, T., Balcázar, J.L., Ruiz-Zarzueka, I., Halaihel, N., Vendrell, D., de Blas, I., Múzquiz, J.L., 2010. Host-microbiota interactions within the fish intestinal ecosystem. Mucosal Immunity 3, 355-60.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41, D590-D596.

Romalde, J.L., 2002. *Photobacterium damselae* subsp. *piscicida*: an integrated view of a bacterial fish pathogen. International Microbiology 5(1), 3-9.

Romero, J., Ringø, E., Merrifield, D.L., 2014. 'The gut microbiota of fish', in D.L. Merrifield and E. Ringø (eds.), Aquaculture nutrition: gut health, probiotics and prebiotics (John Wiley & Sons, Ltd), 75-100.

Ruby, E.G., Urbanowski, M., Campbell, J., Dunn, A., Faini, M., Gunsalus, R., Lostroh, P., Lupp, C., McCann, J., Millikan, D., Schaefer, A., Stabb, E., Stevens, A., Visick, K., Whistler, C., Greenberg, E.P., 2005. Complete genome sequence of *Virbio fischeri*: a symbiotic bacterium with pathogenic congeners. PNAS 102(8), 3004-3009.

Schulze, A.D., Alabi, A.O., Tattersall-Sheldrake, A.R., Miller, K.M., 2006. Bacterial diversity in a marine hatchery: balance between pathogenic and potentially probiotic bacterial strains. Aquaculture 256, 50-73.

Sheppard, M.E., 2005. Aquatic Animal Health Subprogram: Detection and management of health issues in yellowtail kingfish (YTK, *Seriola lalandi*) – the foundation for a health program for Australian finfish aquaculture. FRDC Project No. 2003/216.

Shiina, A., Itoi, S., Washio, S., Sugita, H., 2006. Molecular identification of intestinal microflora in *Takifugu niphobles*. Comparative Biochemistry and Physiology, Part D 1, 128-132.

Sullam, K.E., Essinger, S.D., Lozupone, C.A., O'Connor, M.P., Rosen, G. L., Knight, R., Kilham, S.S., Russell, J.A., 2012. Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. Molecular Ecology 21, 3363-3378.

Sylvain, F-E., Cheaib, B., Llewellyn, M., Correia, T.G., Fagundes, D.B., Val, A.L., Derôme, N., 2016. pH drop impacts differentially skin and gut microbiota of the Amazonian fish tambaqui (*Colossoma macropomum*). Nature Scientific Reports 6, 32032.

Szafranska, A.K., Oxley, A.P.A., Chaves-Moreno, D., Horst, S.A., Roßlenbrolch, S., Peters, G., Goldmann, O., Rohde, M., Sinha, B., Pleper, D.H., Löffler, B., Jauregul, R., Wos-Oxley, M.L., Medina, E., 2014. High-resolution transcriptomic analysis of the adaptive responses of *Staphylococcus aureus* during acute and chronic phases of osteomyelitis. mBio 5(6), e01775-14.

Tendencia, E., 2002. *Vibrio harveyi* isolated from cage-cultured seabass *Lates calcarifer* Bloch in the Philippines. Aquaculture Research 33, 455-458.

Valdes, A.M., Walter, J., Segal, E., Spector, T.D., 2018. Role of the gut microbiota in nutrition and health. BMJ 361, 36-44.

Vanhove, A.S., Duperthuy, M., Charrière, G.M., Le Roux, F., Goudenège, D., Gourbla, B., Kieffer-Jaquinod, S., Couté, Y., Wal, S.N., Destoumieux-Garzón, D., 2015. Outer membrane vesicles are vehicles for the delivery of *Vibrio tasmaniensis* virulence factors to oyster immune cells. Environmental Microbiology 17(4), 1152-65.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* 73(16), 5261-5267.

Wang, W., 2011. Bacterial diseases of crabs: a review. Journal of Invertebrate pathology 106(1), 18-26.

Wang, W., Gu, W., Ding, Z., Ren, Y., Chen, J., Hou, Y., 2005. A novel Spiroplasma pathogen causing systemic infection in the crayfish *Procambarus clarkii* (Crustacea: Decapod), in China. FEMS Microbiology Letters 249, 131-137.

Wang, W., Zhou, Z., He, S., Liu, Y., Cao, Y., Shi, P., Yao, B., Ringø, E., 2010. Identification of the adherent microbiota on the gills and skin of poly-cultured gibel carp (*Carassius auratus gibelio*) and bluntnose black bream (*Megalobrama amblycephala* Yih). Aquaculture Research, 1-10.

Xia, J.H., Lin, G., Fu, G.H., Wan, Z.Y., Lee, M., Wang, L., Liu, X.J., Yue, G.H., 2014. The intestinal microbiome of fish under starvation. BMC Genomics 15, 1-12.

Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30(5), 614-20.

Zhang, M., Sun, Y., Liu, Y., Qiao, F., Chen, L., Liu, W-T, Du, Z., Li, E., 2016. Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference. Aquaculture 454, 72.

Table 3.3.1.3.1. Sample information pertaining to the 2016 Healthy vs Disease Enteritis component.

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
water sample	-	-	-	Lincoln	Bickers	15-002B	3/03/2016	109820	904
water sample	-	-	-	Lincoln	Northern site 1	15-004A	3/03/2016	122248	1086
Healthy	HG	61	4000	Lincoln	Bickers	15-002B	3/03/2016	28374	15
Healthy	MG	61	4000	Lincoln	Bickers	15-002B	3/03/2016	17439	22
Healthy	FG	60	3500	Lincoln	Bickers	15-002B	3/03/2016	3344	32
Healthy	HG	60	3500	Lincoln	Bickers	15-002B	3/03/2016	34729	92
Healthy	MG	60	3500	Lincoln	Bickers	15-002B	3/03/2016	28209	72
Healthy	FG	63	3700	Lincoln	Bickers	15-002B	3/03/2016	22682	74
Healthy	HG	63	3700	Lincoln	Bickers	15-002B	3/03/2016	25900	18
Healthy	MG	63	3700	Lincoln	Bickers	15-002B	3/03/2016	27311	59
Healthy	FG	61	3360	Lincoln	Bickers	15-002B	3/03/2016	24762	42
Healthy	HG	61	3360	Lincoln	Bickers	15-002B	3/03/2016	17575	20
Healthy	MG	61	3360	Lincoln	Bickers	15-002B	3/03/2016	34230	33
Healthy	FG	57	2480	Lincoln	Bickers	15-002B	3/03/2016	19468	35
Healthy	HG	57	2480	Lincoln	Bickers	15-002B	3/03/2016	40315	79
Healthy	MG	57	2480	Lincoln	Bickers	15-002B	3/03/2016	32098	78
Healthy	FG	61	3400	Lincoln	Bickers	15-002B	3/03/2016	42208	25
Healthy	HG	61	3400	Lincoln	Bickers	15-002B	3/03/2016	38672	20
Healthy	MG	61	3400	Lincoln	Bickers	15-002B	3/03/2016	25961	27
Healthy	FG	63	3650	Lincoln	Bickers	15-002B	3/03/2016	11849	35
Healthy	HG	63	3650	Lincoln	Bickers	15-002B	3/03/2016	16811	76
Healthy	FG	56	2300	Lincoln	Bickers	15-002B	3/03/2016	1408	31
Healthy	MG	56	2300	Lincoln	Bickers	15-002B	3/03/2016	11134	30
Healthy	FG	61	3330	Lincoln	Bickers	15-002B	3/03/2016	3391	33
Healthy	MG	61	3330	Lincoln	Bickers	15-002B	3/03/2016	18593	85
Healthy	FG	68	4600	Lincoln	Bickers	15-002B	3/03/2016	54563	37
Healthy	HG	68	4600	Lincoln	Bickers	15-002B	3/03/2016	40013	18
Healthy	MG	68	4600	Lincoln	Bickers	15-002B	3/03/2016	48970	28
Healthy	FG	63	3420	Lincoln	Bickers	15-002B	3/03/2016	15223	41
Healthy	HG	63	3420	Lincoln	Bickers	15-002B	3/03/2016	69595	24
Healthy	MG	63	3420	Lincoln	Bickers	15-002B	3/03/2016	63368	50
Healthy	FG	63	3550	Lincoln	Bickers	15-002B	3/03/2016	52809	50
Healthy	HG	63	3550	Lincoln	Bickers	15-002B	3/03/2016	41268	22
Intermediate	HG	58.5	3080	Lincoln	Northern site	15-004A	3/03/2016	45424	43
Intermediate	HG	54	2250	Lincoln	Northern site	15-004A	3/03/2016	32025	71
Intermediate	FG	58	3000	Lincoln	Northern site	15-004A	3/03/2016	34864	62
Intermediate	HG	58	3000	Lincoln	Northern site	15-004A	3/03/2016	50659	51
Intermediate	MG	58	3000	Lincoln	Northern site	15-004A	3/03/2016	26196	26
Intermediate	FG	59	2910	Lincoln	Northern site	15-004A	3/03/2016	47991	30
Intermediate	HG	59	2910	Lincoln	Northern site	15-004A	3/03/2016	41329	44
Intermediate	MG	59	2910	Lincoln	Northern site	15-004A	3/03/2016	49749	79
Intermediate	FG	59	2800	Lincoln	Northern site	15-004A	3/03/2016	55558	104
Intermediate	HG	59	2800	Lincoln	Northern site	15-004A	3/03/2016	54896	47
Intermediate	MG	59	2800	Lincoln	Northern site	15-004A	3/03/2016	44658	86
Intermediate	FG	61	3270	Lincoln	Northern site	15-004A	3/03/2016	51259	114
Intermediate	HG	61	3270	Lincoln	Northern site	15-004A	3/03/2016	27450	50
Intermediate	MG	61	3270	Lincoln	Northern site	15-004A	3/03/2016	42375	44
Intermediate	FG	64	2600	Lincoln	Northern site	15-004A	3/03/2016	40718	153
Intermediate	HG	64	2600	Lincoln	Northern site	15-004A	3/03/2016	54641	60
Intermediate	HG	62	3440	Lincoln	Northern site	15-004A	3/03/2016	38690	78
Intermediate	MG	62	3440	Lincoln	Northern site	15-004A	3/03/2016	33189	56
Intermediate	HG	60	3250	Lincoln	Northern site	15-004A	3/03/2016	14278	45
Intermediate	MG	60	3250	Lincoln	Northern site	15-004A	3/03/2016	21196	77

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Intermediate	HG	59.5	3100	Lincoln	Northern site	15-004A	3/03/2016	60012	118
Intermediate	FG	62	2890	Lincoln	Northern site	15-004A	3/03/2016	55169	138
Intermediate	HG	62	2890	Lincoln	Northern site	15-004A	3/03/2016	78267	95
Intermediate	FG	59	3000	Lincoln	Northern site	15-004A	3/03/2016	49702	35
Intermediate	HG	59	3000	Lincoln	Northern site	15-004A	3/03/2016	47510	76
Disease	FG	47	1070	Lincoln	Northern site	15-004A	3/03/2016	37249	94
Disease	HG	47	1070	Lincoln	Northern site	15-004A	3/03/2016	39580	137
Disease	MG	47	1070	Lincoln	Northern site	15-004A	3/03/2016	27825	67
Disease	FG	44.5	1370	Lincoln	Northern site	15-004A	3/03/2016	23678	43
Disease	HG	44.5	1370	Lincoln	Northern site	15-004A	3/03/2016	41671	78
Disease	FG	47	1160	Lincoln	Northern site	15-004A	3/03/2016	16023	61
Disease	HG	47	1160	Lincoln	Northern site	15-004A	3/03/2016	39398	26
Disease	MG	47	1160	Lincoln	Northern site	15-004A	3/03/2016	32819	70
Disease	FG	55	1700	Lincoln	Northern site	15-004A	3/03/2016	32864	40
Disease	HG	55	1700	Lincoln	Northern site	15-004A	3/03/2016	14338	20
Disease	MG	55	1700	Lincoln	Northern site	15-004A	3/03/2016	30674	30
Disease	FG	50	1200	Lincoln	Northern site	15-004A	3/03/2016	39041	76
Disease	HG	50	1200	Lincoln	Northern site	15-004A	3/03/2016	70331	80
Disease	MG	50	1200	Lincoln	Northern site	15-004A	3/03/2016	39887	31
Disease	FG	46	1540	Lincoln	Northern site	15-004A	3/03/2016	34182	35
Disease	HG	46	1540	Lincoln	Northern site	15-004A	3/03/2016	46790	26
Disease	MG	46	1540	Lincoln	Northern site	15-004A	3/03/2016	44457	118
Disease	FG	47.5	1250	Lincoln	Northern site	15-004A	3/03/2016	31741	27
Disease	HG	47.5	1250	Lincoln	Northern site	15-004A	3/03/2016	44271	53
Disease	MG	47.5	1250	Lincoln	Northern site	15-004A	3/03/2016	41643	43
Disease	HG	49	1480	Lincoln	Northern site	15-004A	3/03/2016	43962	47
Disease	MG	49	1480	Lincoln	Northern site	15-004A	3/03/2016	25201	57
Disease	FG	52	1250	Lincoln	Northern site	15-004A	3/03/2016	36456	494
Disease	HG	52	1250	Lincoln	Northern site	15-004A	3/03/2016	24953	123
Disease	MG	52	1250	Lincoln	Northern site	15-004A	3/03/2016	63882	172
Disease	FG	54	1410	Lincoln	Northern site	15-004A	3/03/2016	46284	95
Disease	HG	54	1410	Lincoln	Northern site	15-004A	3/03/2016	48912	110
Disease	MG	54	1410	Lincoln	Northern site	15-004A	3/03/2016	35209	73
Disease	FG	50	1710	Lincoln	Northern site	15-004A	3/03/2016	22088	167
Disease	HG	50	1710	Lincoln	Northern site	15-004A	3/03/2016	15487	54

Abbreviations: FG, foregut; HG, hindgut; MG, midgut.

Table 3.3.1.3.2. Summary of sequenced sample parameters for 2016 Healthy vs Disease enteritis component.

Data-set	No. of samples	Total reads	Median library size	Range	Bacterial OTUs post- filtering
2016 Health vs Disease	89	3,348,468	37,960	1,408-122,248	511

Table 3.3.1.3.3. One-way PERMANOVA: Pairwise test between the three health groups.[1]

Health group	*P*	Significant?
H, HI	**0.0001**	Yes
H, D	**0.0001**	Yes
HI, D	0.3107	No

Abbreviations: D, disease; H, healthy; HI, healthy intermediate.
[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.3.4. ANOVA results for diversity indices comparing the three health groups; healthy, healthy intermediate and disease.

Diversity measure	ANOVA summary[1]	Tukey's posthoc test	Adjusted *P*-value
Species richness (S)	F=6.678		
	***P*=0.0020**		
		H vs HI	**0.0374**
		H vs D	**0.0019**
		HI vs D	0.6492
Species evenness (J')	F=13.01		
	***P*<0.0001**		
		H vs HI	**0.0007**
		H vs D	**<0.0001**
		HI vs D	0.7318
Shannon's diversity (H')	F=7.806		
	***P*=0.0008**		
		H vs HI	**0.0115**
		H vs D	**0.0011**
		HI vs D	0.8062
Simpson's diversity (1-λ)	F=7.048		
	***P*=0.0015**		
		H vs HI	**0.0309**
		H vs D	**0.0014**
		HI vs D	0.6391
Delta+ (Δ+)	F=1.696		
	P=0.1897		
Lambda+ (λ+)	F=3.749		
	***P*=0.0276**		
		H vs HI	**0.0500**
		H vs D	0.0594
		HI vs D	0.9865

Abbreviations: D, disease; H, healthy; HI, healthy intermediate.
[1] Where a significant difference ($P < 0.05$) is observed then the Tukey's pairwise test was performed.

Table 3.3.1.3.5. Table ANOVA results for the foregut histology parameters comparing the wild fish ('wild', wild charter) with the three enteritis categories (healthy, healthy intermediate and disease).

Diversity measure	ANOVA summary[1]	Tukey's posthoc test[2]	Adjusted *P*-value
No. of mucous cells (100 µm)	F=6.051		
	***P*=0.0006**		
		WC vs H	**0.0007**
		WC vs D	**0.0234**
Length of villi (µm)	F=1.716		
	P=0.1649		
Thickness of submucosa (µm)	F=5.586		
	***P*=0.0011**		
		H vs HI	**0.0123**
		HI vs D	**0.0021**
Thickness of muscle & serosa (µm)	F=13.27		
	***P*<0.0001**		
		WC vs H	**<0.0001**
		WC vs HI	**<0.0001**
		WC vs D	**<0.0001**
No. of MMCs in submucosa	F=3.2		
	P=0.1808		

Abbreviations: D, disease; H, healthy; HI, healthy intermediate; MMC, melanomacrophage centre; WC, wild charter.
[1] Where a significant difference ($P < 0.05$) is observed then the Tukey's pairwise test was performed.
[2] Only significant pairwise comparisons are shown.

Table 3.3.1.3.6. ANOVA results for the midgut histology parameters comparing the wild fish ('wild', wild charter) with the three enteritis categories (healthy, healthy intermediate and disease).

Diversity measure	ANOVA summary[1]	Tukey's posthoc test[2]	Adjusted *P*-value
No. of mucous cells (100 µm)	F=5.167		
	***P*=0.0018**		
		WC vs H	**0.0011**
		WC vs HI	**0.0034**
		WC vs D	**0.0085**
Length of villi (µm)	F=8.595	W vs H	**0.0004**
	***P*<0.0001**	W vs HI	**0.0013**
		WC vs H	**0.0284**
		H vs D	**0.0050**
		HI vs D	**0.0258**
Thickness of submucosa (µm)	F=5.546		
	***P*=0.0012**		
		H vs HI	**0.0118**
		HI vs D	**0.0006**
Thickness of muscle & serosa (µm)	F=8.234		
	***P*<0.0001**		
		WC vs H	**0.0072**
		WC vs HI	**0.0058**
		WC vs D	**<0.0001**
No. of MMCs in submucosa	F=2.749		
	P=0.5076		

Abbreviations: D, disease; H, healthy; HI, healthy intermediate; MMC, melanomacrophage centre; W, wild; WC, wild charter.
[1] Where a significant difference ($P < 0.05$) is observed then the Tukey's pairwise test was performed.
[2] Only significant pairwise comparisons are shown.

Table 3.3.1.3.7. ANOVA results for the hindgut histology parameters comparing the wild fish ('wild', wild charter) with the three enteritis categories (healthy, healthy intermediate and disease).

Diversity measure	ANOVA summary[1]	Tukey's posthoc test[2]	Adjusted *P*-value
No. of mucous cells (100 µm)	F=3.801		
	***P*=0.0102**		
		WC vs H	**0.0340**
Length of villi (µm)	F=14.31		
	***P*<0.0001**		
		W vs H	**0.0250**
		W vs HI	**0.0437**
		WC vs D	**<0.0001**
		H vs D	**<0.0001**
		HI vs D	**<0.0001**
Thickness of submucosa (µm)	F=1.971		
	P=0.1180		
Thickness of muscle & serosa (µm)	F=24.84		
	***P*<0.0001**		
		W vs D	**0.0330**
		WC vs H	**<0.0001**
		WC vs HI	**<0.0001**
		WC vs D	**<0.0001**
		H vs D	**0.0124**
No. of MMCs in submucosa	F=1.236		
	P=0.3115		

Abbreviations: D, disease; H, healthy; HI, healthy intermediate; MMC, melanomacrophage centre; W, wild; WC, wild charter.
[1] Where a significant difference ($P < 0.05$) is observed then the Tukey's pairwise test was performed.
[2] Only significant pairwise comparisons are shown.

Table 3.3.1.3.8. Sample information pertaining to Gastrointestinal Health component (2016 gut enteritis healthy vs disease vs coccidiosis).

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
water sample	-	-	-	Lincoln	Bickers	15-002B	3/03/2016	99278	352
water sample	-	-	-	Lincoln	Northern site 1	15-004A	3/03/2016	114606	378
water sample	-	-	-	Arno Bay	AB3-AB Site 2	AB16-7	21/01/2016	92729	274
Enteritis - H	MG	61	4000	Lincoln	Bickers	15-002B	3/03/2016	28060	14
Enteritis - H	HG	61	4000	Lincoln	Bickers	15-002B	3/03/2016	17288	20
Enteritis - H	FG	60	3500	Lincoln	Bickers	15-002B	3/03/2016	3475	25
Enteritis - H	MG	60	3500	Lincoln	Bickers	15-002B	3/03/2016	35516	46
Enteritis - H	HG	60	3500	Lincoln	Bickers	15-002B	3/03/2016	28952	38
Enteritis - H	FG	63	3700	Lincoln	Bickers	15-002B	3/03/2016	24666	37
Enteritis - H	MG	63	3700	Lincoln	Bickers	15-002B	3/03/2016	25574	16
Enteritis - H	HG	63	3700	Lincoln	Bickers	15-002B	3/03/2016	28106	34
Enteritis - H	FG	61	3360	Lincoln	Bickers	15-002B	3/03/2016	24670	33
Enteritis - H	MG	61	3360	Lincoln	Bickers	15-002B	3/03/2016	17608	17
Enteritis - H	HG	61	3360	Lincoln	Bickers	15-002B	3/03/2016	34292	25
Enteritis - H	FG	57	2480	Lincoln	Bickers	15-002B	3/03/2016	19370	22
Enteritis - H	MG	57	2480	Lincoln	Bickers	15-002B	3/03/2016	40016	45
Enteritis - H	HG	57	2480	Lincoln	Bickers	15-002B	3/03/2016	31714	40
Enteritis - H	FG	61	3400	Lincoln	Bickers	15-002B	3/03/2016	41771	21
Enteritis - H	MG	61	3400	Lincoln	Bickers	15-002B	3/03/2016	38374	19
Enteritis - H	HG	61	3400	Lincoln	Bickers	15-002B	3/03/2016	25662	25
Enteritis - H	FG	63	3650	Lincoln	Bickers	15-002B	3/03/2016	11764	25
Enteritis - H	HG	63	3650	Lincoln	Bickers	15-002B	3/03/2016	17590	32
Enteritis - H	FG	56	2300	Lincoln	Bickers	15-002B	3/03/2016	1397	22
Enteritis - H	MG	56	2300	Lincoln	Bickers	15-002B	3/03/2016	11431	25
Enteritis - H	FG	61	3330	Lincoln	Bickers	15-002B	3/03/2016	3382	29
Enteritis - H	MG	61	3330	Lincoln	Bickers	15-002B	3/03/2016	15986	40
Enteritis - H	FG	68	4600	Lincoln	Bickers	15-002B	3/03/2016	55804	23
Enteritis - H	MG	68	4600	Lincoln	Bickers	15-002B	3/03/2016	40654	16
Enteritis - H	HG	68	4600	Lincoln	Bickers	15-002B	3/03/2016	50393	24
Enteritis - H	FG	63	3420	Lincoln	Bickers	15-002B	3/03/2016	15704	30
Enteritis - H	MG	63	3420	Lincoln	Bickers	15-002B	3/03/2016	70631	23
Enteritis - H	HG	63	3420	Lincoln	Bickers	15-002B	3/03/2016	63284	32
Enteritis - H	FG	63	3550	Lincoln	Bickers	15-002B	3/03/2016	52380	33
Enteritis - H	HG	63	3550	Lincoln	Bickers	15-002B	3/03/2016	40866	19
Enteritis - HI	HG	58.5	3080	Lincoln	Northern site	15-004A	3/03/2016	45122	33
Enteritis - HI	HG	54	2250	Lincoln	Northern site	15-004A	3/03/2016	31792	41
Enteritis - HI	FG	58	3000	Lincoln	Northern site	15-004A	3/03/2016	34643	34
Enteritis - HI	MG	58	3000	Lincoln	Northern site	15-004A	3/03/2016	50290	41
Enteritis - HI	HG	58	3000	Lincoln	Northern site	15-004A	3/03/2016	26057	21
Enteritis - HI	FG	59	2910	Lincoln	Northern site	15-004A	3/03/2016	47639	24
Enteritis - HI	MG	59	2910	Lincoln	Northern site	15-004A	3/03/2016	41227	36
Enteritis - HI	HG	59	2910	Lincoln	Northern site	15-004A	3/03/2016	49604	50
Enteritis - HI	FG	59	2800	Lincoln	Northern site	15-004A	3/03/2016	56599	51
Enteritis - HI	MG	59	2800	Lincoln	Northern site	15-004A	3/03/2016	55067	35
Enteritis - HI	HG	59	2800	Lincoln	Northern site	15-004A	3/03/2016	45992	47
Enteritis - HI	FG	61	3270	Lincoln	Northern site	15-004A	3/03/2016	50510	34
Enteritis - HI	MG	61	3270	Lincoln	Northern site	15-004A	3/03/2016	27719	34
Enteritis - HI	HG	61	3270	Lincoln	Northern site	15-004A	3/03/2016	42234	27
Enteritis - HI	FG	64	2600	Lincoln	Northern site	15-004A	3/03/2016	37963	34
Enteritis - HI	HG	64	2600	Lincoln	Northern site	15-004A	3/03/2016	54418	42
Enteritis - HI	MG	62	3440	Lincoln	Northern site	15-004A	3/03/2016	39095	43
Enteritis - HI	HG	62	3440	Lincoln	Northern site	15-004A	3/03/2016	33125	29

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Enteritis - HI	MG	60	3250	Lincoln	Northern site	15-004A	3/03/2016	14154	27
Enteritis - HI	HG	60	3250	Lincoln	Northern site	15-004A	3/03/2016	20981	35
Enteritis - HI	FG	59.5	3100	Lincoln	Northern site	15-004A	3/03/2016	15054	30
Enteritis - HI	HG	59.5	3100	Lincoln	Northern site	15-004A	3/03/2016	59257	51
Enteritis - HI	FG	62	2890	Lincoln	Northern site	15-004A	3/03/2016	55112	62
Enteritis - HI	HG	62	2890	Lincoln	Northern site	15-004A	3/03/2016	78287	57
Enteritis - HI	FG	59	3000	Lincoln	Northern site	15-004A	3/03/2016	50372	32
Enteritis - HI	HG	59	3000	Lincoln	Northern site	15-004A	3/03/2016	48900	44
Enteritis - D	FG	47	1070	Lincoln	Northern site	15-004A	3/03/2016	37423	46
Enteritis - D	MG	47	1070	Lincoln	Northern site	15-004A	3/03/2016	38651	55
Enteritis - D	HG	47	1070	Lincoln	Northern site	15-004A	3/03/2016	27640	40
Enteritis - D	FG	44.5	1370	Lincoln	Northern site	15-004A	3/03/2016	23684	26
Enteritis - D	HG	44.5	1370	Lincoln	Northern site	15-004A	3/03/2016	41820	48
Enteritis - D	FG	47	1160	Lincoln	Northern site	15-004A	3/03/2016	15835	40
Enteritis - D	MG	47	1160	Lincoln	Northern site	15-004A	3/03/2016	39216	25
Enteritis - D	HG	47	1160	Lincoln	Northern site	15-004A	3/03/2016	32515	50
Enteritis - D	FG	55	1700	Lincoln	Northern site	15-004A	3/03/2016	32584	36
Enteritis - D	MG	55	1700	Lincoln	Northern site	15-004A	3/03/2016	14372	21
Enteritis - D	HG	55	1700	Lincoln	Northern site	15-004A	3/03/2016	30425	28
Enteritis - D	FG	50	1200	Lincoln	Northern site	15-004A	3/03/2016	38764	35
Enteritis - D	MG	50	1200	Lincoln	Northern site	15-004A	3/03/2016	70021	49
Enteritis - D	HG	50	1200	Lincoln	Northern site	15-004A	3/03/2016	39709	27
Enteritis - D	FG	46	1540	Lincoln	Northern site	15-004A	3/03/2016	33984	24
Enteritis - D	MG	46	1540	Lincoln	Northern site	15-004A	3/03/2016	46550	22
Enteritis - D	HG	46	1540	Lincoln	Northern site	15-004A	3/03/2016	43810	38
Enteritis - D	FG	47.5	1250	Lincoln	Northern site	15-004A	3/03/2016	31550	22
Enteritis - D	MG	47.5	1250	Lincoln	Northern site	15-004A	3/03/2016	44029	26
Enteritis - D	HG	47.5	1250	Lincoln	Northern site	15-004A	3/03/2016	41388	28
Enteritis - D	MG	49	1480	Lincoln	Northern site	15-004A	3/03/2016	43939	34
Enteritis - D	HG	49	1480	Lincoln	Northern site	15-004A	3/03/2016	24987	26
Enteritis - D	FG	52	1250	Lincoln	Northern site	15-004A	3/03/2016	28707	136
Enteritis - D	MG	52	1250	Lincoln	Northern site	15-004A	3/03/2016	25133	63
Enteritis - D	HG	52	1250	Lincoln	Northern site	15-004A	3/03/2016	63101	75
Enteritis - D	FG	54	1410	Lincoln	Northern site	15-004A	3/03/2016	46200	56
Enteritis - D	MG	54	1410	Lincoln	Northern site	15-004A	3/03/2016	49078	50
Enteritis - D	HG	54	1410	Lincoln	Northern site	15-004A	3/03/2016	34995	46
Enteritis - D	FG	50	1710	Lincoln	Northern site	15-004A	3/03/2016	25451	51
Enteritis - D	HG	50	1710	Lincoln	Northern site	15-004A	3/03/2016	15407	36
Coccidiosis - H	FG	32.5	500	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	12193	55
Coccidiosis - H	MG	32.5	500	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	30082	67
Coccidiosis - H	HG	32.5	500	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	29548	56
Coccidiosis - H	FG	34.0	600	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	24633	71
Coccidiosis - H	MG	34.0	600	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	37429	53
Coccidiosis - H	FG	35.0	600	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	5398	84
Coccidiosis - H	MG	35.0	600	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	41499	70
Coccidiosis - H	FG	35.0	530	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	2904	61
Coccidiosis - H	HG	35.0	530	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	42039	64
Coccidiosis - H	FG	32.0	595	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	22979	81
Coccidiosis - H	MG	32.0	595	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	40394	104
Coccidiosis - H	HG	32.0	595	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	51353	84
Coccidiosis - H	FG	32.0	600	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	4449	76
Coccidiosis - H	MG	32.0	600	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	40078	81
Coccidiosis - H	HG	32.0	600	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	33450	69
Coccidiosis - D	FG	26.0	180	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	785	43

Sample type	Gut region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Coccidiosis - D	MG	26.0	180	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	4311	48
Coccidiosis - D	HG	26.0	180	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	3821	69
Coccidiosis - D	FG	27.5	300	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	34071	87
Coccidiosis - D	MG	27.5	300	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	53064	91
Coccidiosis - D	HG	27.5	300	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	55847	89
Coccidiosis - D	FG	24.5	150	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	17313	33
Coccidiosis - D	MG	24.5	150	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	34866	38
Coccidiosis - D	HG	24.5	150	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	38127	33
Coccidiosis - D	FG	22.0	170	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	3651	65
Coccidiosis - D	MG	22.0	170	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	44066	61
Coccidiosis - D	HG	22.0	170	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	56493	71
Coccidiosis - D	FG	26.0	230	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	18420	71
Coccidiosis - D	MG	26.0	230	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	13890	74
Coccidiosis - D	HG	26.0	230	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	20206	80
Coccidiosis - D	FG	26.5	210	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	5431	39
Coccidiosis - D	MG	26.5	210	Arno Bay	AB3-AB Site 2	AB16-8	18/07/2016	20883	23

Abbreviations: D, disease; FG, foregut; H, healthy; HG, hindgut; HI, healthy intermediate; MG, midgut.

Table 3.3.1.3.9. Summary of sequenced sample parameters for Gastrointestinal Health (2016 gut enteritis healthy vs disease vs coccidiosis).

Data-set	No. of samples	Total reads	Median library size	Range	Bacterial OTUs post- filtering
Gastrointestinal Health	122	4,663,108	35,603	829-118,473	562

Table 3.3.1.3.10. One-way PERMANOVA: Pairwise test between the Gastrointestinal Health samples.[1]

Health group	*P*	Significant?
CH, CD	**0.0002**	Yes
CH, EH	**0.0001**	Yes
CH, EHI	**0.0001**	Yes
CH, ED	**0.0001**	Yes
CD, EH	**0.0001**	Yes
CD, EHI	**0.0001**	Yes
CD, ED	**0.0001**	Yes
EH, EHI	**0.0001**	Yes
EH, ED	**0.0001**	Yes
EHI, ED	0.3177	No

Abbreviations: CD, coccidiosis disease; CH, coccidiosis healthy; ED, enteritis disease; EH, enteritis healthy; EHI, enteritis healthy intermediate

[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.3.11. Two-way PERMANOVA (crossed design): Main test to determine if there is a significant difference in microbiome composition of coccidiosis samples between the three gut regions (FG, MG, HG).[1]

Source	df	SS	MS	Pseudo-F	*P*
Coccidiosis health class	1	17347	17347	7.2101	**0.0003**
Gut region	2	3807.8	1903.9	0.79134	0.6223
Coccidiosis × gut	2	4437.9	2219	0.92228	0.4967
Residual	26	62554	2405.9		
Total	31	88489			

[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.3.12. Those bacterial taxa that have the greatest contribution towards the observed difference between coccidiosis healthy (Δ) and coccidiosis disease (O) health classes.[1]

Bacterial taxa (RDP similarity S_ab score)_OTU no.	Av. abundance coccidiosis healthy (Δ)	Av. abundance coccidiosis disease (O)	% Contribution	Highest abundant group
Vibrio sp. V776/*Aliivibrio finisterrensis* (1.000)_OTU 4	16.06	31.88	20.74	O
Anabaena cylindrica (0.376)_OTU 15	7.67	21.64	15.17	O
Vibrio tasmaniensis/*V. pomeroyi* (1.000)_OTU 31	3.01	19.76	11.76	O
Synechococcus sp. (0.978)_OTU 29	7.72	1.11	4.53	Δ
Synechococcus sp. (1.000)_OTU 23	6.14	0.94	3.6	Δ
Pseudoalteromonas sp. (1.000)_OTU 40	5.46	0.16	3.35	Δ
Geobacillus stearothermophilus/*G. thermoparaffinivorans* (1.000)_OTU 45	4.22	1.02	2.67	Δ
Campylobacter hyointestinalis subsp. *lawsonii* (0.673)_ OTU 44	4.2	0.47	2.67	Δ
Bacillus smithii (1.000)_OTU 83	3.51	0.64	2.19	Δ
Pseudomonas veronii/*P. azotoformans*/*P. chlororaphis* subsp. *aureofaciens* (1.000)_OTU 14	3.39	0.36	2.04	Δ
Brevinema andersonii (0.632)_OTU 2	0.01	3.2	2.01	O
Bacillus sp./*Geobacillus stearothermophilus* (0.977)_OTU 234	3.17	0.61	1.96	Δ
Cetobacterium somerae (0.596)_OTU 25	2.57	0.4	1.66	Δ

[1] The discernible bacterial taxa were derived using the Similarity Percentage (SIMPER) algorithm in the PRIMER program, setting a cut-off at 75% cumulative contribution, in order to give only the top few bacterial taxa that contribute to the difference between the groups.

Table 3.3.1.3.13. Unpaired t-test results with Welch's correction for diversity indices comparing the two coccidiosis health groups; coccidiosis healthy vs coccidiosis disease.[1,2]

Diversity measure	t-test summary	Significant?
Species richness (S)	*P*=0.1170	No
Species evenness (J')	***P*=0.0032**	Yes
Shannon's diversity (H')	***P*=0.0032**	Yes
Simpson's diversity (1-λ)	***P*=0.0365**	Yes
Delta+ (Δ+)	*P*=0.9845	No
Lambda+ (λ+)	*P*=0.5067	No

[1] Significant difference denoted by $P < 0.05$, bolded if significant.
[2] ANOVA results for diversity indices comparing the three enteritis health groups presented in Table 3.3.1.3.4.

Table 3.3.1.3.14. Sample information pertaining to the Healthy vs Disease Extension component.

Sample type	Gut/swab region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
2016 sampling - scrapings									
water sample	-	-	-	Lincoln	Bickers	15-002B	3/03/2016	99047	355
water sample	-	-	-	Lincoln	Northern site 1	15-004A	3/03/2016	116571	367
Enteritis - H	MG	61	4000	Lincoln	Bickers	15-002B	3/03/2016	17606	21
Enteritis - H	HG	61	4000	Lincoln	Bickers	15-002B	3/03/2016	28407	17
Enteritis - H	FG	60	3500	Lincoln	Bickers	15-002B	3/03/2016	3472	28
Enteritis - H	MG	60	3500	Lincoln	Bickers	15-002B	3/03/2016	28852	48
Enteritis - H	HG	60	3500	Lincoln	Bickers	15-002B	3/03/2016	34647	53
Enteritis - H	FG	63	3700	Lincoln	Bickers	15-002B	3/03/2016	24771	49
Enteritis - H	MG	63	3700	Lincoln	Bickers	15-002B	3/03/2016	28175	42
Enteritis - H	HG	63	3700	Lincoln	Bickers	15-002B	3/03/2016	25564	18
Enteritis - H	FG	61	3360	Lincoln	Bickers	15-002B	3/03/2016	24642	31
Enteritis - H	MG	61	3360	Lincoln	Bickers	15-002B	3/03/2016	34257	25
Enteritis - H	HG	61	3360	Lincoln	Bickers	15-002B	3/03/2016	17544	17
Enteritis - H	FG	57	2480	Lincoln	Bickers	15-002B	3/03/2016	19382	32
Enteritis - H	MG	57	2480	Lincoln	Bickers	15-002B	3/03/2016	31745	52
Enteritis - H	HG	57	2480	Lincoln	Bickers	15-002B	3/03/2016	40008	59
Enteritis - H	FG	61	3400	Lincoln	Bickers	15-002B	3/03/2016	41794	24
Enteritis - H	MG	61	3400	Lincoln	Bickers	15-002B	3/03/2016	25679	25
Enteritis - H	HG	61	3400	Lincoln	Bickers	15-002B	3/03/2016	38450	22
Enteritis - H	FG	63	3650	Lincoln	Bickers	15-002B	3/03/2016	13400	30
Enteritis - H	HG	63	3650	Lincoln	Bickers	15-002B	3/03/2016	19900	38
Enteritis - H	FG	56	2300	Lincoln	Bickers	15-002B	3/03/2016	1398	24
Enteritis - H	MG	56	2300	Lincoln	Bickers	15-002B	3/03/2016	11307	26
Enteritis - H	FG	61	3330	Lincoln	Bickers	15-002B	3/03/2016	3382	32
Enteritis - H	MG	61	3330	Lincoln	Bickers	15-002B	3/03/2016	13353	39
Enteritis - H	FG	68	4600	Lincoln	Bickers	15-002B	3/03/2016	55783	27
Enteritis - H	MG	68	4600	Lincoln	Bickers	15-002B	3/03/2016	50423	25
Enteritis - H	HG	68	4600	Lincoln	Bickers	15-002B	3/03/2016	40664	18
Enteritis - H	FG	63	3420	Lincoln	Bickers	15-002B	3/03/2016	15817	33
Enteritis - H	MG	63	3420	Lincoln	Bickers	15-002B	3/03/2016	63252	36
Enteritis - H	HG	63	3420	Lincoln	Bickers	15-002B	3/03/2016	71163	23
Enteritis - H	FG	63	3550	Lincoln	Bickers	15-002B	3/03/2016	52398	33
Enteritis - H	HG	63	3550	Lincoln	Bickers	15-002B	3/03/2016	40844	19
Enteritis - HI	HG	58.5	3080	Lincoln	Northern site	15-004A	3/03/2016	45139	30
Enteritis - HI	HG	54	2250	Lincoln	Northern site	15-004A	3/03/2016	31814	57
Enteritis - HI	FG	58	3000	Lincoln	Northern site	15-004A	3/03/2016	34647	39
Enteritis - HI	MG	58	3000	Lincoln	Northern site	15-004A	3/03/2016	26063	25
Enteritis - HI	HG	58	3000	Lincoln	Northern site	15-004A	3/03/2016	50291	45
Enteritis - HI	FG	59	2910	Lincoln	Northern site	15-004A	3/03/2016	47645	25
Enteritis - HI	MG	59	2910	Lincoln	Northern site	15-004A	3/03/2016	49524	56
Enteritis - HI	HG	59	2910	Lincoln	Northern site	15-004A	3/03/2016	41012	42
Enteritis - HI	FG	59	2800	Lincoln	Northern site	15-004A	3/03/2016	57103	62
Enteritis - HI	MG	59	2800	Lincoln	Northern site	15-004A	3/03/2016	46244	62
Enteritis - HI	HG	59	2800	Lincoln	Northern site	15-004A	3/03/2016	55290	47
Enteritis - HI	FG	61	3270	Lincoln	Northern site	15-004A	3/03/2016	50809	50
Enteritis - HI	MG	61	3270	Lincoln	Northern site	15-004A	3/03/2016	42520	35
Enteritis - HI	HG	61	3270	Lincoln	Northern site	15-004A	3/03/2016	32806	43
Enteritis - HI	FG	64	2600	Lincoln	Northern site	15-004A	3/03/2016	38355	51

Sample type	Gut/swab region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Enteritis - HI	HG	64	2600	Lincoln	Northern site	15-004A	3/03/2016	54458	46
Enteritis - HI	MG	62	3440	Lincoln	Northern site	15-004A	3/03/2016	33134	34
Enteritis - HI	HG	62	3440	Lincoln	Northern site	15-004A	3/03/2016	39088	50
Enteritis - HI	MG	60	3250	Lincoln	Northern site	15-004A	3/03/2016	22973	48
Enteritis - HI	HG	60	3250	Lincoln	Northern site	15-004A	3/03/2016	16084	37
Enteritis - HI	FG	59.5	3100	Lincoln	Northern site	15-004A	3/03/2016	15131	31
Enteritis - HI	HG	59.5	3100	Lincoln	Northern site	15-004A	3/03/2016	59369	78
Enteritis - HI	FG	62	2890	Lincoln	Northern site	15-004A	3/03/2016	55262	73
Enteritis - HI	HG	62	2890	Lincoln	Northern site	15-004A	3/03/2016	78215	64
Enteritis - HI	FG	59	3000	Lincoln	Northern site	15-004A	3/03/2016	50437	34
Enteritis - HI	HG	59	3000	Lincoln	Northern site	15-004A	3/03/2016	49143	55
Enteritis - D	FG	47	1070	Lincoln	Northern site	15-004A	3/03/2016	40593	60
Enteritis - D	MG	47	1070	Lincoln	Northern site	15-004A	3/03/2016	27661	50
Enteritis - D	HG	47	1070	Lincoln	Northern site	15-004A	3/03/2016	32879	77
Enteritis - D	FG	44.5	1370	Lincoln	Northern site	15-004A	3/03/2016	23699	30
Enteritis - D	HG	44.5	1370	Lincoln	Northern site	15-004A	3/03/2016	41840	58
Enteritis - D	FG	47	1160	Lincoln	Northern site	15-004A	3/03/2016	15844	41
Enteritis - D	MG	47	1160	Lincoln	Northern site	15-004A	3/03/2016	32502	50
Enteritis - D	HG	47	1160	Lincoln	Northern site	15-004A	3/03/2016	39237	29
Enteritis - D	FG	55	1700	Lincoln	Northern site	15-004A	3/03/2016	32949	43
Enteritis - D	MG	55	1700	Lincoln	Northern site	15-004A	3/03/2016	30787	31
Enteritis - D	HG	55	1700	Lincoln	Northern site	15-004A	3/03/2016	16265	23
Enteritis - D	FG	50	1200	Lincoln	Northern site	15-004A	3/03/2016	38817	45
Enteritis - D	MG	50	1200	Lincoln	Northern site	15-004A	3/03/2016	39726	27
Enteritis - D	HG	50	1200	Lincoln	Northern site	15-004A	3/03/2016	70078	59
Enteritis - D	FG	46	1540	Lincoln	Northern site	15-004A	3/03/2016	33990	31
Enteritis - D	MG	46	1540	Lincoln	Northern site	15-004A	3/03/2016	43854	58
Enteritis - D	HG	46	1540	Lincoln	Northern site	15-004A	3/03/2016	46555	26
Enteritis - D	FG	47.5	1250	Lincoln	Northern site	15-004A	3/03/2016	31594	28
Enteritis - D	MG	47.5	1250	Lincoln	Northern site	15-004A	3/03/2016	41393	29
Enteritis - D	HG	47.5	1250	Lincoln	Northern site	15-004A	3/03/2016	43988	36
Enteritis - D	MG	49	1480	Lincoln	Northern site	15-004A	3/03/2016	25021	36
Enteritis - D	HG	49	1480	Lincoln	Northern site	15-004A	3/03/2016	43926	36
Enteritis - D	FG	52	1250	Lincoln	Northern site	15-004A	3/03/2016	31063	148
Enteritis - D	MG	52	1250	Lincoln	Northern site	15-004A	3/03/2016	64491	95
Enteritis - D	HG	52	1250	Lincoln	Northern site	15-004A	3/03/2016	30582	72
Enteritis - D	FG	54	1410	Lincoln	Northern site	15-004A	3/03/2016	46271	69
Enteritis - D	MG	54	1410	Lincoln	Northern site	15-004A	3/03/2016	34972	49
Enteritis - D	HG	54	1410	Lincoln	Northern site	15-004A	3/03/2016	49266	64
Enteritis - D	FG	50	1710	Lincoln	Northern site	15-004A	3/03/2016	29733	66
Enteritis - D	HG	50	1710	Lincoln	Northern site	15-004A	3/03/2016	20999	41
2017 sampling - scrapings									
Enteritis - H	HG	34	670	Arno Bay	Arno Bay	17-4	10/02/2017	71948	102
Enteritis - H	HG	34.5	550	Arno Bay	Arno Bay	17-4	10/02/2017	40123	73
Enteritis - H	HG	36	600	Arno Bay	Arno Bay	17-4	10/02/2017	70918	121
Enteritis - H	HG	35	640	Arno Bay	Arno Bay	17-4	10/02/2017	95851	84
Enteritis - H	HG	32	450	Arno Bay	Arno Bay	17-4	10/02/2017	68355	137
Enteritis - H	HG	35	600	Arno Bay	Arno Bay	17-4	10/02/2017	143867	130
Enteritis - H	HG	36.5	620	Arno Bay	Arno Bay	17-4	10/02/2017	96153	65
Enteritis - H	HG	26.5	240	Arno Bay	Arno Bay	17-8	10/02/2017	182447	155
Enteritis - H	HG	25	250	Arno Bay	Arno Bay	17-8	10/02/2017	71087	196

Sample type	Gut/swab region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Enteritis - H	HG	25	220	Arno Bay	Arno Bay	17-8	10/02/2017	50117	158
Enteritis - H	HG	26	300	Arno Bay	Arno Bay	17-8	10/02/2017	51016	183
Enteritis - H	HG	29	300	Arno Bay	Arno Bay	17-8	10/02/2017	73239	169
Enteritis - H	HG	24	210	Arno Bay	Arno Bay	17-8	10/02/2017	208971	125
Enteritis - H	HG	26.5	220	Arno Bay	Arno Bay	17-8	10/02/2017	34025	149
Enteritis - H	HG	23.5	190	Arno Bay	Arno Bay	17-8	10/02/2017	65887	138
Enteritis - H	HG	40	1360	Lincoln	Point Boston	17-2	27/02/2017	91732	28
Enteritis - H	HG	41	1230	Lincoln	Point Boston	17-2	27/02/2017	59388	56
Enteritis - H	HG	39.5	1070	Lincoln	Point Boston	17-2	27/02/2017	93635	67
Enteritis - H	HG	39	1160	Lincoln	Point Boston	17-2	27/02/2017	84036	22
Enteritis - H	HG	38	1140	Lincoln	Point Boston	17-2	27/02/2017	84103	61
Enteritis - H	HG	37	1100	Lincoln	Point Boston	17-2	27/02/2017	77999	44
Enteritis - H	HG	42	1100	Lincoln	Point Boston	17-2	27/02/2017	64697	78
Enteritis - H	HG	37.5	1110	Lincoln	Point Boston	17-2	27/02/2017	78184	30
Enteritis - H	HG	37	1040	Lincoln	Point Boston	17-2	27/02/2017	45877	38
Enteritis - H	HG	43	1320	Lincoln	Point Boston	17-2	27/02/2017	80096	71
Enteritis - D	HG	36.5	600	Lincoln	Point Boston	17-2	27/02/2017	76634	44
Enteritis - D	HG	34	560	Lincoln	Point Boston	17-2	27/02/2017	101428	66
Enteritis - D	HG	33.5	530	Lincoln	Point Boston	17-2	27/02/2017	210100	65
Enteritis - D	HG	32.5	460	Lincoln	Point Boston	17-2	27/02/2017	54713	51
Enteritis - D	HG	34.5	730	Lincoln	Point Boston	17-2	27/02/2017	84019	63
Enteritis - D	HG	36.5	800	Lincoln	Point Boston	17-2	27/02/2017	76485	37
Enteritis - D	HG	32.5	460	Lincoln	Point Boston	17-2	27/02/2017	65943	44
Enteritis - D	HG	32.5	540	Lincoln	Point Boston	17-2	27/02/2017	72885	46
Enteritis - D	HG	33	500	Lincoln	Point Boston	17-2	27/02/2017	115288	31
Enteritis - H	HG	34	720	Lincoln	Point Boston	17-3	27/02/2017	70744	50
Enteritis - H	HG	35	680	Lincoln	Point Boston	17-3	27/02/2017	62347	40
Enteritis - H	HG	35.5	660	Lincoln	Point Boston	17-3	27/02/2017	54613	64
Enteritis - H	HG	35.5	800	Lincoln	Point Boston	17-3	27/02/2017	79813	47
Enteritis - H	HG	36	730	Lincoln	Point Boston	17-3	27/02/2017	57609	53
Enteritis - H	HG	35	780	Lincoln	Point Boston	17-3	27/02/2017	75037	25
Enteritis - H	HG	40	890	Lincoln	Point Boston	17-3	27/02/2017	47459	62
Enteritis - H	HG	37	820	Lincoln	Point Boston	17-3	27/02/2017	58898	32
Enteritis - H	HG	36	830	Lincoln	Point Boston	17-3	27/02/2017	27891	78
Enteritis - H	HG	35	850	Lincoln	Point Boston	17-3	27/02/2017	92407	63
Enteritis - D	HG	31	350	Lincoln	Point Boston	17-3	27/02/2017	82331	43
Enteritis - D	HG	32	510	Lincoln	Point Boston	17-3	27/02/2017	94580	44
Enteritis - D	HG	32	520	Lincoln	Point Boston	17-3	27/02/2017	77168	52
Enteritis - D	HG	32	550	Lincoln	Point Boston	17-3	27/02/2017	47572	25
Enteritis - D	HG	28	430	Lincoln	Point Boston	17-3	27/02/2017	28263	39
Enteritis - D	HG	33	540	Lincoln	Point Boston	17-3	27/02/2017	72947	41
Enteritis - D	HG	34	600	Lincoln	Point Boston	17-3	27/02/2017	87489	33
Enteritis - D	HG	29	460	Lincoln	Point Boston	17-3	27/02/2017	66838	25
Enteritis - D	HG	28.5	320	Lincoln	Point Boston	17-3	27/02/2017	41738	21
Enteritis - D	HG	28	370	Lincoln	Point Boston	17-3	27/02/2017	50291	19
Enteritis - H	HG	58	2880	Lincoln	Bickers	16-4GS	28/02/2017	61035	101
Enteritis - H	HG	61	3110	Lincoln	Bickers	16-4GS	28/02/2017	88116	64
Enteritis - H	HG	63	3930	Lincoln	Bickers	16-4GS	28/02/2017	78211	32
Enteritis - H	HG	56	3200	Lincoln	Bickers	16-4GS	28/02/2017	87388	27
Enteritis - H	HG	60	3650	Lincoln	Bickers	16-4GS	28/02/2017	71462	35
Enteritis - H	HG	60	3700	Lincoln	Bickers	16-4GS	28/02/2017	84202	30

Sample type	Gut/swab region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Enteritis - H	HG	61	3520	Lincoln	Bickers	16-4GS	28/02/2017	75101	14
Enteritis - H	HG	58	3290	Lincoln	Bickers	16-4GS	28/02/2017	36311	23
Enteritis - D	HG	51	1700	Lincoln	Bickers	16-4GS	28/02/2017	64475	42
Enteritis - D	HG	50.5	1830	Lincoln	Bickers	16-4GS	28/02/2017	63180	83
Enteritis - D	HG	49	1380	Lincoln	Bickers	16-4GS	28/02/2017	77540	93
Enteritis - D	HG	50.5	1900	Lincoln	Bickers	16-4GS	28/02/2017	47650	78
Enteritis - D	HG	54.5	2100	Lincoln	Bickers	16-4GS	28/02/2017	97253	94
Enteritis - D	HG	54	1740	Lincoln	Bickers	16-4GS	28/02/2017	55005	79
Enteritis - D	HG	52	1620	Lincoln	Bickers	16-4GS	28/02/2017	75432	57
Enteritis - D	HG	52.5	1930	Lincoln	Bickers	16-4GS	28/02/2017	78739	62
Enteritis - D	HG	47	1020	Lincoln	Bickers	16-4GS	28/02/2017	48042	38
Enteritis - H	HG	60.5	3450	Lincoln	Bickers	16-3A	28/02/2017	64076	51
Enteritis - H	HG	62.5	3950	Lincoln	Bickers	16-3A	28/02/2017	59132	40
Enteritis - H	HG	57.5	3750	Lincoln	Bickers	16-3A	28/02/2017	54341	45
Enteritis - H	HG	63	3400	Lincoln	Bickers	16-3A	28/02/2017	77514	54
Enteritis - H	HG	60	3700	Lincoln	Bickers	16-3A	28/02/2017	40432	68
Enteritis - H	HG	61.5	3570	Lincoln	Bickers	16-3A	28/02/2017	50380	21
Enteritis - H	HG	61	3700	Lincoln	Bickers	16-3A	28/02/2017	43881	44
Enteritis - H	HG	62.5	3810	Lincoln	Bickers	16-3A	28/02/2017	59021	51
Enteritis - H	HG	60	3500	Lincoln	Bickers	16-3A	28/02/2017	44533	41
Enteritis - D	HG	55	1980	Lincoln	Bickers	16-3A	28/02/2017	48689	78
Enteritis - D	HG	53	1800	Lincoln	Bickers	16-3A	28/02/2017	42920	62
Enteritis - D	HG	50	1770	Lincoln	Bickers	16-3A	28/02/2017	59329	55
Enteritis - D	HG	52.5	1560	Lincoln	Bickers	16-3A	28/02/2017	61638	28
Enteritis - D	HG	55	1800	Lincoln	Bickers	16-3A	28/02/2017	32623	84
Enteritis - D	HG	49	1820	Lincoln	Bickers	16-3A	28/02/2017	41572	36
Enteritis - D	HG	50.5	1160	Lincoln	Bickers	16-3A	28/02/2017	88923	87
Enteritis - D	HG	49	1570	Lincoln	Bickers	16-3A	28/02/2017	71939	62
Enteritis - D	HG	52	1510	Lincoln	Bickers	16-3A	28/02/2017	46352	59
Enteritis - D	HG	49	1290	Lincoln	Bickers	16-3A	28/02/2017	85784	66
Enteritis - H	HG	43.5	1060	Arno Bay	Arno Bay	17-4	28/04/2017	17957	66
Enteritis - H	HG	48.5	1860	Arno Bay	Arno Bay	17-4	28/04/2017	70659	70
Enteritis - H	HG	45.5	1360	Arno Bay	Arno Bay	17-4	28/04/2017	9013	60
Enteritis - H	HG	47	1700	Arno Bay	Arno Bay	17-4	28/04/2017	58597	74
Enteritis - H	HG	46	1550	Arno Bay	Arno Bay	17-4	28/04/2017	12745	46
Enteritis - H	HG	46.5	1600	Arno Bay	Arno Bay	17-4	28/04/2017	46725	59
Enteritis - H	HG	43.5	1260	Arno Bay	Arno Bay	17-4	28/04/2017	94848	46
Enteritis - H	HG	42.5	1140	Arno Bay	Arno Bay	17-4	28/04/2017	74813	58
Enteritis - H	HG	44.5	1410	Arno Bay	Arno Bay	17-4	28/04/2017	44730	67
Enteritis - H	HG	46	1630	Arno Bay	Arno Bay	17-4	28/04/2017	41267	66
Enteritis - H	HG	43	1220	Arno Bay	Arno Bay	17-4	28/04/2017	73785	79
Enteritis - H	HG	47	1680	Arno Bay	Arno Bay	17-4	28/04/2017	41303	36
Enteritis - H	HG	45.5	1630	Arno Bay	Arno Bay	17-4	28/04/2017	73786	57
Enteritis - D[1]	HG	36.5	600	Arno Bay	Arno Bay	17-4	28/04/2017	63297	51
Enteritis - D	HG	46	1260	Arno Bay	Arno Bay	17-4	28/04/2017	96426	50
Enteritis - D	HG	45.5	1260	Arno Bay	Arno Bay	17-4	28/04/2017	33939	21
Enteritis - D	HG	45.5	1250	Arno Bay	Arno Bay	17-4	28/04/2017	66450	46
Enteritis - D	HG	46	1600	Arno Bay	Arno Bay	17-4	28/04/2017	55827	24
Enteritis - H	HG	40.5	900	Lincoln	Northern Site	17-8	11/04/2017	15129	39
Enteritis - H	HG	37	750	Lincoln	Northern Site	17-8	11/04/2017	94548	44
Enteritis - H	HG	39	860	Lincoln	Northern Site	17-8	11/04/2017	6797	41

Sample type	Gut/swab region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Enteritis - H[1]	HG	37.5	800	Lincoln	Northern Site	17-8	11/04/2017	848	50
Enteritis - H	HG	38	830	Lincoln	Northern Site	17-8	11/04/2017	220888	63
Enteritis - H	HG	37.5	790	Lincoln	Northern Site	17-8	11/04/2017	118224	71
Enteritis - H[1]	HG	37.5	850	Lincoln	Northern Site	17-8	11/04/2017	64	18
Enteritis - D	HG	36	630	Lincoln	Northern Site	17-8	11/04/2017	10801	28
Enteritis - D	HG	37	730	Lincoln	Northern Site	17-8	11/04/2017	97892	93
Enteritis - D	HG	33	470	Lincoln	Northern Site	17-8	11/04/2017	10869	22
Enteritis - D	HG	30	270	Lincoln	Northern Site	17-8	11/04/2017	4419	31
Enteritis - D	HG	32	400	Lincoln	Northern Site	17-8	11/04/2017	4497	37
Enteritis - D	HG	30.5	300	Lincoln	Northern Site	17-8	11/04/2017	76351	115
Enteritis - D	HG	29	290	Lincoln	Northern Site	17-8	11/04/2017	13522	22
2016 sampling swabs									
Enteritis - H	SK	61	4000	Lincoln	Bickers	15-002B	3/03/2016	25894	365
Enteritis - H	GL	61	4000	Lincoln	Bickers	15-002B	3/03/2016	29613	290
Enteritis - H	SK	60	3500	Lincoln	Bickers	15-002B	3/03/2016	26155	362
Enteritis - H	GL	60	3500	Lincoln	Bickers	15-002B	3/03/2016	28559	308
Enteritis - H	SK	63	3700	Lincoln	Bickers	15-002B	3/03/2016	17244	324
Enteritis - H	GL	63	3700	Lincoln	Bickers	15-002B	3/03/2016	35422	343
Enteritis - H	SK	61	3360	Lincoln	Bickers	15-002B	3/03/2016	49469	371
Enteritis - H	GL	61	3360	Lincoln	Bickers	15-002B	3/03/2016	27776	300
Enteritis - H	SK	57	2480	Lincoln	Bickers	15-002B	3/03/2016	26646	381
Enteritis - H	GL	57	2480	Lincoln	Bickers	15-002B	3/03/2016	48524	368
Enteritis - H	SK	61	3400	Lincoln	Bickers	15-002B	3/03/2016	34966	341
Enteritis - H	GL	61	3400	Lincoln	Bickers	15-002B	3/03/2016	27936	344
Enteritis - H	SK	63	3650	Lincoln	Bickers	15-002B	3/03/2016	19069	300
Enteritis - H	GL	63	3650	Lincoln	Bickers	15-002B	3/03/2016	25720	365
Enteritis - H	SK	56	2300	Lincoln	Bickers	15-002B	3/03/2016	35522	385
Enteritis - H	GL	56	2300	Lincoln	Bickers	15-002B	3/03/2016	34496	382
Enteritis - H	SK	61	3330	Lincoln	Bickers	15-002B	3/03/2016	27108	338
Enteritis - H	GL	61	3330	Lincoln	Bickers	15-002B	3/03/2016	32699	363
Enteritis - H	SK	68	4600	Lincoln	Bickers	15-002B	3/03/2016	54185	430
Enteritis - H	GL	68	4600	Lincoln	Bickers	15-002B	3/03/2016	36760	280
Enteritis - H	SK	63	3420	Lincoln	Bickers	15-002B	3/03/2016	43885	411
Enteritis - H	GL	63	3420	Lincoln	Bickers	15-002B	3/03/2016	51540	343
Enteritis - H	SK	63	3550	Lincoln	Bickers	15-002B	3/03/2016	17364	340
Enteritis - H	GL	63	3550	Lincoln	Bickers	15-002B	3/03/2016	44452	377
Enteritis - HI	SK	59.5	3100	Lincoln	Northern site1	15-004A	3/03/2016	31393	151
Enteritis - HI	GL	59.5	3100	Lincoln	Northern site1	15-004A	3/03/2016	32170	206
Enteritis - HI	SK	62	2890	Lincoln	Northern site1	15-004A	3/03/2016	28656	351
Enteritis - HI	GL	62	2890	Lincoln	Northern site1	15-004A	3/03/2016	27929	280
Enteritis - HI	SK	59	3000	Lincoln	Northern site1	15-004A	3/03/2016	37205	130
Enteritis - HI	GL	59	3000	Lincoln	Northern site1	15-004A	3/03/2016	40752	253
Enteritis - HI	SK	58.5	3080	Lincoln	Northern site1	15-004A	3/03/2016	34819	334
Enteritis - HI	GL	58.5	3080	Lincoln	Northern site1	15-004A	3/03/2016	26308	243
Enteritis - HI	SK	54	2250	Lincoln	Northern site1	15-004A	3/03/2016	27925	200
Enteritis - HI	GL	54	2250	Lincoln	Northern site1	15-004A	3/03/2016	50125	276
Enteritis - HI	SK	58	3000	Lincoln	Northern site1	15-004A	3/03/2016	26412	372
Enteritis - HI	GL	58	3000	Lincoln	Northern site1	15-004A	3/03/2016	51621	340
Enteritis - HI	SK	59	2910	Lincoln	Northern site1	15-004A	3/03/2016	39142	359
Enteritis - HI	GL	59	2910	Lincoln	Northern site1	15-004A	3/03/2016	35997	314
Enteritis - HI	SK	59	2800	Lincoln	Northern site1	15-004A	3/03/2016	26307	239

Sample type	Gut/swab region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Enteritis - HI	GL	59	2800	Lincoln	Northern site1	15-004A	3/03/2016	16780	272
Enteritis - HI	SK	61	3270	Lincoln	Northern site1	15-004A	3/03/2016	31442	346
Enteritis - HI	GL	61	3270	Lincoln	Northern site1	15-004A	3/03/2016	37215	403
Enteritis - HI	SK	64	2600	Lincoln	Northern site1	15-004A	3/03/2016	40641	457
Enteritis - HI	GL	64	2600	Lincoln	Northern site1	15-004A	3/03/2016	29531	343
Enteritis - HI	SK	62	3440	Lincoln	Northern site1	15-004A	3/03/2016	45968	343
Enteritis - HI	GL	62	3440	Lincoln	Northern site1	15-004A	3/03/2016	33133	365
Enteritis - HI	SK	60	3250	Lincoln	Northern site1	15-004A	3/03/2016	28689	290
Enteritis - HI	GL	60	3250	Lincoln	Northern site1	15-004A	3/03/2016	29180	327
Enteritis - D	SK	47	1070	Lincoln	Northern site1	15-004A	3/03/2016	36839	286
Enteritis - D	GL	47	1070	Lincoln	Northern site1	15-004A	3/03/2016	35235	330
Enteritis - D	SK	44.5	1370	Lincoln	Northern site1	15-004A	3/03/2016	19358	356
Enteritis - D	GL	44.5	1370	Lincoln	Northern site1	15-004A	3/03/2016	35749	308
Enteritis - D	SK	47	1160	Lincoln	Northern site1	15-004A	3/03/2016	30717	397
Enteritis - D	GL	47	1160	Lincoln	Northern site1	15-004A	3/03/2016	47134	365
Enteritis - D	SK	55	1700	Lincoln	Northern site1	15-004A	3/03/2016	48568	391
Enteritis - D	GL	55	1700	Lincoln	Northern site1	15-004A	3/03/2016	37651	370
Enteritis - D	SK	50	1200	Lincoln	Northern site1	15-004A	3/03/2016	54390	421
Enteritis - D	GL	50	1200	Lincoln	Northern site1	15-004A	3/03/2016	18967	423
Enteritis - D	SK	49	1510	Lincoln	Northern site1	15-004A	3/03/2016	32036	432
Enteritis - D	GL	49	1510	Lincoln	Northern site1	15-004A	3/03/2016	20592	437
Enteritis - D	SK	46	1540	Lincoln	Northern site1	15-004A	3/03/2016	31499	242
Enteritis - D	GL	46	1540	Lincoln	Northern site1	15-004A	3/03/2016	24012	311
Enteritis - D	SK	47.5	1250	Lincoln	Northern site1	15-004A	3/03/2016	22146	384
Enteritis - D	GL	47.5	1250	Lincoln	Northern site1	15-004A	3/03/2016	19455	409
Enteritis - D	SK	49	1480	Lincoln	Northern site1	15-004A	3/03/2016	43167	349
Enteritis - D	GL	49	1480	Lincoln	Northern site1	15-004A	3/03/2016	26028	345
Enteritis - D	SK	52	1250	Lincoln	Northern site1	15-004A	3/03/2016	45577	414
Enteritis - D	GL	52	1250	Lincoln	Northern site1	15-004A	3/03/2016	23242	345
Enteritis - D	SK	54	1410	Lincoln	Northern site1	15-004A	3/03/2016	39599	478
Enteritis - D	GL	54	1410	Lincoln	Northern site1	15-004A	3/03/2016	33730	313
Enteritis - D	SK	50	1710	Lincoln	Northern site1	15-004A	3/03/2016	35968	436
Enteritis - D	GL	50	1710	Lincoln	Northern site1	15-004A	3/03/2016	27451	386
2017 sampling swabs									
Enteritis - H	SK	32	500	Arno Bay	Arno Bay	17-4	10/02/2017	90389	360
Enteritis - H	GL	32	500	Arno Bay	Arno Bay	17-4	10/02/2017	74145	217
Enteritis - H	SK	34	670	Arno Bay	Arno Bay	17-4	10/02/2017	69765	403
Enteritis - H	GL	34	670	Arno Bay	Arno Bay	17-4	10/02/2017	66709	207
Enteritis - H	SK	36.5	650	Arno Bay	Arno Bay	17-4	10/02/2017	47289	333
Enteritis - H	GL	36.5	650	Arno Bay	Arno Bay	17-4	10/02/2017	61468	179
Enteritis - H	SK	34.5	550	Arno Bay	Arno Bay	17-4	10/02/2017	59140	349
Enteritis - H	GL	34.5	550	Arno Bay	Arno Bay	17-4	10/02/2017	107829	217
Enteritis - H	SK	37	690	Arno Bay	Arno Bay	17-4	10/02/2017	54049	390
Enteritis - H	GL	37	690	Arno Bay	Arno Bay	17-4	10/02/2017	41329	214
Enteritis - H	SK	36	600	Arno Bay	Arno Bay	17-4	10/02/2017	43108	378
Enteritis - H	GL	36	600	Arno Bay	Arno Bay	17-4	10/02/2017	66378	199
Enteritis - H	SK	35	640	Arno Bay	Arno Bay	17-4	10/02/2017	49182	359
Enteritis - H	GL	35	640	Arno Bay	Arno Bay	17-4	10/02/2017	76101	229
Enteritis - H	SK	32	450	Arno Bay	Arno Bay	17-4	10/02/2017	54900	310
Enteritis - H	GL	32	450	Arno Bay	Arno Bay	17-4	10/02/2017	82295	186
Enteritis - H	SK	35	600	Arno Bay	Arno Bay	17-4	10/02/2017	70082	356

Sample type	Gut/swab region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Enteritis - H	GL	35	600	Arno Bay	Arno Bay	17-4	10/02/2017	80428	205
Enteritis - H	SK	36.5	620	Arno Bay	Arno Bay	17-4	10/02/2017	71824	379
Enteritis - H	GL	36.5	620	Arno Bay	Arno Bay	17-4	10/02/2017	53205	212
Enteritis - H	SK	26.5	240	Arno Bay	Arno Bay	17-8	10/02/2017	59221	364
Enteritis - H	GL	26.5	240	Arno Bay	Arno Bay	17-8	10/02/2017	47458	249
Enteritis - H	SK	25.5	240	Arno Bay	Arno Bay	17-8	10/02/2017	38973	382
Enteritis - H	GL	25.5	240	Arno Bay	Arno Bay	17-8	10/02/2017	72276	230
Enteritis - H	SK	25	250	Arno Bay	Arno Bay	17-8	10/02/2017	47514	399
Enteritis - H	GL	25	250	Arno Bay	Arno Bay	17-8	10/02/2017	36477	286
Enteritis - H	SK	25	220	Arno Bay	Arno Bay	17-8	10/02/2017	44676	432
Enteritis - H	GL	25	220	Arno Bay	Arno Bay	17-8	10/02/2017	61974	291
Enteritis - H	SK	26	300	Arno Bay	Arno Bay	17-8	10/02/2017	64105	458
Enteritis - H	GL	26	300	Arno Bay	Arno Bay	17-8	10/02/2017	101096	281
Enteritis - H	SK	29	300	Arno Bay	Arno Bay	17-8	10/02/2017	95296	470
Enteritis - H	GL	29	300	Arno Bay	Arno Bay	17-8	10/02/2017	79114	265
Enteritis - H	SK	24	210	Arno Bay	Arno Bay	17-8	10/02/2017	79371	246
Enteritis - H	GL	24	210	Arno Bay	Arno Bay	17-8	10/02/2017	90688	210
Enteritis - H	SK	25	260	Arno Bay	Arno Bay	17-8	10/02/2017	82223	408
Enteritis - H	GL	25	260	Arno Bay	Arno Bay	17-8	10/02/2017	76342	203
Enteritis - H	SK	26.5	220	Arno Bay	Arno Bay	17-8	10/02/2017	45996	388
Enteritis - H	GL	26.5	220	Arno Bay	Arno Bay	17-8	10/02/2017	42045	216
Enteritis - H	SK	23.5	190	Arno Bay	Arno Bay	17-8	10/02/2017	35997	342
Enteritis - H	GL	23.5	190	Arno Bay	Arno Bay	17-8	10/02/2017	150420	264
Enteritis - H	SK	40	1360	Lincoln	Point Boston	17-2	27/02/2017	76298	446
Enteritis - H	GL	40	1360	Lincoln	Point Boston	17-2	27/02/2017	79518	465
Enteritis - H	SK	41	1230	Lincoln	Point Boston	17-2	27/02/2017	100274	334
Enteritis - H	GL	41	1230	Lincoln	Point Boston	17-2	27/02/2017	83763	393
Enteritis - H	SK	39.5	1070	Lincoln	Point Boston	17-2	27/02/2017	134309	479
Enteritis - H	GL	39.5	1070	Lincoln	Point Boston	17-2	27/02/2017	79900	347
Enteritis - H	SK	39	1160	Lincoln	Point Boston	17-2	27/02/2017	63254	435
Enteritis - H	GL	39	1160	Lincoln	Point Boston	17-2	27/02/2017	64572	354
Enteritis - H	SK	38	1140	Lincoln	Point Boston	17-2	27/02/2017	56207	426
Enteritis - H	GL	38	1140	Lincoln	Point Boston	17-2	27/02/2017	40161	323
Enteritis - H	SK	37	1100	Lincoln	Point Boston	17-2	27/02/2017	88719	455
Enteritis - H	GL	37	1100	Lincoln	Point Boston	17-2	27/02/2017	48456	284
Enteritis - H	SK	42	1100	Lincoln	Point Boston	17-2	27/02/2017	54876	365
Enteritis - H	GL	42	1100	Lincoln	Point Boston	17-2	27/02/2017	45628	236
Enteritis - H	SK	37.5	1110	Lincoln	Point Boston	17-2	27/02/2017	80059	424
Enteritis - H	GL	37.5	1110	Lincoln	Point Boston	17-2	27/02/2017	50118	222
Enteritis - H	SK	37	1040	Lincoln	Point Boston	17-2	27/02/2017	50681	389
Enteritis - H	GL	37	1040	Lincoln	Point Boston	17-2	27/02/2017	50515	378
Enteritis - H	SK	43	1320	Lincoln	Point Boston	17-2	27/02/2017	46841	443
Enteritis - H	GL	43	1320	Lincoln	Point Boston	17-2	27/02/2017	54288	343
Enteritis - D	SK	36.5	600	Lincoln	Point Boston	17-2	27/02/2017	56929	459
Enteritis - D	GL	36.5	600	Lincoln	Point Boston	17-2	27/02/2017	32026	147
Enteritis - D	SK	34	560	Lincoln	Point Boston	17-2	27/02/2017	55183	407
Enteritis - D	GL	34	560	Lincoln	Point Boston	17-2	27/02/2017	101055	271
Enteritis - D	SK	33.5	530	Lincoln	Point Boston	17-2	27/02/2017	103961	444
Enteritis - D	GL	33.5	530	Lincoln	Point Boston	17-2	27/02/2017	49421	309
Enteritis - D	SK	32.5	460	Lincoln	Point Boston	17-2	27/02/2017	54050	469
Enteritis - D	GL	32.5	460	Lincoln	Point Boston	17-2	27/02/2017	90630	349

Sample type	Gut/swab region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Enteritis - D	SK	34.5	730	Lincoln	Point Boston	17-2	27/02/2017	71112	471
Enteritis - D	GL	34.5	730	Lincoln	Point Boston	17-2	27/02/2017	57330	248
Enteritis - D	SK	36.5	800	Lincoln	Point Boston	17-2	27/02/2017	146936	496
Enteritis - D	GL	36.5	800	Lincoln	Point Boston	17-2	27/02/2017	99189	318
Enteritis - D	SK	32.5	460	Lincoln	Point Boston	17-2	27/02/2017	106808	379
Enteritis - D	GL	32.5	460	Lincoln	Point Boston	17-2	27/02/2017	117972	247
Enteritis - D	SK	32.5	540	Lincoln	Point Boston	17-2	27/02/2017	85900	431
Enteritis - D	GL	32.5	540	Lincoln	Point Boston	17-2	27/02/2017	75703	175
Enteritis - D	SK	33	500	Lincoln	Point Boston	17-2	27/02/2017	62794	386
Enteritis - D	GL	33	500	Lincoln	Point Boston	17-2	27/02/2017	52595	192
Enteritis - D	SK	34	580	Lincoln	Point Boston	17-2	27/02/2017	57754	428
Enteritis - D	GL	34	580	Lincoln	Point Boston	17-2	27/02/2017	70004	350
Enteritis - H	SK	34	720	Lincoln	Point Boston	17-3	27/02/2017	104846	351
Enteritis - H	GL	34	720	Lincoln	Point Boston	17-3	27/02/2017	90921	271
Enteritis - H	SK	35	680	Lincoln	Point Boston	17-3	27/02/2017	67426	344
Enteritis - H	GL	35	680	Lincoln	Point Boston	17-3	27/02/2017	67422	301
Enteritis - H	SK	35.5	660	Lincoln	Point Boston	17-3	27/02/2017	72649	455
Enteritis - H	GL	35.5	660	Lincoln	Point Boston	17-3	27/02/2017	107129	331
Enteritis - H	SK	35.5	800	Lincoln	Point Boston	17-3	27/02/2017	54668	397
Enteritis - H	GL	35.5	800	Lincoln	Point Boston	17-3	27/02/2017	87068	288
Enteritis - H	SK	36	730	Lincoln	Point Boston	17-3	27/02/2017	64404	396
Enteritis - H	GL	36	730	Lincoln	Point Boston	17-3	27/02/2017	61904	274
Enteritis - H	SK	35	780	Lincoln	Point Boston	17-3	27/02/2017	78186	386
Enteritis - H	GL	35	780	Lincoln	Point Boston	17-3	27/02/2017	70894	250
Enteritis - H	SK	40	890	Lincoln	Point Boston	17-3	27/02/2017	64229	377
Enteritis - H	GL	40	890	Lincoln	Point Boston	17-3	27/02/2017	56179	301
Enteritis - H	SK	37	820	Lincoln	Point Boston	17-3	27/02/2017	102916	419
Enteritis - H	GL	37	820	Lincoln	Point Boston	17-3	27/02/2017	169011	290
Enteritis - H	SK	36	830	Lincoln	Point Boston	17-3	27/02/2017	165950	375
Enteritis - H	GL	36	830	Lincoln	Point Boston	17-3	27/02/2017	156467	343
Enteritis - H	SK	35	850	Lincoln	Point Boston	17-3	27/02/2017	66661	355
Enteritis - H	GL	35	850	Lincoln	Point Boston	17-3	27/02/2017	66664	357
Enteritis - D	SK	31	350	Lincoln	Point Boston	17-3	27/02/2017	119921	472
Enteritis - D	GL	31	350	Lincoln	Point Boston	17-3	27/02/2017	116979	344
Enteritis - D	SK	32	510	Lincoln	Point Boston	17-3	27/02/2017	109739	371
Enteritis - D	GL	32	510	Lincoln	Point Boston	17-3	27/02/2017	159168	380
Enteritis - D	SK	32	520	Lincoln	Point Boston	17-3	27/02/2017	114691	521
Enteritis - D	GL	32	520	Lincoln	Point Boston	17-3	27/02/2017	98119	422
Enteritis - D	SK	32	550	Lincoln	Point Boston	17-3	27/02/2017	187921	584
Enteritis - D	GL	32	550	Lincoln	Point Boston	17-3	27/02/2017	154911	458
Enteritis - D	SK	28	430	Lincoln	Point Boston	17-3	27/02/2017	167638	535
Enteritis - D	GL	28	430	Lincoln	Point Boston	17-3	27/02/2017	129259	389
Enteritis - D	SK	33	540	Lincoln	Point Boston	17-3	27/02/2017	100879	484
Enteritis - D	GL	33	540	Lincoln	Point Boston	17-3	27/02/2017	85477	353
Enteritis - D	SK	34	600	Lincoln	Point Boston	17-3	27/02/2017	122126	485
Enteritis - D	GL	34	600	Lincoln	Point Boston	17-3	27/02/2017	159254	332
Enteritis - D	SK	29	460	Lincoln	Point Boston	17-3	27/02/2017	131327	444
Enteritis - D	GL	29	460	Lincoln	Point Boston	17-3	27/02/2017	139010	337
Enteritis - D	SK	28.5	320	Lincoln	Point Boston	17-3	27/02/2017	147445	390
Enteritis - D	GL	28.5	320	Lincoln	Point Boston	17-3	27/02/2017	87846	320
Enteritis - D	SK	28	370	Lincoln	Point Boston	17-3	27/02/2017	110467	376

Sample type	Gut/swab region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Enteritis - D	GL	28	370	Lincoln	Point Boston	17-3	27/02/2017	83941	250
Enteritis - H	SK	58	2880	Lincoln	Bickers	16-4GS	28/02/2017	58375	408
Enteritis - H	GL	58	2880	Lincoln	Bickers	16-4GS	28/02/2017	79620	343
Enteritis - H	SK	61	3110	Lincoln	Bickers	16-4GS	28/02/2017	72452	380
Enteritis - H	GL	61	3110	Lincoln	Bickers	16-4GS	28/02/2017	102455	401
Enteritis - H	SK	63	3930	Lincoln	Bickers	16-4GS	28/02/2017	114336	375
Enteritis - H	GL	63	3930	Lincoln	Bickers	16-4GS	28/02/2017	111629	396
Enteritis - H	SK	56	3200	Lincoln	Bickers	16-4GS	28/02/2017	95220	505
Enteritis - H	GL	56	3200	Lincoln	Bickers	16-4GS	28/02/2017	103207	401
Enteritis - H	SK	60	3650	Lincoln	Bickers	16-4GS	28/02/2017	57390	336
Enteritis - H	GL	60	3650	Lincoln	Bickers	16-4GS	28/02/2017	98959	417
Enteritis - H	SK	60	3700	Lincoln	Bickers	16-4GS	28/02/2017	79024	324
Enteritis - H	GL	60	3700	Lincoln	Bickers	16-4GS	28/02/2017	83326	406
Enteritis - H	SK	61	3520	Lincoln	Bickers	16-4GS	28/02/2017	59523	262
Enteritis - H	GL	61	3520	Lincoln	Bickers	16-4GS	28/02/2017	85166	337
Enteritis - H	SK	58	3290	Lincoln	Bickers	16-4GS	28/02/2017	92094	345
Enteritis - H	GL	58	3290	Lincoln	Bickers	16-4GS	28/02/2017	98935	398
Enteritis - H	SK	62	3330	Lincoln	Bickers	16-4GS	28/02/2017	65683	347
Enteritis - H	GL	62	3330	Lincoln	Bickers	16-4GS	28/02/2017	92228	398
Enteritis - D	SK	51	1700	Lincoln	Bickers	16-4GS	28/02/2017	128024	452
Enteritis - D	GL	51	1700	Lincoln	Bickers	16-4GS	28/02/2017	86765	415
Enteritis - D	SK	50.5	1830	Lincoln	Bickers	16-4GS	28/02/2017	82340	410
Enteritis - D	GL	50.5	1830	Lincoln	Bickers	16-4GS	28/02/2017	95805	439
Enteritis - D	SK	49	1380	Lincoln	Bickers	16-4GS	28/02/2017	88187	330
Enteritis - D	GL	49	1380	Lincoln	Bickers	16-4GS	28/02/2017	85798	332
Enteritis - D	SK	50.5	1900	Lincoln	Bickers	16-4GS	28/02/2017	82894	355
Enteritis - D	GL	50.5	1900	Lincoln	Bickers	16-4GS	28/02/2017	84398	385
Enteritis - D	SK	54.5	2100	Lincoln	Bickers	16-4GS	28/02/2017	106084	337
Enteritis - D	GL	54.5	2100	Lincoln	Bickers	16-4GS	28/02/2017	53006	388
Enteritis - D	SK	54	1740	Lincoln	Bickers	16-4GS	28/02/2017	72498	354
Enteritis - D	GL	54	1740	Lincoln	Bickers	16-4GS	28/02/2017	118085	403
Enteritis - D	SK	52	1620	Lincoln	Bickers	16-4GS	28/02/2017	71754	312
Enteritis - D	GL	52	1620	Lincoln	Bickers	16-4GS	28/02/2017	97972	427
Enteritis - D	SK	52.5	1930	Lincoln	Bickers	16-4GS	28/02/2017	78251	314
Enteritis - D	GL	52.5	1930	Lincoln	Bickers	16-4GS	28/02/2017	119234	429
Enteritis - D	SK	47	1020	Lincoln	Bickers	16-4GS	28/02/2017	69125	354
Enteritis - D	GL	47	1020	Lincoln	Bickers	16-4GS	28/02/2017	91502	378
Enteritis - H	SK	60.5	3450	Lincoln	Bickers	16-3A	28/02/2017	65265	440
Enteritis - H	GL	60.5	3450	Lincoln	Bickers	16-3A	28/02/2017	87010	385
Enteritis - H	SK	62.5	3950	Lincoln	Bickers	16-3A	28/02/2017	73168	334
Enteritis - H	GL	62.5	3950	Lincoln	Bickers	16-3A	28/02/2017	69080	428
Enteritis - H	SK	57.5	3750	Lincoln	Bickers	16-3A	28/02/2017	69324	424
Enteritis - H	GL	57.5	3750	Lincoln	Bickers	16-3A	28/02/2017	121363	465
Enteritis - H	SK	63	3400	Lincoln	Bickers	16-3A	28/02/2017	67706	465
Enteritis - H	GL	63	3400	Lincoln	Bickers	16-3A	28/02/2017	120714	383
Enteritis - H	SK	61.5	3900	Lincoln	Bickers	16-3A	28/02/2017	96368	444
Enteritis - H	GL	61.5	3900	Lincoln	Bickers	16-3A	28/02/2017	95142	381
Enteritis - H	SK	60	3700	Lincoln	Bickers	16-3A	28/02/2017	66058	420
Enteritis - H	GL	60	3700	Lincoln	Bickers	16-3A	28/02/2017	57705	252
Enteritis - H	SK	61.5	3570	Lincoln	Bickers	16-3A	28/02/2017	72270	482
Enteritis - H	GL	61.5	3570	Lincoln	Bickers	16-3A	28/02/2017	58178	357

Sample type	Gut/swab region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Enteritis - H	SK	61	3700	Lincoln	Bickers	16-3A	28/02/2017	52860	439
Enteritis - H	GL	61	3700	Lincoln	Bickers	16-3A	28/02/2017	90186	417
Enteritis - H	SK	62.5	3810	Lincoln	Bickers	16-3A	28/02/2017	72372	535
Enteritis - H	GL	62.5	3810	Lincoln	Bickers	16-3A	28/02/2017	133859	427
Enteritis - H	SK	60	3500	Lincoln	Bickers	16-3A	28/02/2017	160530	541
Enteritis - H	GL	60	3500	Lincoln	Bickers	16-3A	28/02/2017	113128	426
Enteritis - D	SK	55	1980	Lincoln	Bickers	16-3A	28/02/2017	68965	455
Enteritis - D	GL	55	1980	Lincoln	Bickers	16-3A	28/02/2017	170789	508
Enteritis - D	SK	53	1800	Lincoln	Bickers	16-3A	28/02/2017	89585	449
Enteritis - D	GL	53	1800	Lincoln	Bickers	16-3A	28/02/2017	64670	425
Enteritis - D	SK	50	1770	Lincoln	Bickers	16-3A	28/02/2017	116474	441
Enteritis - D	GL	50	1770	Lincoln	Bickers	16-3A	28/02/2017	109869	393
Enteritis - D	SK	52.5	1560	Lincoln	Bickers	16-3A	28/02/2017	70535	417
Enteritis - D	GL	52.5	1560	Lincoln	Bickers	16-3A	28/02/2017	88725	484
Enteritis - D	SK	55	1800	Lincoln	Bickers	16-3A	28/02/2017	87436	448
Enteritis - D	GL	55	1800	Lincoln	Bickers	16-3A	28/02/2017	142452	470
Enteritis - D	SK	49	1820	Lincoln	Bickers	16-3A	28/02/2017	59070	313
Enteritis - D	GL	49	1820	Lincoln	Bickers	16-3A	28/02/2017	111405	382
Enteritis - D	SK	50.5	1160	Lincoln	Bickers	16-3A	28/02/2017	43378	260
Enteritis - D	GL	50.5	1160	Lincoln	Bickers	16-3A	28/02/2017	73181	455
Enteritis - D	SK	49	1570	Lincoln	Bickers	16-3A	28/02/2017	46506	373
Enteritis - D	GL	49	1570	Lincoln	Bickers	16-3A	28/02/2017	56017	342
Enteritis - D	SK	52	1510	Lincoln	Bickers	16-3A	28/02/2017	62645	376
Enteritis - D	GL	52	1510	Lincoln	Bickers	16-3A	28/02/2017	57356	466
Enteritis - D	SK	49	1290	Lincoln	Bickers	16-3A	28/02/2017	45902	305
Enteritis - D	GL	49	1290	Lincoln	Bickers	16-3A	28/02/2017	61660	426
Enteritis - H	SK	43.5	1060	Arno Bay	Arno Bay	17-4	28/04/2017	40155	365
Enteritis - H	GL	43.5	1060	Arno Bay	Arno Bay	17-4	28/04/2017	46162	177
Enteritis - H	SK	48.5	1860	Arno Bay	Arno Bay	17-4	28/04/2017	41071	376
Enteritis - H	GL	48.5	1860	Arno Bay	Arno Bay	17-4	28/04/2017	45480	153
Enteritis - H	SK	45.5	1360	Arno Bay	Arno Bay	17-4	28/04/2017	43516	419
Enteritis - H	GL	45.5	1360	Arno Bay	Arno Bay	17-4	28/04/2017	73760	191
Enteritis - H	SK	47	1700	Arno Bay	Arno Bay	17-4	28/04/2017	46496	442
Enteritis - H	GL	47	1700	Arno Bay	Arno Bay	17-4	28/04/2017	39687	231
Enteritis - H	SK	46	1550	Arno Bay	Arno Bay	17-4	28/04/2017	53004	305
Enteritis - H	GL	46	1550	Arno Bay	Arno Bay	17-4	28/04/2017	38787	115
Enteritis - H	SK	46.5	1600	Arno Bay	Arno Bay	17-4	28/04/2017	35851	212
Enteritis - H	GL	46.5	1600	Arno Bay	Arno Bay	17-4	28/04/2017	46504	139
Enteritis - H	SK	43.5	1260	Arno Bay	Arno Bay	17-4	28/04/2017	35798	363
Enteritis - H	GL	43.5	1260	Arno Bay	Arno Bay	17-4	28/04/2017	39601	165
Enteritis - H	SK	42.5	1140	Arno Bay	Arno Bay	17-4	28/04/2017	21664	303
Enteritis - H	GL	42.5	1140	Arno Bay	Arno Bay	17-4	28/04/2017	66235	136
Enteritis - H	SK	44.5	1130	Arno Bay	Arno Bay	17-4	28/04/2017	67637	245
Enteritis - H	GL	44.5	1130	Arno Bay	Arno Bay	17-4	28/04/2017	59557	123
Enteritis - H	SK	44.5	1410	Arno Bay	Arno Bay	17-4	28/04/2017	38590	384
Enteritis - H	GL	44.5	1410	Arno Bay	Arno Bay	17-4	28/04/2017	63733	188
Enteritis - H	SK	46	1630	Arno Bay	Arno Bay	17-4	28/04/2017	22520	168
Enteritis - H	GL	46	1630	Arno Bay	Arno Bay	17-4	28/04/2017	59292	174
Enteritis - H	SK	43	1220	Arno Bay	Arno Bay	17-4	28/04/2017	55151	234
Enteritis - H	GL	43	1220	Arno Bay	Arno Bay	17-4	28/04/2017	119864	237
Enteritis - H	SK	47	1680	Arno Bay	Arno Bay	17-4	28/04/2017	99620	370

Sample type	Gut/swab region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Enteritis - H	GL	47	1680	Arno Bay	Arno Bay	17-4	28/04/2017	120244	278
Enteritis - H	SK	45.5	1630	Arno Bay	Arno Bay	17-4	28/04/2017	88504	408
Enteritis - H	GL	45.5	1630	Arno Bay	Arno Bay	17-4	28/04/2017	93778	270
Enteritis - D	SK	36.5	600	Arno Bay	Arno Bay	17-4	28/04/2017	48479	361
Enteritis - D	GL	36.5	600	Arno Bay	Arno Bay	17-4	28/04/2017	48964	170
Enteritis - D	SK	46	1260	Arno Bay	Arno Bay	17-4	28/04/2017	21885	336
Enteritis - D	GL	46	1260	Arno Bay	Arno Bay	17-4	28/04/2017	55243	196
Enteritis - D	SK	45.5	1260	Arno Bay	Arno Bay	17-4	28/04/2017	49180	138
Enteritis - D	GL	45.5	1260	Arno Bay	Arno Bay	17-4	28/04/2017	50649	124
Enteritis - D	SK	41.5	1130	Arno Bay	Arno Bay	17-4	28/04/2017	17675	266
Enteritis - D	GL	41.5	1130	Arno Bay	Arno Bay	17-4	28/04/2017	38024	157
Enteritis - D	SK	45.5	1250	Arno Bay	Arno Bay	17-4	28/04/2017	54499	347
Enteritis - D	GL	45.5	1250	Arno Bay	Arno Bay	17-4	28/04/2017	46712	132
Enteritis - D	SK	46	1600	Arno Bay	Arno Bay	17-4	28/04/2017	29202	204
Enteritis - D	GL	46	1600	Arno Bay	Arno Bay	17-4	28/04/2017	46474	138
Enteritis - H	SK	35.5	830	Lincoln	Northern Site	17-8	11/04/2017	45387	353
Enteritis - H	GL	35.5	830	Lincoln	Northern Site	17-8	11/04/2017	60161	189
Enteritis - H	SK	40.5	900	Lincoln	Northern Site	17-8	11/04/2017	59044	367
Enteritis - H	GL	40.5	900	Lincoln	Northern Site	17-8	11/04/2017	113065	235
Enteritis - H	SK	39.5	810	Lincoln	Northern Site	17-8	11/04/2017	58240	432
Enteritis - H	GL	39.5	810	Lincoln	Northern Site	17-8	11/04/2017	25665	139
Enteritis - H	SK	37	750	Lincoln	Northern Site	17-8	11/04/2017	42129	302
Enteritis - H	GL	37	750	Lincoln	Northern Site	17-8	11/04/2017	44725	201
Enteritis - H	SK	39	860	Lincoln	Northern Site	17-8	11/04/2017	33516	268
Enteritis - H	GL	39	860	Lincoln	Northern Site	17-8	11/04/2017	46111	208
Enteritis - H	SK	37.5	800	Lincoln	Northern Site	17-8	11/04/2017	49918	422
Enteritis - H	GL	37.5	800	Lincoln	Northern Site	17-8	11/04/2017	34599	180
Enteritis - H	SK	38	830	Lincoln	Northern Site	17-8	11/04/2017	41942	354
Enteritis - H	GL	38	830	Lincoln	Northern Site	17-8	11/04/2017	30100	161
Enteritis - H	SK	37.5	790	Lincoln	Northern Site	17-8	11/04/2017	40701	237
Enteritis - H	GL	37.5	790	Lincoln	Northern Site	17-8	11/04/2017	79550	216
Enteritis - H	SK	37.5	850	Lincoln	Northern Site	17-8	11/04/2017	78330	473
Enteritis - H	GL	37.5	850	Lincoln	Northern Site	17-8	11/04/2017	68001	226
Enteritis - H	SK	39.5	780	Lincoln	Northern Site	17-8	11/04/2017	29075	267
Enteritis - H	GL	39.5	780	Lincoln	Northern Site	17-8	11/04/2017	46982	224
Enteritis - D	SK	36	630	Lincoln	Northern Site	17-8	11/04/2017	25768	238
Enteritis - D	GL	36	630	Lincoln	Northern Site	17-8	11/04/2017	37708	198
Enteritis - D	SK	37	730	Lincoln	Northern Site	17-8	11/04/2017	37529	246
Enteritis - D	GL	37	730	Lincoln	Northern Site	17-8	11/04/2017	31531	195
Enteritis - D	SK	28	320	Lincoln	Northern Site	17-8	11/04/2017	40664	407
Enteritis - D	GL	28	320	Lincoln	Northern Site	17-8	11/04/2017	57627	257
Enteritis - D	SK	28.5	300	Lincoln	Northern Site	17-8	11/04/2017	51157	434
Enteritis - D	GL	28.5	300	Lincoln	Northern Site	17-8	11/04/2017	59624	251
Enteritis - D	SK	33	470	Lincoln	Northern Site	17-8	11/04/2017	29284	467
Enteritis - D	GL	33	470	Lincoln	Northern Site	17-8	11/04/2017	34458	227
Enteritis - D	SK	30	270	Lincoln	Northern Site	17-8	11/04/2017	28018	442
Enteritis - D	GL	30	270	Lincoln	Northern Site	17-8	11/04/2017	90558	291
Enteritis - D	SK	32	400	Lincoln	Northern Site	17-8	11/04/2017	22484	421
Enteritis - D	GL	32	400	Lincoln	Northern Site	17-8	11/04/2017	33199	222
Enteritis - D	SK	30.5	300	Lincoln	Northern Site	17-8	11/04/2017	25120	430
Enteritis - D	GL	30.5	300	Lincoln	Northern Site	17-8	11/04/2017	45488	235

Sample type	Gut/swab region	Fork length (cm)	Weight (g)	Location	Site	Cage ID	Date sample collected	library size	# bacterial OTUs
Enteritis - D	SK	29	270	Lincoln	Northern Site	17-8	11/04/2017	28389	352
Enteritis - D	GL	29	270	Lincoln	Northern Site	17-8	11/04/2017	37438	199
Enteritis - D	SK	29	290	Lincoln	Northern Site	17-8	11/04/2017	33856	331
Enteritis - D	GL	29	290	Lincoln	Northern Site	17-8	11/04/2017	34164	169

Abbreviations: D, disease; FG, foregut; GL, gill; H, healthy; HG, hindgut; HI, healthy intermediate; MG, midgut; SK, skin.
[1] Samples removed due to low sequence reads.

Table 3.3.1.3.15. Sample information pertaining to the Healthy vs Disease Extension component.

Experiment data-set	No. of samples	Total reads	Median library size	Range	Bacterial OTUs post- filtering
Healthy vs disease extension					
i) gut scrapings + SW	218	11,819,766	49,821	1,398-220,888	593
ii) gill swabs	177	12,023,824	59,624	16,780-170,789	787
iii) skin swabs	177	10,873,759	54,668	17,244-187,921	923

Abbreviations: SW, seawater.

Table 3.3.1.3.16. Summary of the three data subsets that were analysed from the 2016 and 2017 enteritis sampling events.

Subset	Year Class	Run	Site (cage ID)	Feed manufacturer, feed type (days on feed prior to sampling)	Healthy fish mean weight (kg)	Disease fish mean weight (kg)
1. Temporal scale – same feed type and fish size, variable site[1] (for disease samples), year class and feed manufacturer	2015 (sampled 2 Mar 16)	1/2	Bickers/NS[1] (15-002B/15-004A)	A, 9mm (276 d/307 d)	3.4	1.4
	2016 (sampled 28 Feb 17)	2	Bickers (16-4GS)	B, 9mm (151 d)	3.5	1.7
	2016 (sampled 28 Feb 17)	2	Bickers (16-3A)	C, 9mm (120 d)	3.6	1.6
2. Same feed manufacturer and feed type – year class, site and fish size variable	2016	2	Bickers (16-4GS)	B, 9mm (151 d)	3.5	1.7
	2017	2	AB (17-4)	B, 9mm (44d)	1.4	1.2
	2017	1	PB (17-2)	B, 9mm (25 d)	1.2	0.4
	2017	3	NS (17-8)	B, 9mm (6 d)	0.8	0.2
3. Same feed manufacturer, site and year class – feed type and fish size variable	2017	1	PB (17-2)	B, 9mm (25 d)	1.2	0.6
	2017	2	PB (17-3)	B, 6mm (54 d)	0.8	0.5

Abbreviations: AB, Arno Bay; NS, Northern Site; PB, Point Boston.
[1] All sea-cages containing healthy fish were located at the same site (Bickers), however for the 2016 disease samples, sea-cage 15-004A at Northern Site was sampled (~6 km from Bickers).

Table 3.3.1.3.17. One-way PERMANOVA: Pairwise test between the healthy before and healthy after samples from two sea-cages.[1]

Health status, site, sea-cage	*P*	Significant?
Before AB 17-4, After AB 17-4	**0.0003**	Yes
Before AB 17-8, After NS 17-8	**0.0004**	Yes
Before AB 17-4, Before AB 17-8	**0.0008**	Yes
Before AB 17-4, After NS 17-8	0.1043	No
After AB 17-4, Before AB 17-8	**0.0001**	Yes
After AB 17-4, After NS 17-8	**0.0033**	Yes

Abbreviations: AB, Arno Bay; NS, Northern Site; PB.
[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.3.18. One-way PERMANOVA: Pairwise test between the healthy samples, disease samples and healthy vs disease samples for data subset 1 'temporal scale'.[1]

Health status, site, sea-cage	*P*	Significant?
H Bi 15-002B, H Bi 16-4GS	0.4238	No
H Bi 15-002B, H Bi 16-3A	0.5186	No
H Bi 16-4GS, H Bi 16-3A	0.8893	No
D NS 15-004A, D Bi 16-4GS	0.0610	No
D NS 15-004A, D Bi 16-3A	**0.0003**	Yes
D Bi 16-4GS, D Bi 16-3A	0.1051	No
H Bi 15-002B, D NS 15-004A	**0.0168**	Yes
H Bi 16-4GS, D Bi 16-4GS	0.8494	No
H Bi 16-3A, D Bi 16-3A	**0.0225**	Yes

Abbreviations: Bi, Bickers; D, Disease; H, Healthy; NS, Northern Site.
[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.3.19. Those bacterial taxa that have the greatest contribution towards the observed difference between each enteritis health class.[1]

A. D NS 15-004A, D Bi 16-3A

Bacterial taxa (RDP similarity S_ab score)_OTU no.	**Av. abundance D NS 15-004A (Δ)**	**Av. abundance D Bi 16-3A (O)**	**% Contribution**	**Highest abundant group**
Mycoplasma insons (0.428)_OTU 1	72.79	11.00	39.29	Δ
Vibrio sp. V776/*Aliivibrio finisterrensis* (1.000)_OTU 4	8.85	24.64	15.64	O
Photobacterium sp. (0.875)_OTU 10950	5.10	17.21	10.99	O
Pseudoalteromonas atlantica (1.000)_OTU 37	0.01	8.65	5.15	O
Brevinema andersonii (0.700)_OTU 25	6.76	1.32	4.45	Δ

B. H Bi 16-3A, D Bi 16-3A

Bacterial taxa (RDP similarity S_ab score)_OTU no.	**Av. abundance H Bi 16-3A (Δ)**	**Av. abundance D Bi 16-3A (O)**	**% Contribution**	**Highest abundant group**
Mycoplasma insons (0.428)_OTU 1	45.27	11.00	25.89	Δ
Vibrio sp. V776/*Aliivibrio finisterrensis* (1.000)_OTU 4	16.41	24.64	17.90	O
Photobacterium sp. (0.875)_OTU 10950	0.35	17.21	10.01	O
Photobacterium phosphoreum/*P. iliopiscarium* (1.000)_OTU 28	10.21	0.39	6.04	Δ
Pseudoalteromonas atlantica (1.000)_OTU 37	1.41	8.65	5.24	O
Anabaena cylindrica (0.377)_OTU 16	7.20	2.02	4.94	Δ
Anabaena cylindrica (0.350)_OTU12360	7.78	0.65	4.79	Δ
Synechococcus sp. (1.000)_OTU 32	0.30	6.37	3.68	O

C. H Bi 15-002B, D NS 15-004A

Bacterial taxa (RDP similarity S_ab score)_OTU no.	**Av. abundance H Bi 15-002B (Δ)**	**Av. abundance D NS 15-004A (O)**	**% Contribution**	**Highest abundant group**
Mycoplasma insons (0.428)_OTU 1	30.02	72.79	38.26	O
Vibrio sp. V776/*Aliivibrio finisterrensis* (1.000)_OTU 4	21.89	8.85	18.24	Δ
Photobacterium damselae subsp. *damselae*/*P. leiognathi* (1.000)_OTU 6	12.99	1.63	9.58	Δ
Brevinema andersonii (0.700)_OTU 25	7.62	6.76	8.85	Δ
Photobacterium phosphoreum/*P. iliopiscarium* (1.000)_OTU 28	9.89	1.32	7.15	Δ

[1] The discernible bacterial taxa were derived using the Similarity Percentage (SIMPER) algorithm in the PRIMER program, setting a cut-off at 75% cumulative contribution, in order to give only the top few bacterial taxa that contribute to the difference between the groups.

Table 3.3.1.3.20. One-way PERMANOVA: Pairwise test between the healthy samples, disease samples and healthy vs disease samples for data subset 2 'same feed manufacturer and feed type'.[1]

Health status, site, sea-cage	*P*	Significant?
H Bi 16-4GS, H AB 17-4	**0.0002**	Yes
H Bi 16-4GS, H PB 17-2	**0.0121**	Yes
H Bi 16-4GS, H NS 17-8	**0.0003**	Yes
H AB 17-4, H PB 17-2	**0.0007**	Yes
H AB 17-4, H NS 17-8	**0.0037**	Yes
H PB 17-2, H NS 17-8	**0.0020**	Yes
D Bi 16-4GS, D AB 17-4	**0.0007**	Yes
D Bi 16-4GS, D PB 17-2	**0.0011**	Yes
D Bi 16-4GS, D NS 17-8	**0.0008**	Yes
D AB 17-4, D PB 17-2	0.2069	No
D AB 17-4, D NS 17-8	**0.0048**	Yes
D PB 17-2, D NS 17-8	**0.0068**	Yes
H Bi 16-4GS, D Bi 16-4GS	0.8527	No
H AB 17-4, D AB 17-4	**0.0030**	Yes
H PB 17-2, D PB 17-2	0.0679	No
H NS 17-8, D NS 17-8	0.1852	No

Abbreviations: AB, Arno Bay; Bi, Bickers; D, Disease; H, Healthy; NS, Northern Site; PB, Point Boston.
[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.3.21. Those bacterial taxa that have the greatest contribution towards the observed difference between healthy and disease fish in sea-cage 17-4.[1]

Bacterial taxa (RDP similarity S_ab score)_OTU no.	Av. abundance H AB 17-4 (Δ)	Av. abundance D AB 17-4 (O)	% Contribution	Highest abundant group
Vibrio harveyi/*Aliivibrio fischeri* (1.000)_OTU 10	14.16	67.20	1.80	O
Photobacterium sp. (0.875)_OTU 10950[2]	25.18	10.20	0.91	Δ
Photobacterium damselae subsp. *damselae*/*P. leiognathi* (1.000)_OTU 6	15.49	0.73	0.53	Δ
Neorickettsia helminthoeca (0.533)_OTU 26	9.99	0.66	0.63	Δ

Abbreviations: AB, Arno Bay; D, disease; H, healthy.

[1] The discernible bacterial taxa were derived using the Similarity Percentage (SIMPER) algorithm in the PRIMER program, setting a cut-off at 75% cumulative contribution, in order to give only the top few bacterial taxa that contribute to the difference between the groups.

[2] Although OTU 10950 was enriched in the healthy compared to disease samples, when comparing the healthy before to the disease fish from this sea-cage, average abundance increased from 0.01 to 10.20.

Table 3.3.1.3.22. One-way PERMANOVA: Pairwise test between the healthy samples, disease samples and healthy vs disease samples for data subset 3 'same feed manufacturer, site and year class'.[1]

Health status, site, sea-cage	*P*	Significant?
H PB 17-2, H PB 17-3	0.3265	No
D PB 17-2, D PB 17-3	0.2051	No
H PB 17-2, D PB 17-2	0.0625	No
H PB 17-3, D PB 17-3	**0.0032**	Yes

Abbreviations: D, Disease; H, Healthy; PB, Point Boston.
[1] Significant difference denoted by $P < 0.05$, bolded if significant.

Table 3.3.1.3.23. Those bacterial taxa that have the greatest contribution towards the observed difference between healthy and disease fish in sea-cage 17-3.[1]

Bacterial taxa (RDP similarity S_ab score)_OTU no.	Av. abundance H PB 17-3 (Δ)	Av. abundance D PB 17-3 (O)	% Contribution	Highest abundant group
Vibrio harveyi/*Aliivibrio fischeri* (1.000)_OTU 10	1.05	42.22	24.09	O
Photobacterium sp. (0.875)_OTU 10950	19.53	40.38	23.97	O
Vibrio sp. V776/*Aliivibrio finisterrensis* (1.000)_OTU 4	32.89	7.27	19.97	Δ
Mycoplasma insons (0.428)_OTU 1	20.48	0.02	11.91	Δ

Abbreviations: D, disease; H, healthy; PB, Point Boston.

[1] The discernible bacterial taxa were derived using the Similarity Percentage (SIMPER) algorithm in the PRIMER program, setting a cut-off at 75% cumulative contribution, in order to give only the top few bacterial taxa that contribute to the difference between the groups.

Table 3.3.1.3.24. Greatest contribution of *Photobacterium* sp. (OTU 10950) towards the observed difference between healthy (Δ) and disease (O) (enteritis) fish at each site and sea-cage.[1]

Site and sea-cage	Av. abundance Healthy fish (Δ)	Av. abundance Disease fish (O)	Highest abundant group
2016 Bickers/Northern Site	0.93	2.81	O
Arno Bay 17-4[2]	0.01	10.20	O
Arno Bay/Northern Site 17-8	1.67	8.76	O
Point Boston 17-2	11.72	13.45	O
Point Boston 17-3	19.53	40.38	O
Bickers 16-4GS	8.46	9.84	O
Bickers 16-3A	0.35	17.21	O

[1] Derived using the Similarity Percentage (SIMPER) algorithm in the PRIMER program.
[2] Healthy before samples compared to disease samples from sea-cage 17-4.

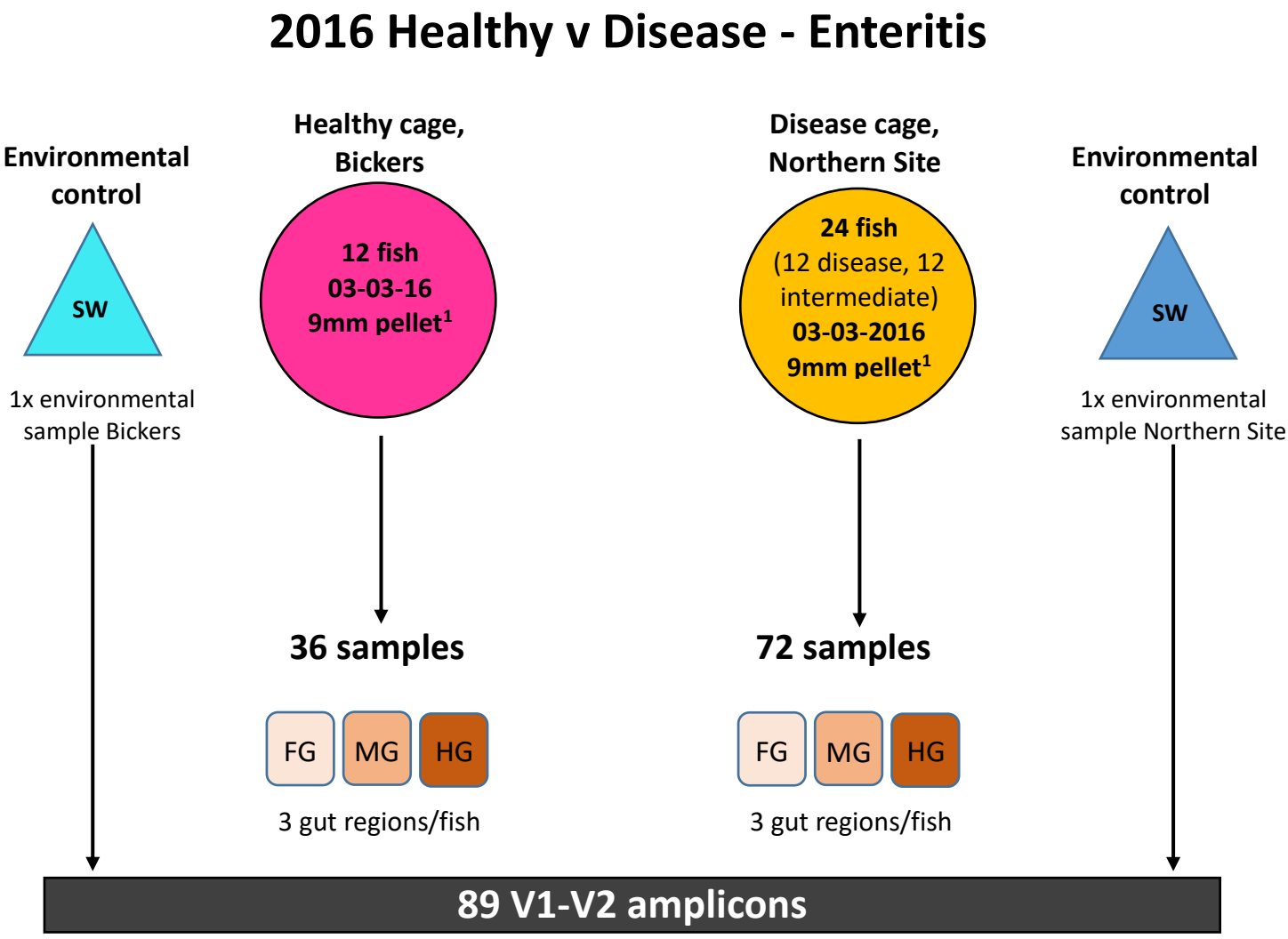


Figure 3.3.1.3.1. Experimental design for the 2016 health vs disease enteritis component at Port Lincoln.
Abbreviations: FG, foregut; HG, hindgut; MG, midgut; SW, seawater.
[1] Pellet manufactured by same feed company.

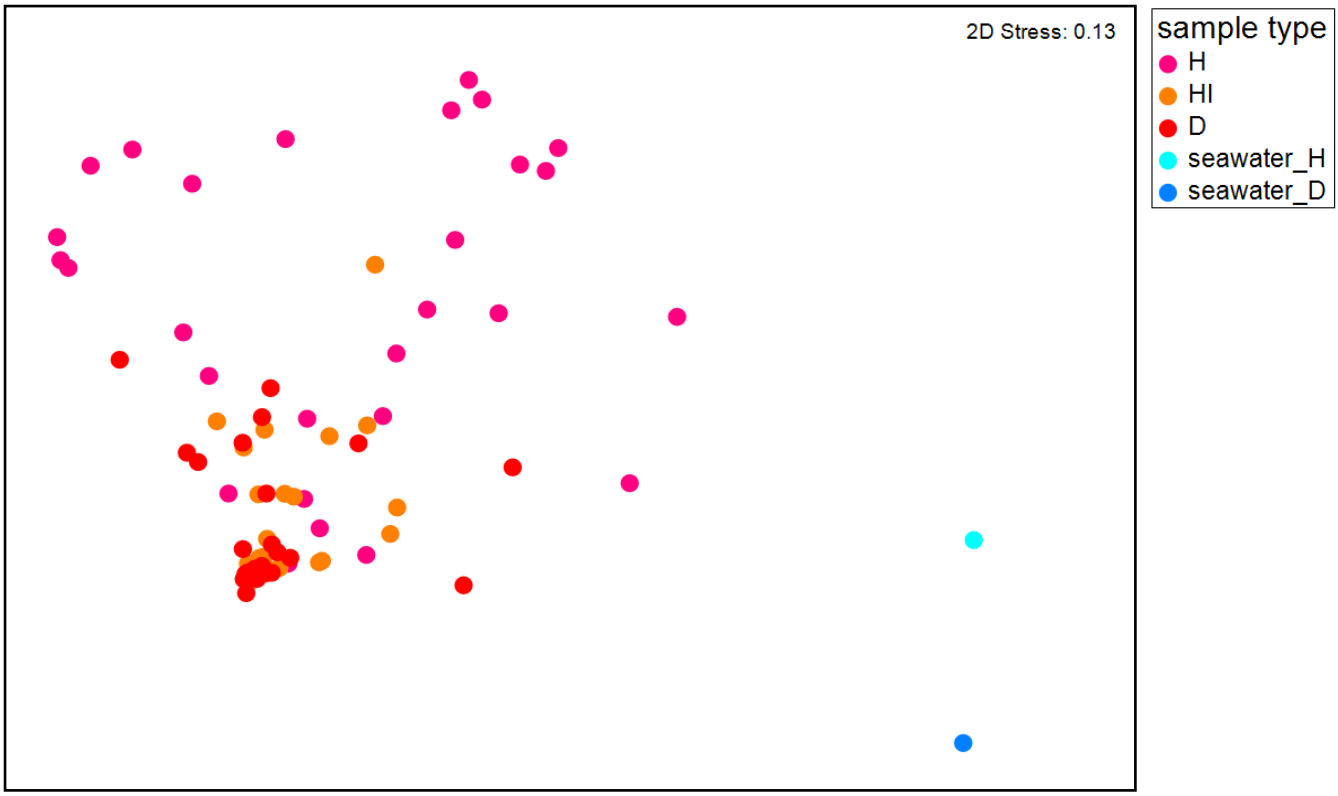


Figure 3.3.1.3.2. Difference between the global community structure of all 89 samples for the 2016 healthy vs disease component as analysed by non-metric multidimensional scaling (nMDS).[1]
Abbreviations: D, disease; H, healthy; HI, healthy intermediate.
[1] Gut scrapings from the three gut regions (fore-, mid- and hindgut) of 12 healthy, 12 healthy intermediate and 12 disease YTK along with a water sample from the healthy sea-cage site and disease sea-cage site.

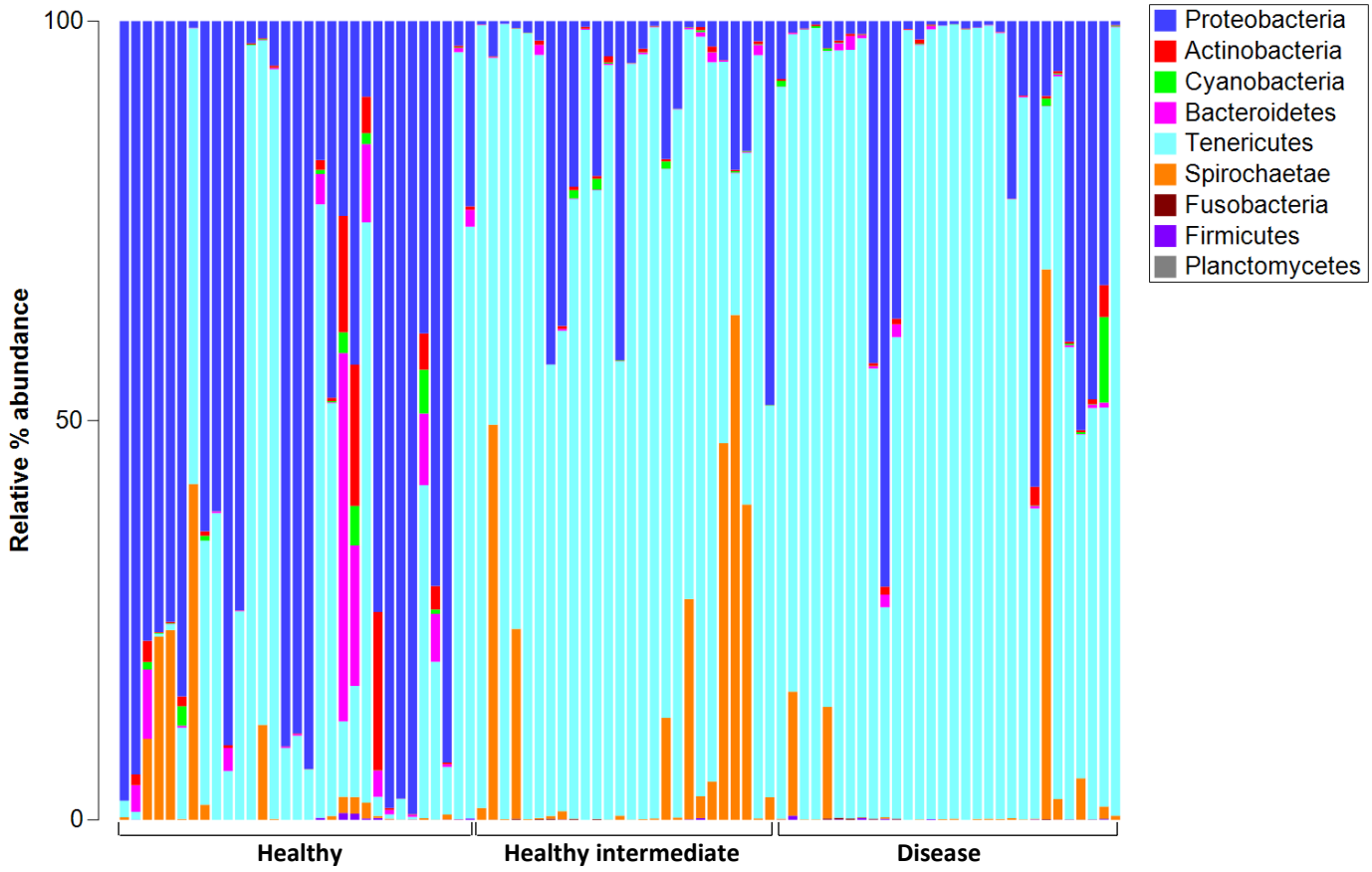


Figure 3.3.1.3.3. Relative percent abundance of bacterial phyla associated with gut scrapings from YTK from the three enteritis groups; healthy, healthy intermediate and disease.

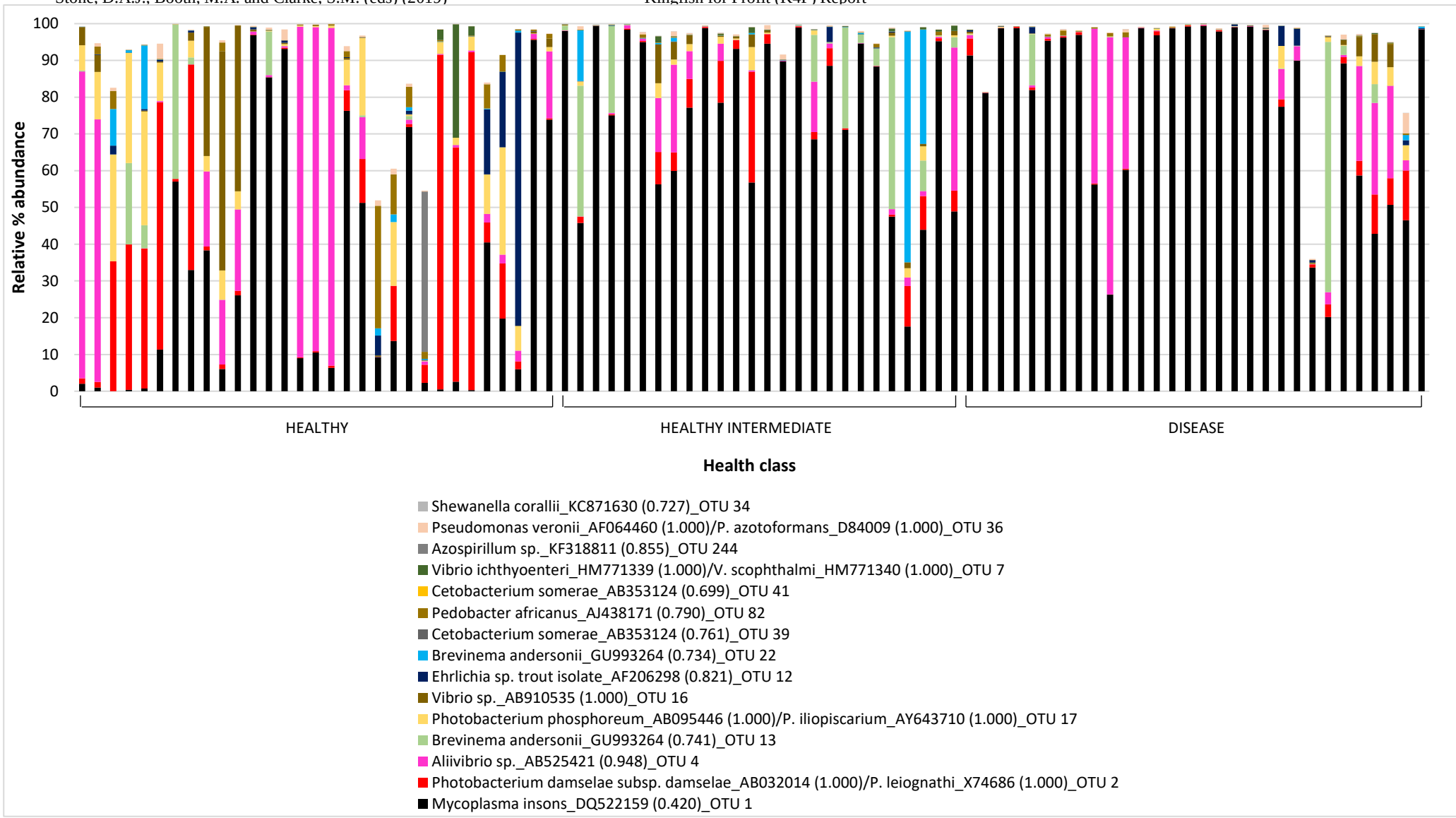


Figure 3.3.1.3.4. Relative percent abundance of the 15 most abundant bacterial OTUs in the gut scraping samples from the three entertitis groups; healthy, healthy intermediate and disease.

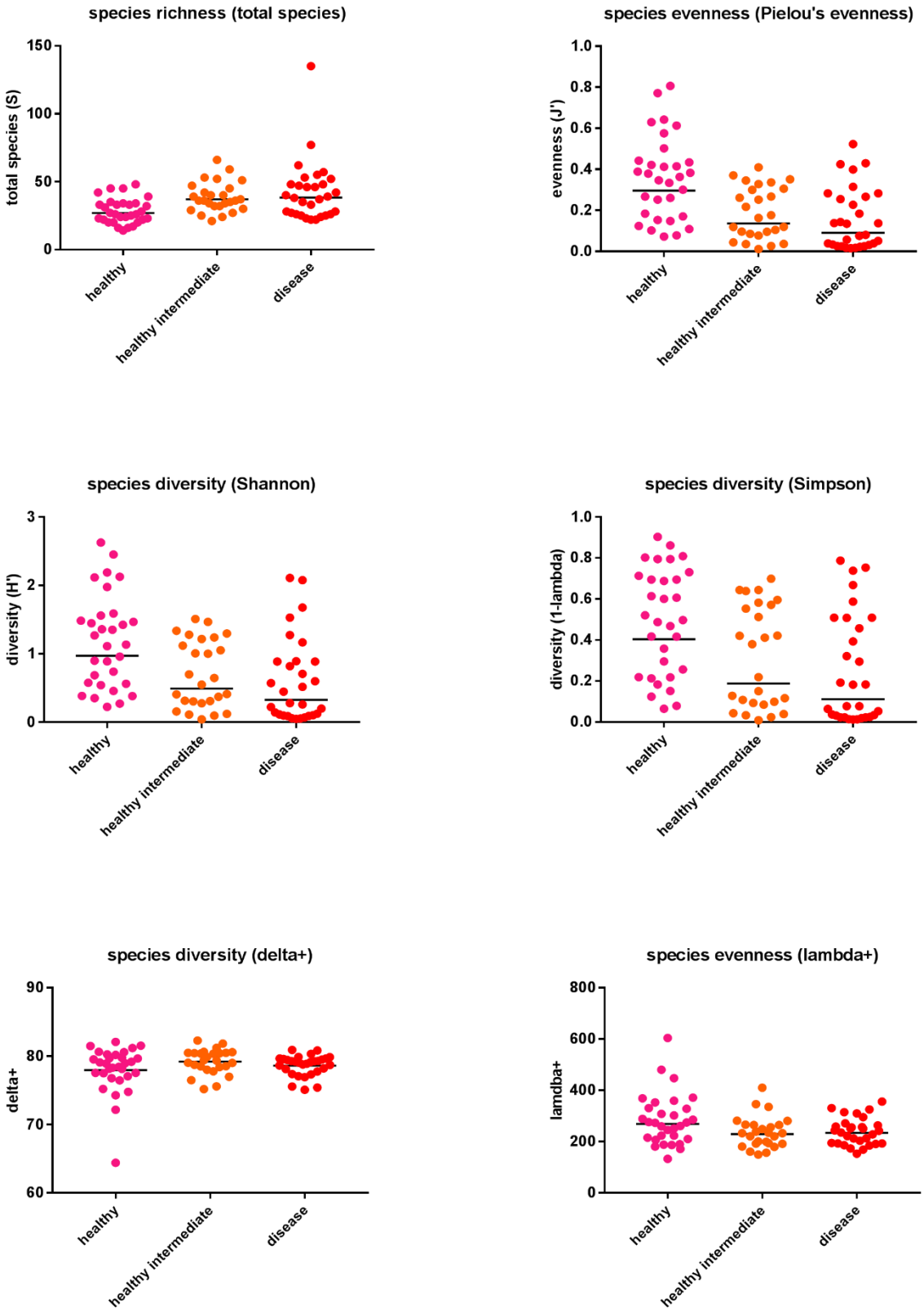


Figure 3.3.1.3.5. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for enteritis samples.[1]

[1] Mean values are plotted for each of the groups of interest (healthy vs healthy intermediate vs disease).

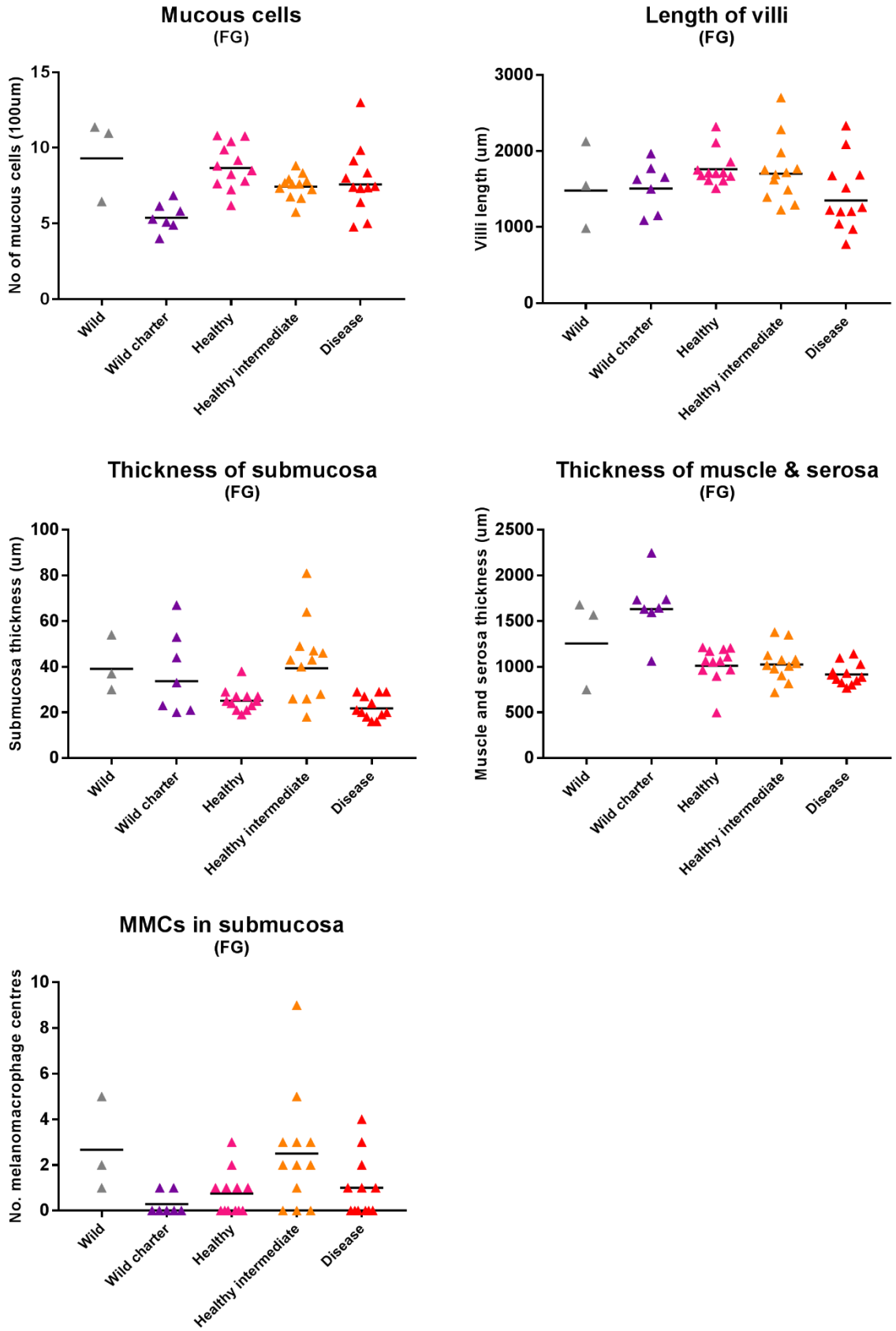


Figure 3.3.1.3.6. Histological parameters evaluated from the foregut tissue of wild, wild charter, healthy, healthy intermediate and disease fish (enteritis cohort).
Abbreviations: FG, foregut; MMCs, melanomacrophage centres.

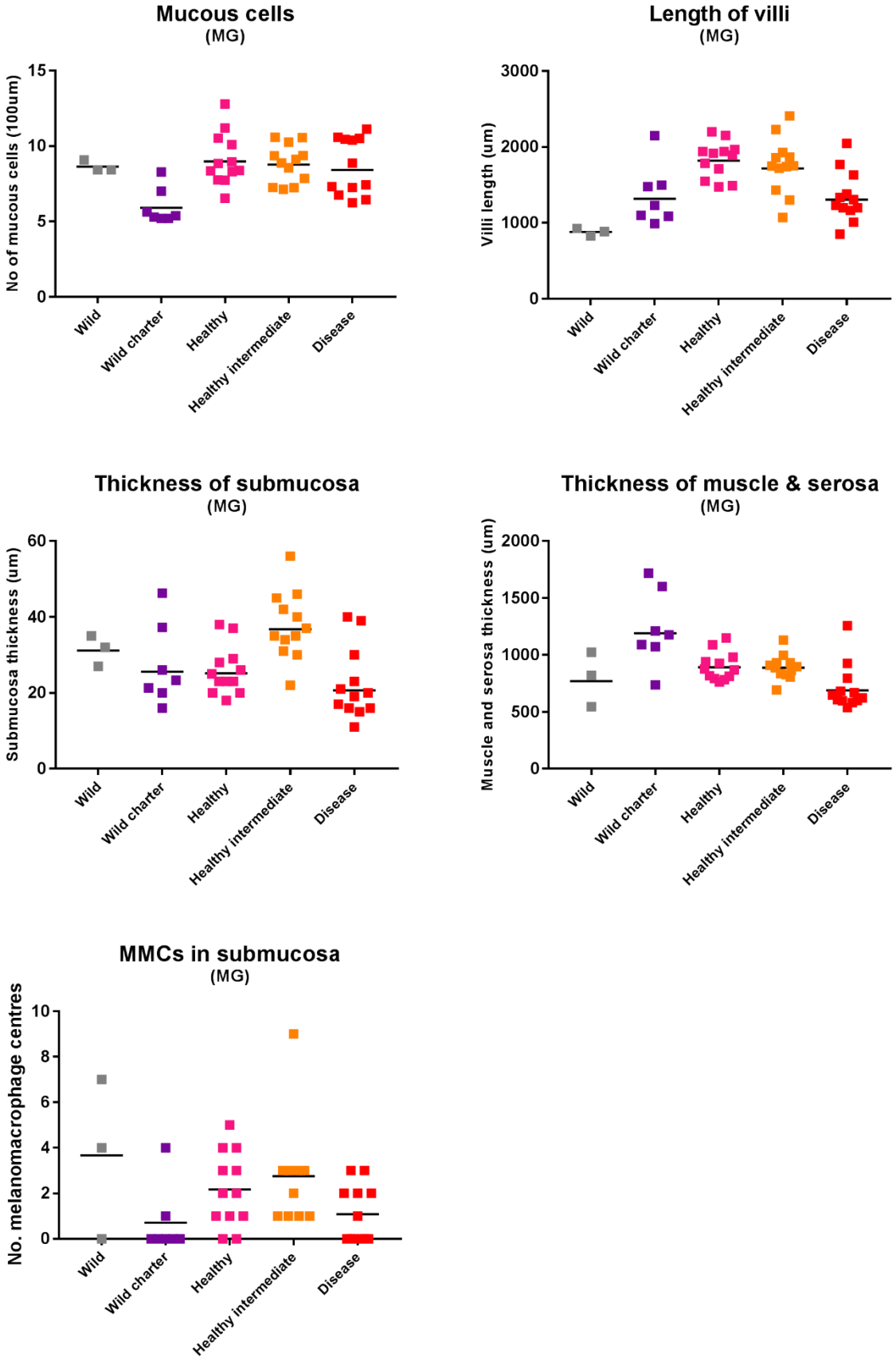


Figure 3.3.1.3.7. Histological parameters evaluated from the midgut tissue of wild, wild charter, healthy, healthy intermediate and disease fish (enteritis cohort).
Abbreviations: MG, midgut; MMCs, melanomacrophage centres.

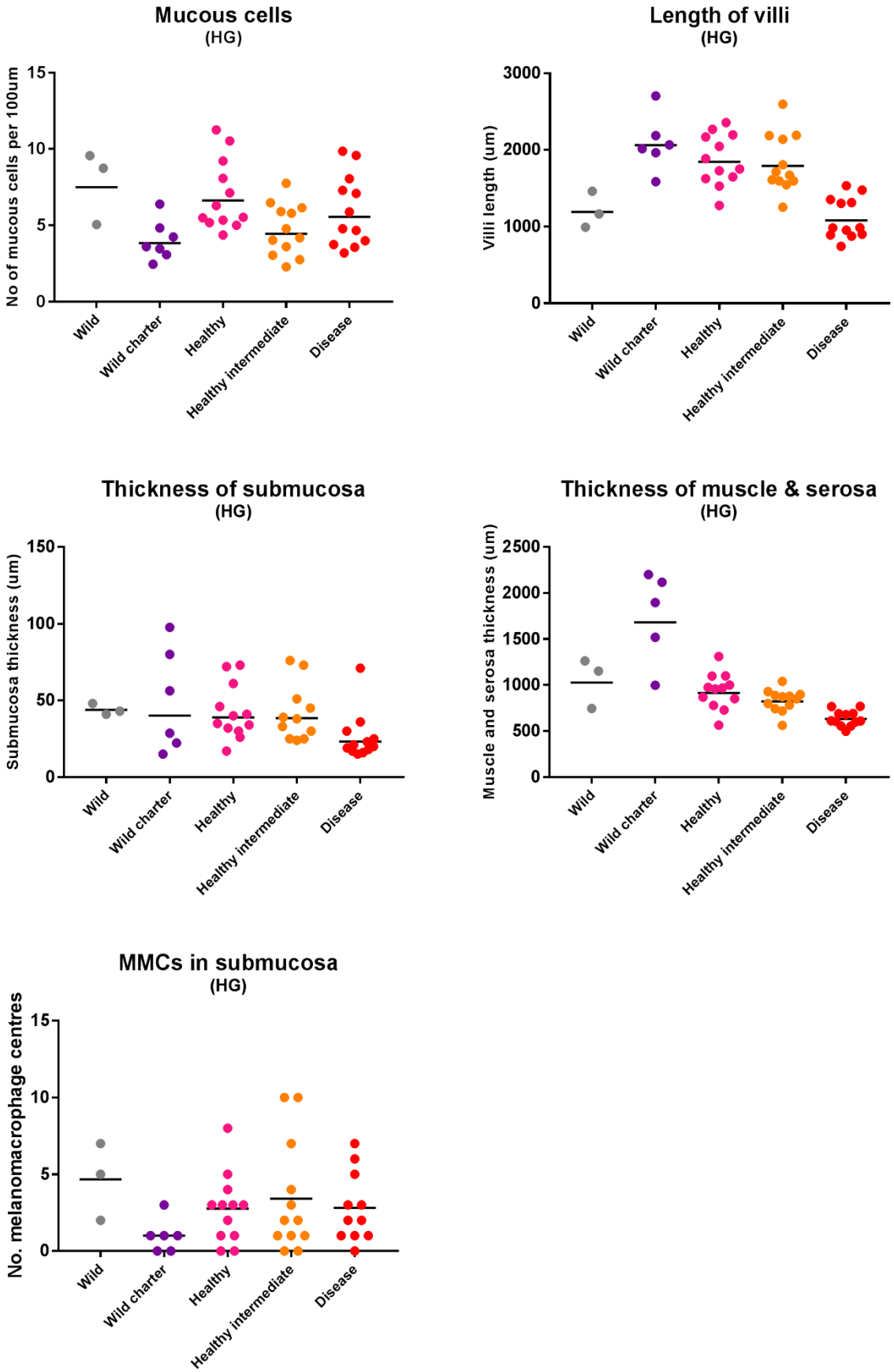


Figure 3.3.1.3.8. Histological parameters evaluated from the hindgut tissue of wild, wild charter, healthy, healthy intermediate and disease fish (enteritis cohort).

Abbreviations: HG, hindgut; MMCs, melanomacrophage centre

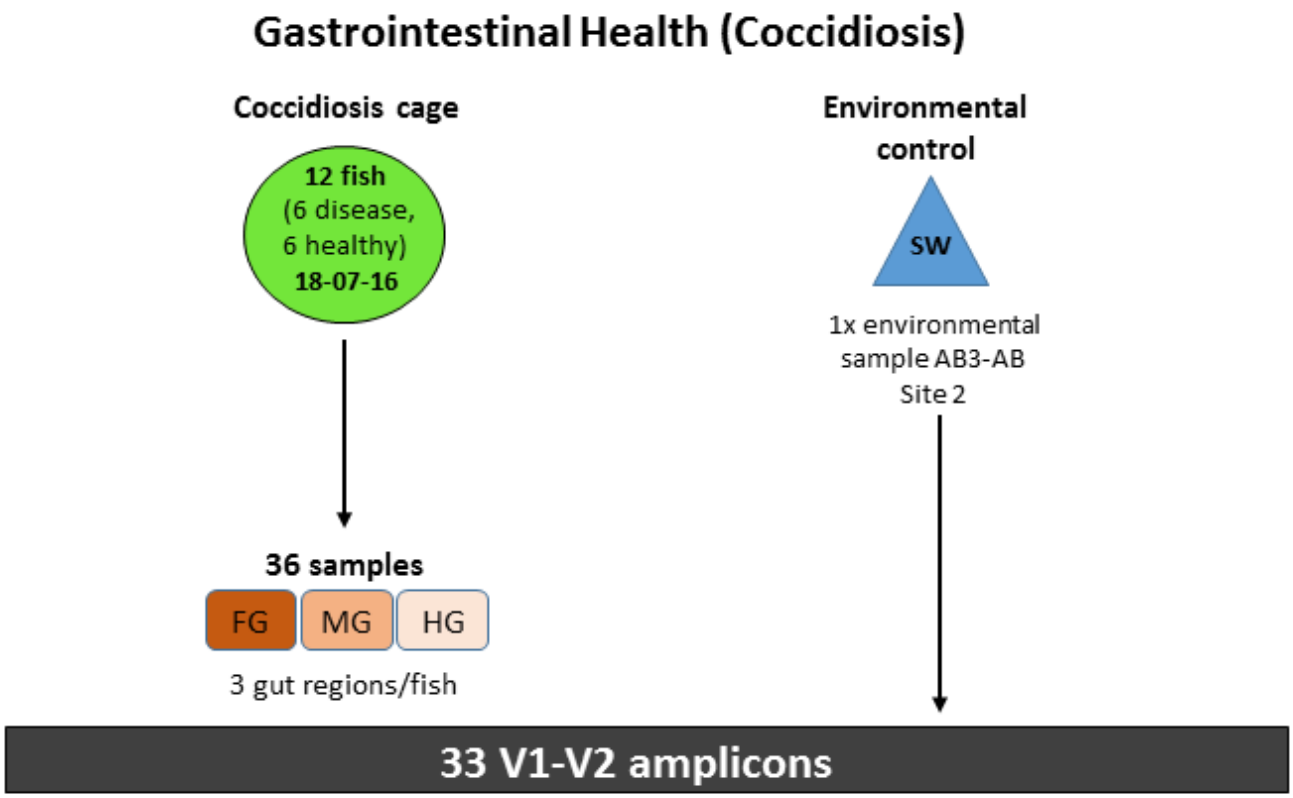


Figure 3.3.1.3.9. Experimental design for the coccidiosis gastrointestinal health component at Arno Bay.

Abbreviations: AB, Arno Bay; FG, foregut; HG, hindgut; MG, midgut; SW, seawater.

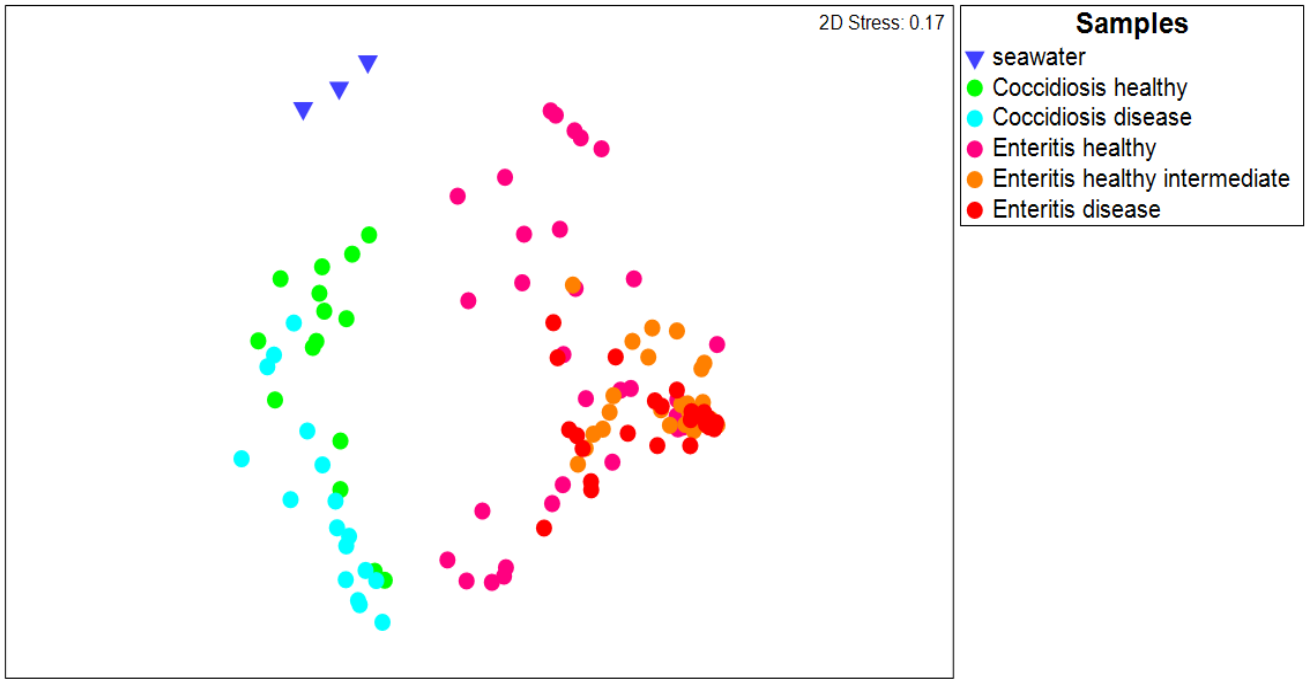


Figure 3.3.1.3.10. Difference between the global community structure of all 122 samples for the gastrointestinal health component (2016 gut enteritis vs coccidiosis) as analysed by non-metric multidimensional scaling (nMDS).[1]

[1] Gut scrapings from the three gut regions (fore-, mid- and hindgut) of 12 healthy, 12 healthy intermediate and 12 disease YTK (2016 gut enteritis, Port Lincoln), six healthy and six disease YTK (coccidiosis, Arno Bay) and three environmental water samples from the enteritis healthy sea-cage site, enteritis disease sea-cage site and coccidiosis sea-cage site.

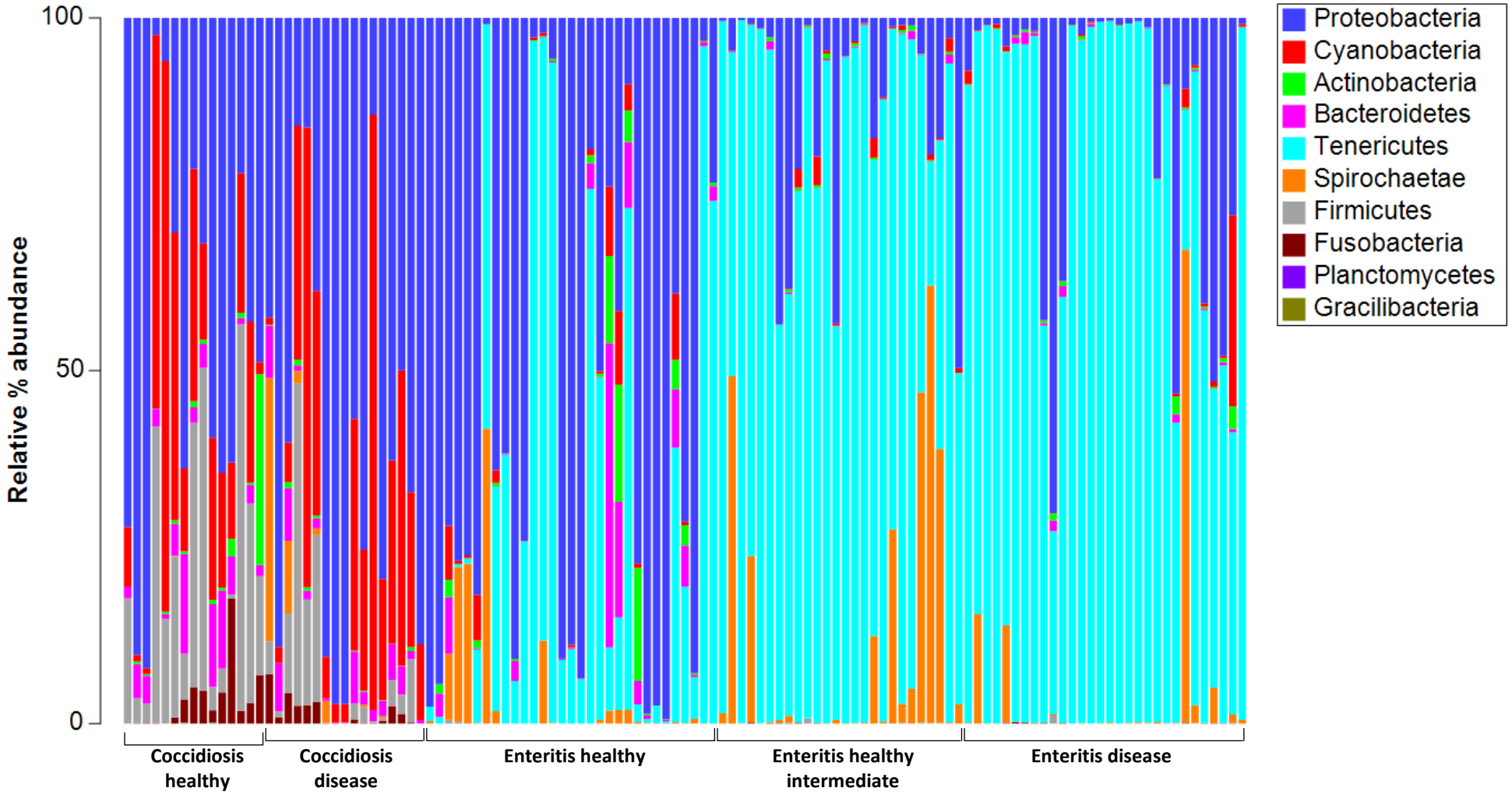


Figure 3.3.1.3.11. Relative percent abundance of bacterial phyla associated with gut scrapings from YTK from the gastrointestinal health component (coccidiosis healthy, coccidiosis disease, enteritis healthy, enteritis healthy intermediate, enteritis disease).

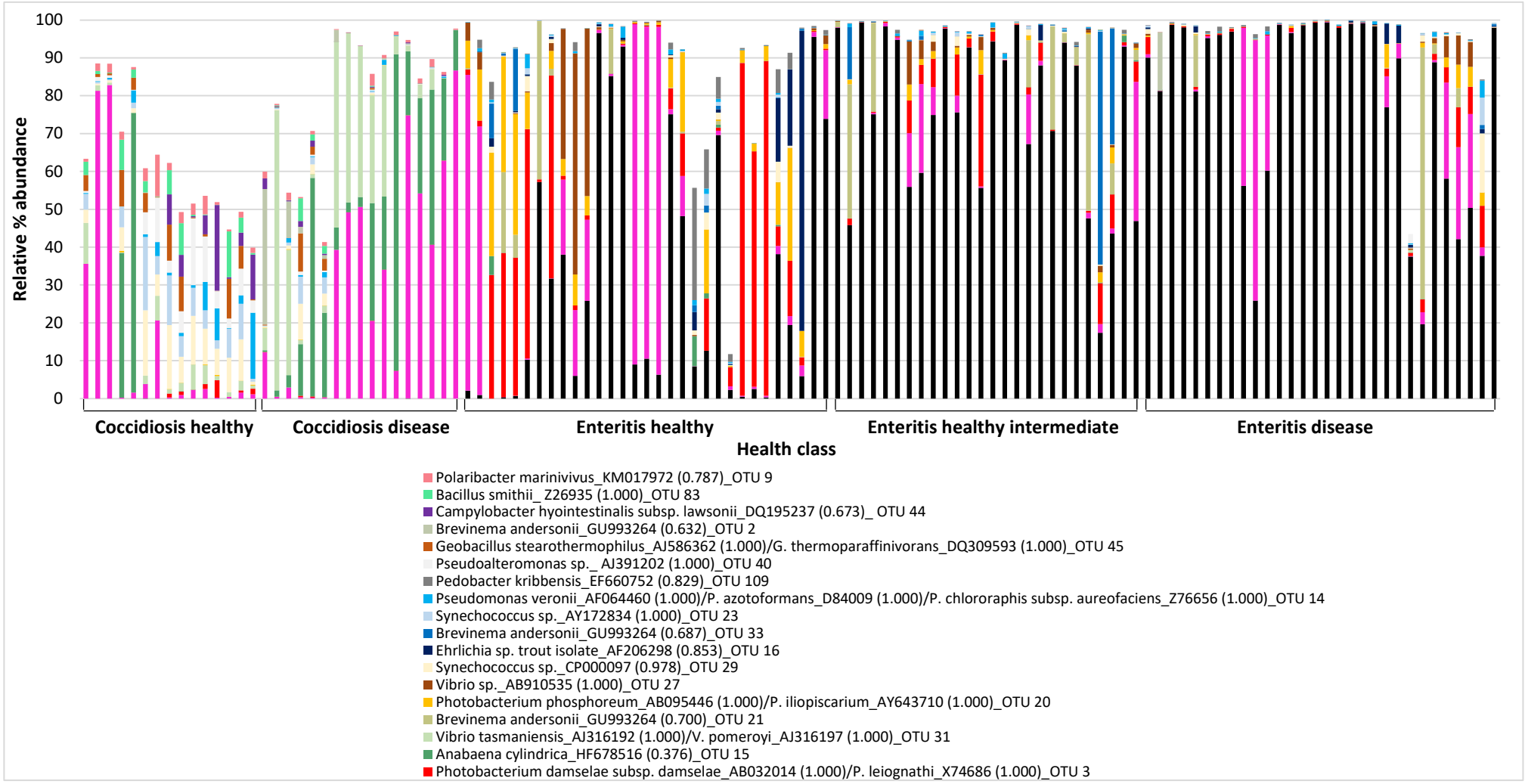


Figure 3.3.1.3.12. Relative percent abundance of the 20 most abundant bacterial OTUs in the samples representing coccidiosis healthy, coccidiosis disease, enteritis healthy, enteritis healthy intermediate and enteritis disease.

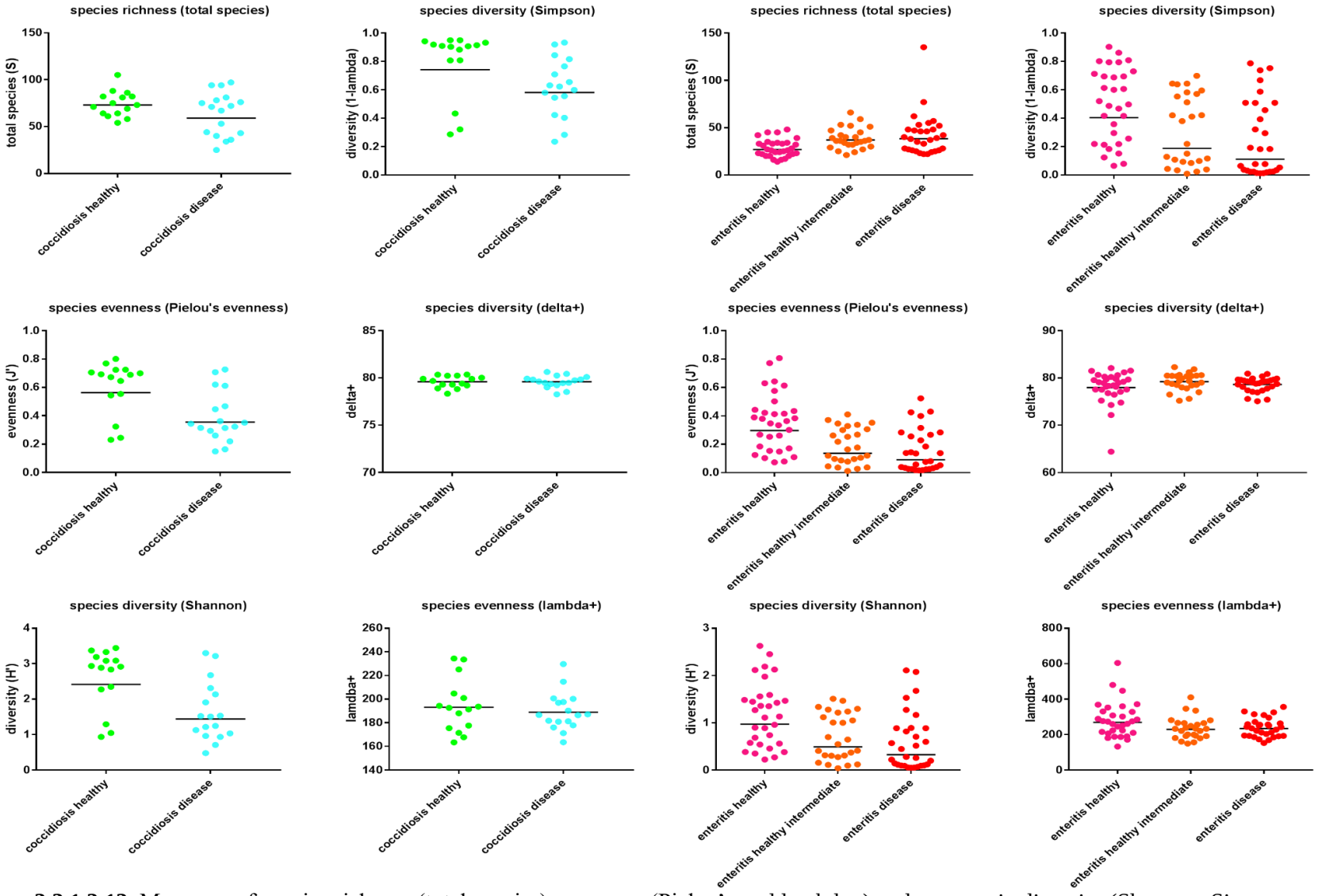
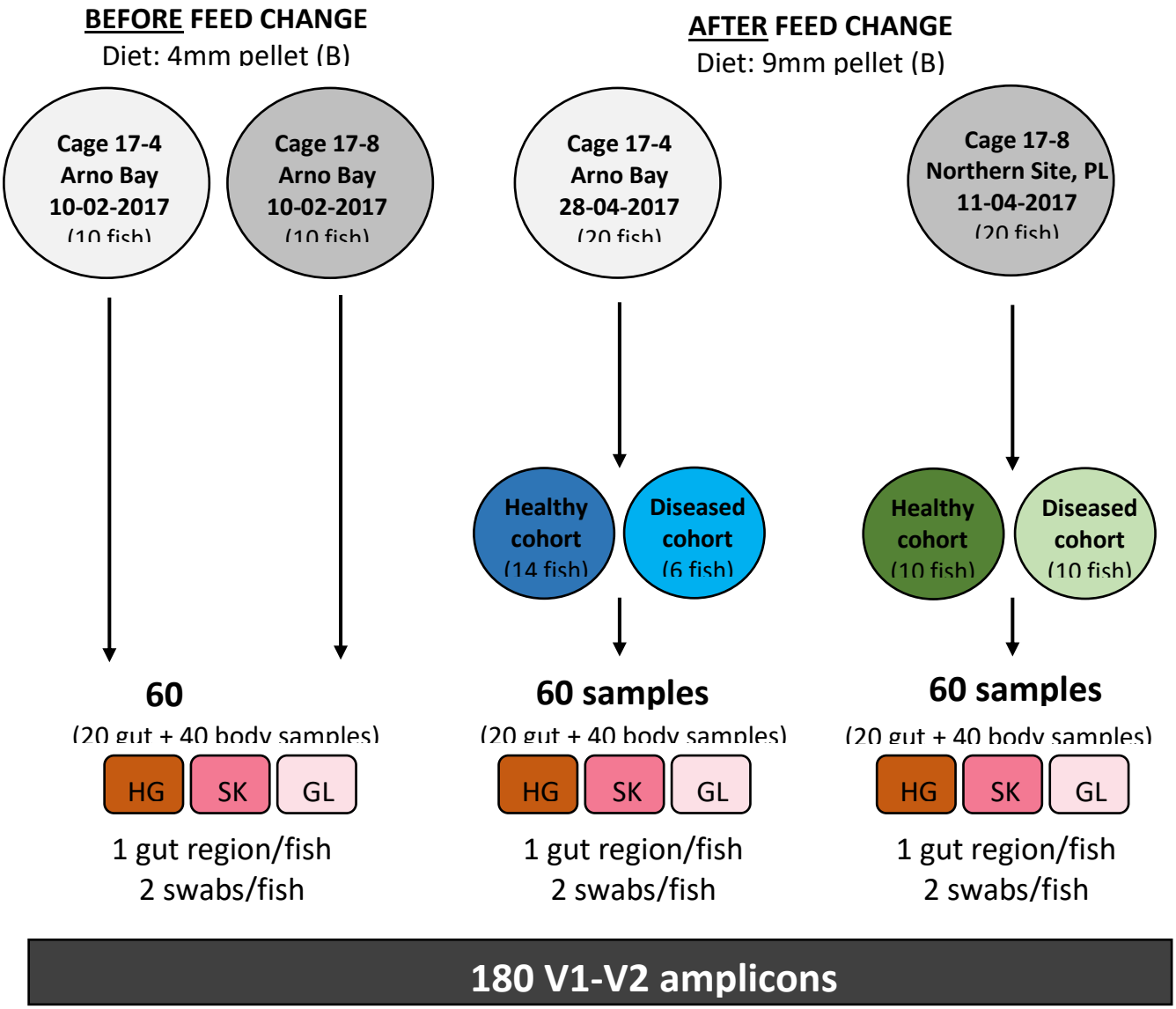


Figure 3.3.1.3.13. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for coccidiosis and enteritis samples.[1]

[1] Mean values are plotted for each of the groups of interest (coccidiosis vs enteritis).

A) 2017 enteritis sampling: ARNO BAY



180 V1-V2 amplicons

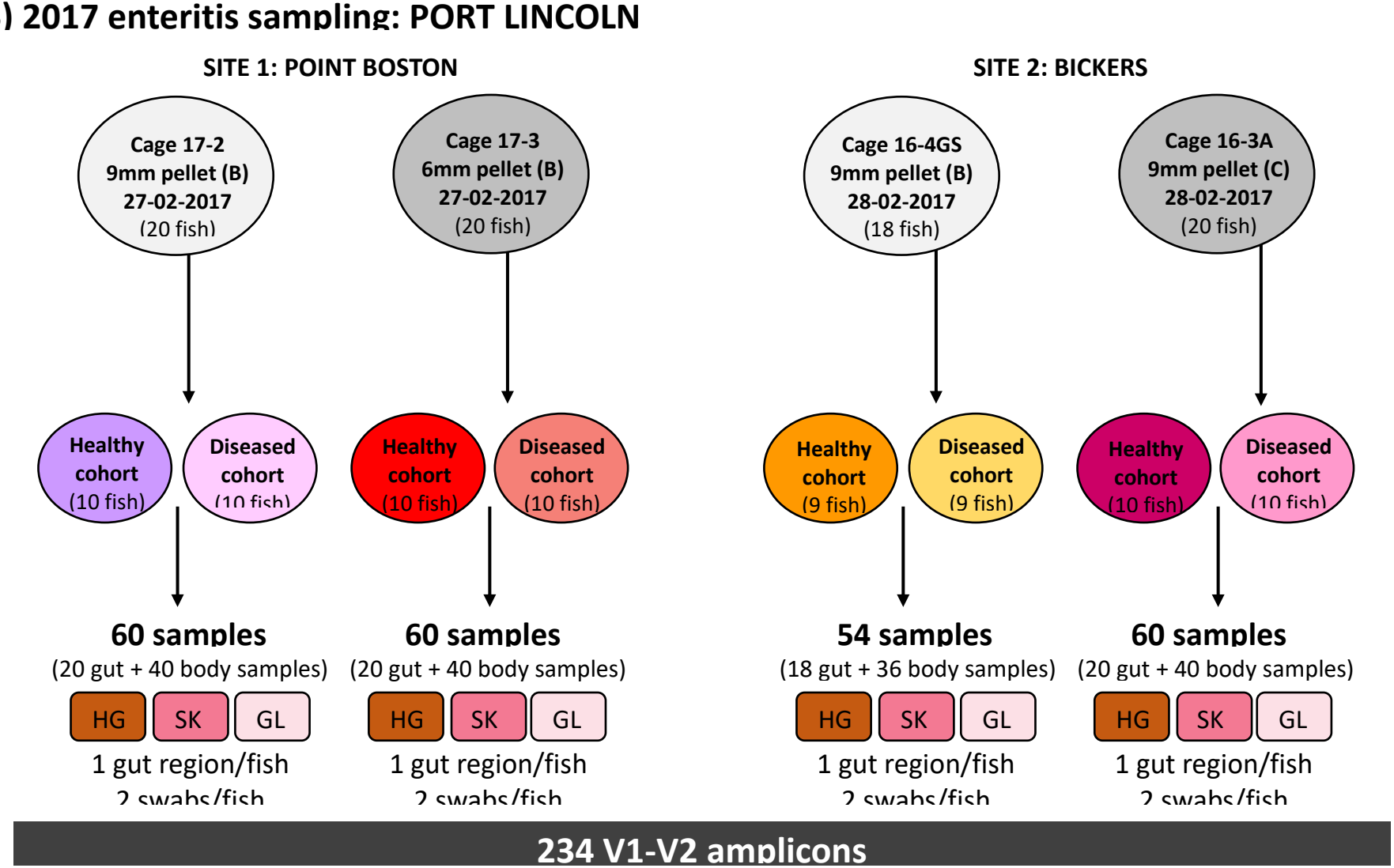


Figure 3.3.1.3.14. Experimental design for the 2017 gastrointestinal health extension (including swab samples) at A) Arno Bay before and after diet changes and B) Port Lincoln where poor health had been recorded.
Abbreviations: A, feed company A; B, feed company B; C, feed company C; GL, gill swab; HG, hindgut; SK, skin swab.

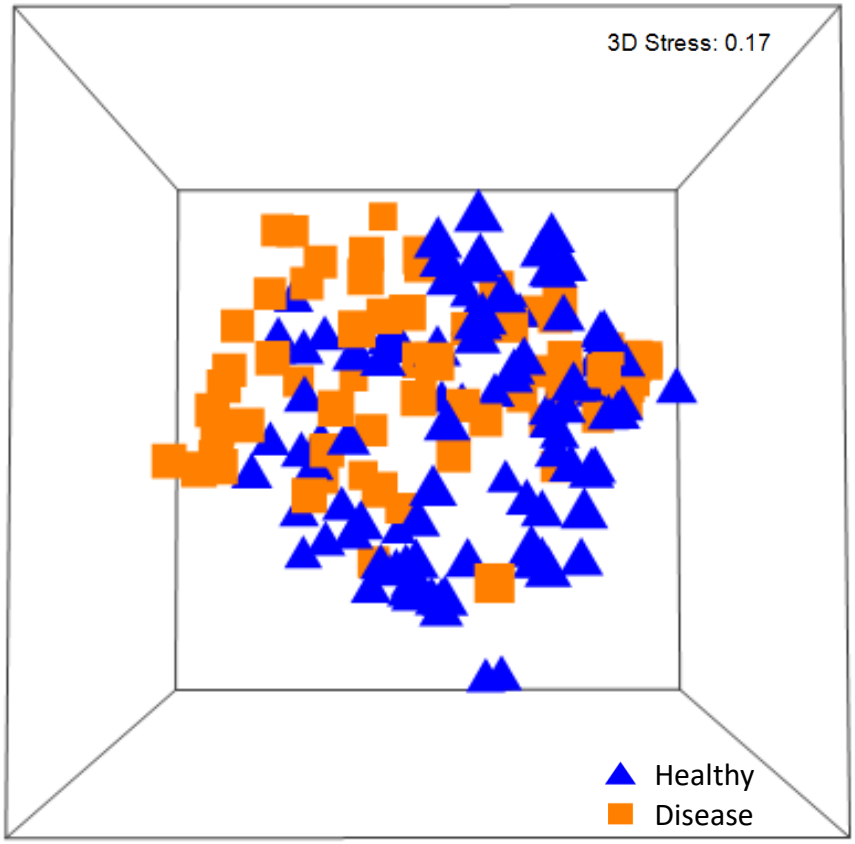
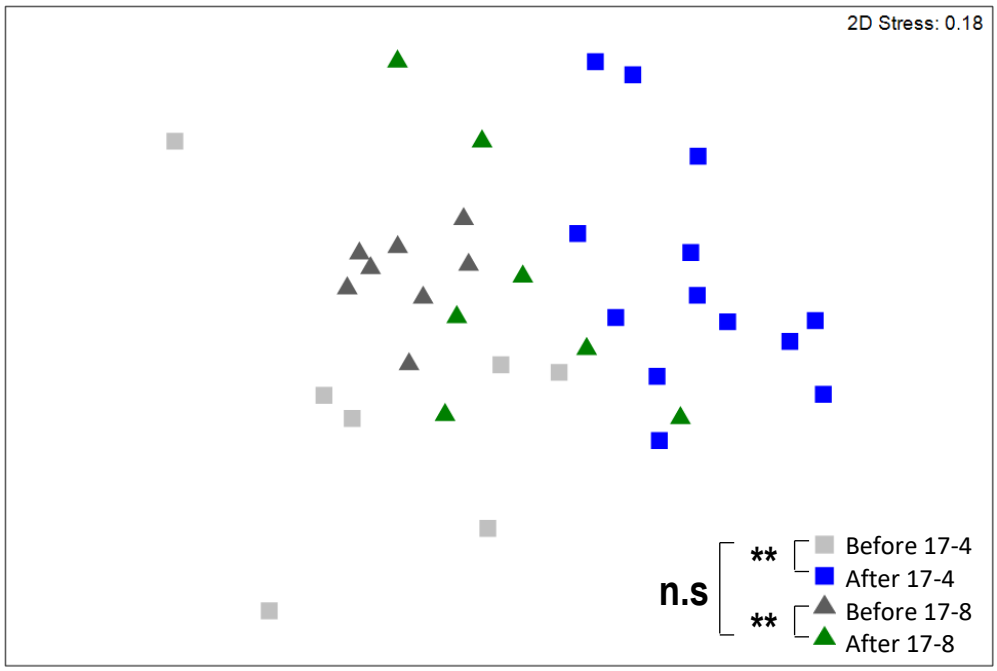
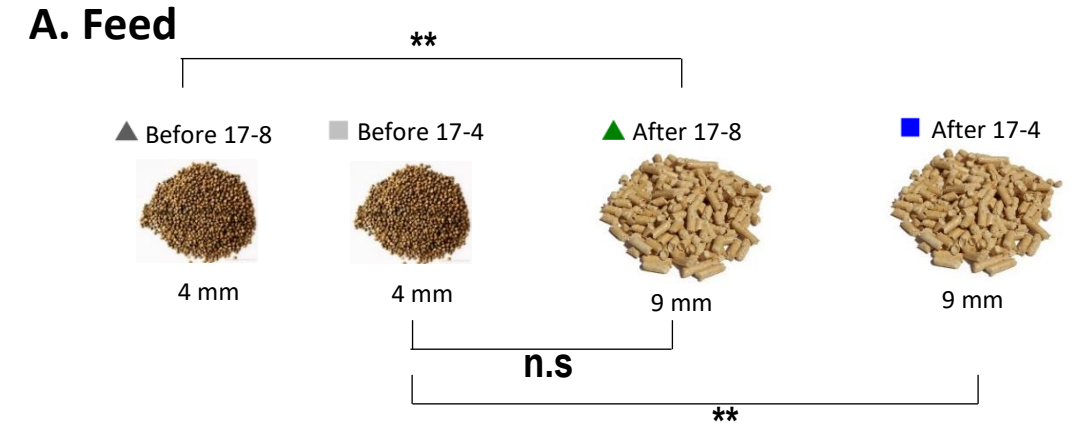


Figure 3.3.1.3.15. Difference between the global bacterial community structure of all 218 healthy vs disease gut scraping samples (2016 and 2017 dataset combined) as analysed by non-metric multidimensional scaling (nMDS) – 3D plot shown.





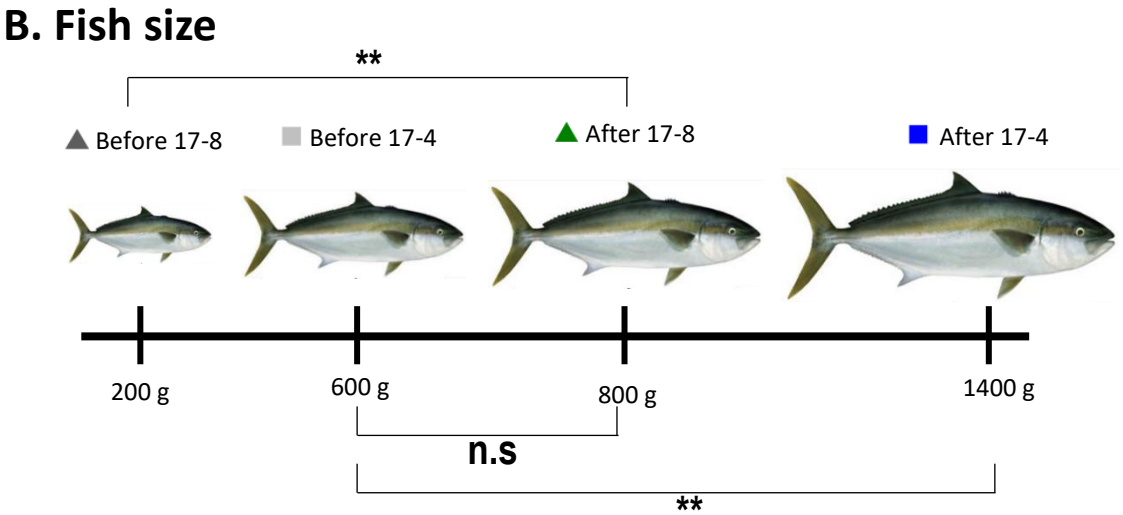


Figure 3.3.1.3.16. Difference between the global bacterial community structure of 'healthy before' and 'healthy after' YTK collected from two Arno Bay/Northern Site sea-cages (17-4 and 17-8) as analysed by non-metric multidimensional scaling (nMDS) (top plot), with A) feed type (pellet size, 4mm vs 9mm) and B) fish size (mean weights) shown for each cage.[1]

[1] Asterisk represent significant $P < 0.05$ as analysed by one-way PERMANOVA pairwise test, n.s. is not significant – see Table 3.3.1.3.17 for further detail

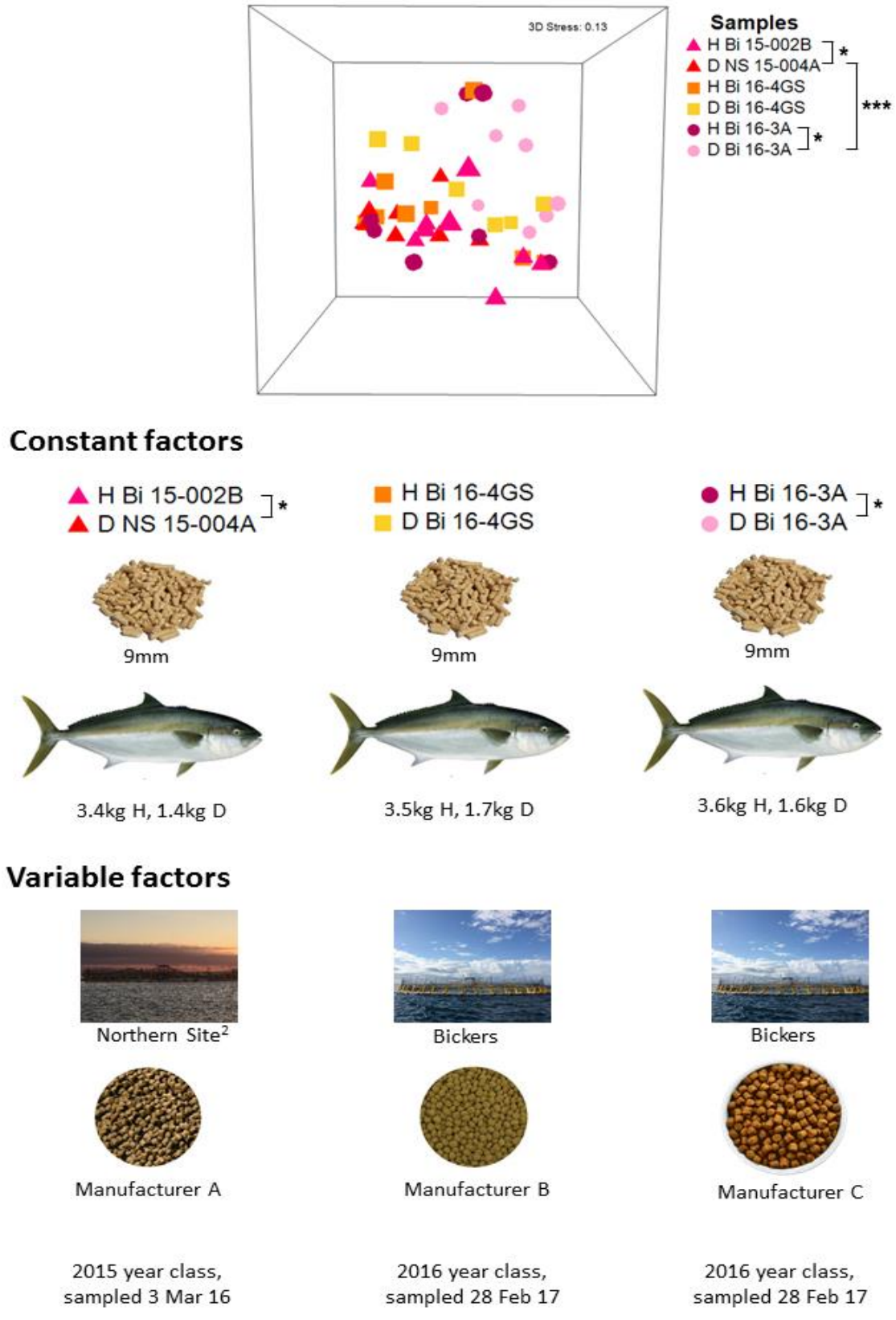


Figure 3.3.1.3.17. Difference between the global bacterial community structure of data subset 1 'temporal scale' as analysed by non-metric multidimensional scaling (nMDS), with the constant and variable factors shown.[1]

Abbreviations: Bi, Bickers; D, Disease; H, Healthy; NS, Northern Site.

[1] Asterisk represent significance $P < 0.05$ as analysed by one-way PERMANOVA pairwise test.

[2] Disease samples from Northern Site, healthy fish from Bickers.

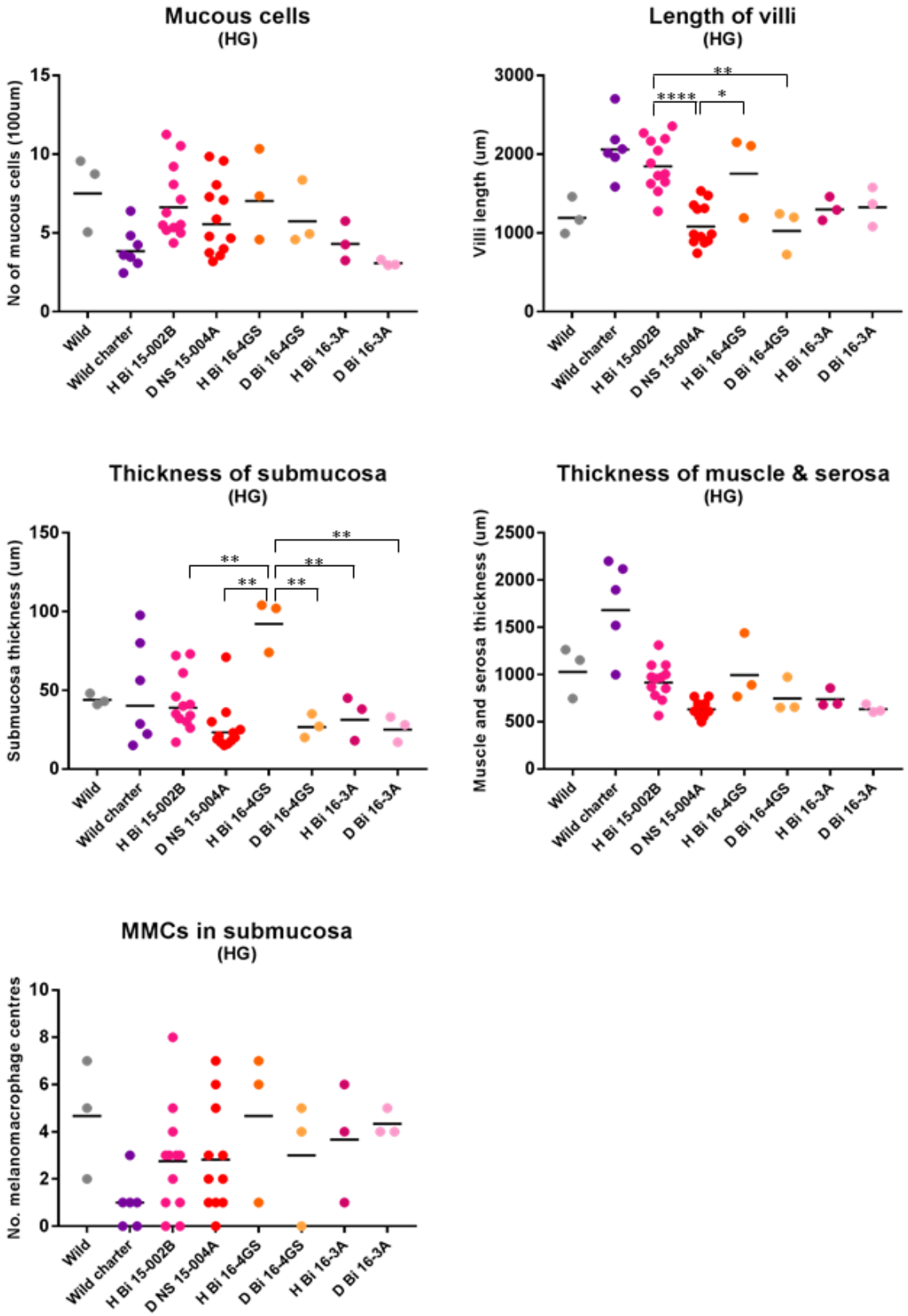


Figure 3.3.1.3.18. Histological parameters evaluated from hindgut tissue of wild and healthy vs disease fish corresponding to data subset 1 'temporal scale'.[1]

Abbreviations: Bi, Bickers; D, Disease; H, Healthy; HG, hindgut; MMCs, melanomacrophage centres; NS, Northern Site.

[1] Asterisk represent significance $P < 0.05$ as analysed by one-way ANOVA with Tukey's pairwise comparisons. Significance not shown for wild fish with the health classes.

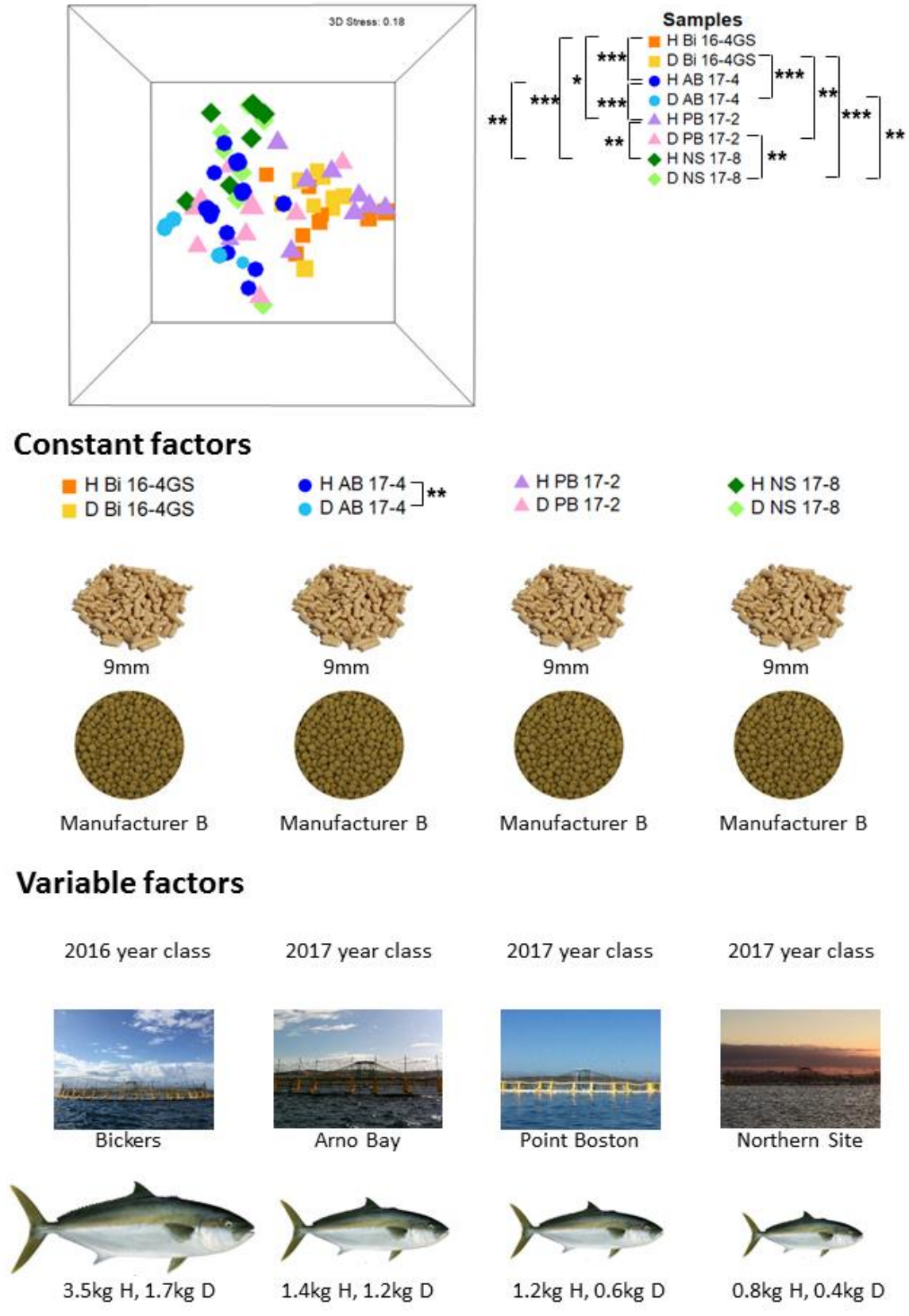


Figure 3.3.1.3.19. Difference between the global bacterial community structure of data subset 2 'same feed manufacturer and feed type' as analysed by non-metric multidimensional scaling (nMDS), with the constant and variable factors shown.[1]

Abbreviations: AB, Arno Bay; Bi, Bickers; D, Disease; H, Healthy; NS, Northern Site; PB, Point Boston.

[1] Asterisk represent significance $P < 0.05$ as analysed by one-way PERMANOVA pairwise test

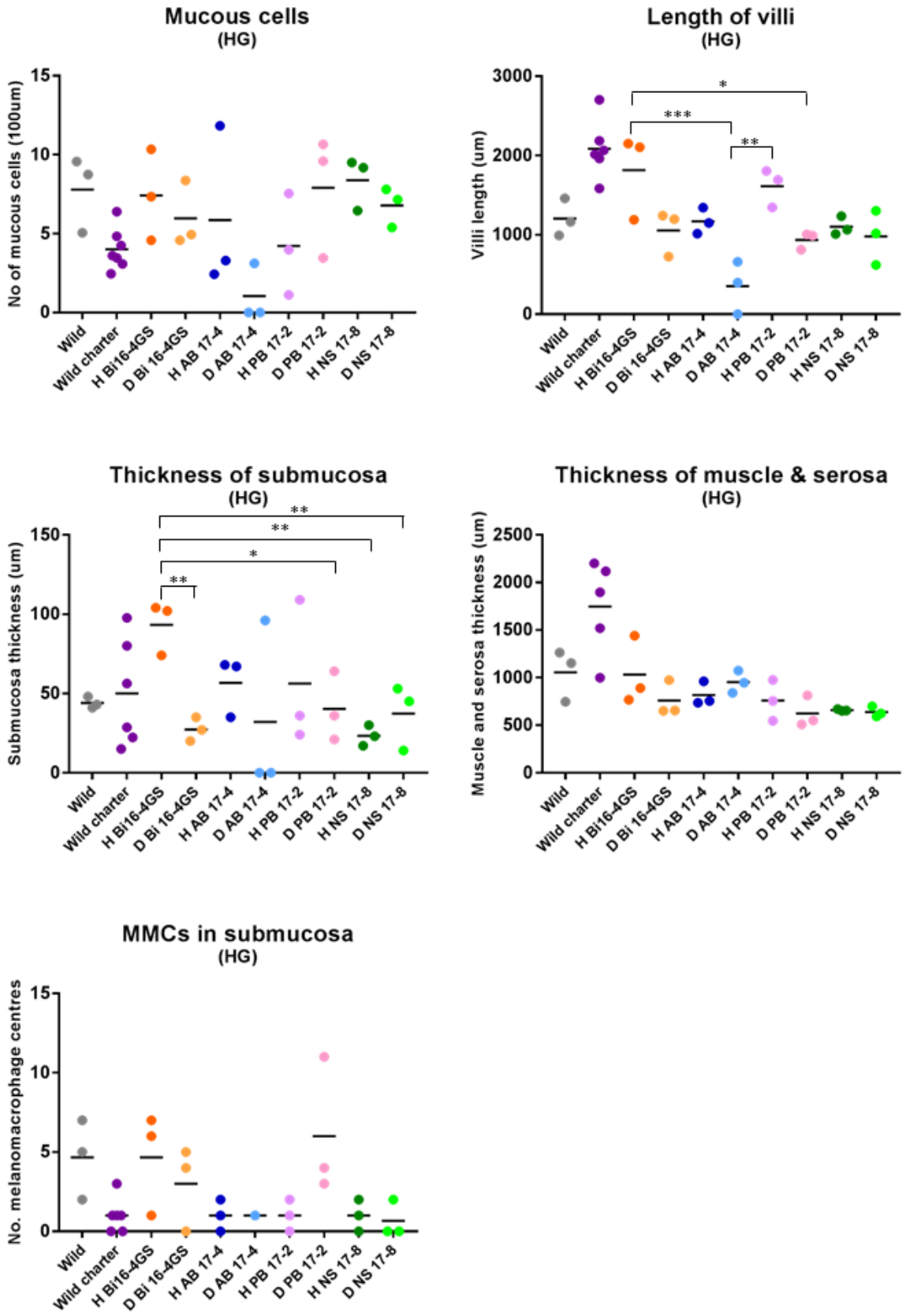


Figure 3.3.1.3.20. Histological parameters evaluated from hindgut tissue of wild and healthy vs disease fish corresponding to data subset 2 'same feed manufacturer and feed type'.[1]

Abbreviations: AB, Arno Bay; Bi, Bickers; D, Disease; H, Healthy; HG, hindgut; MMCs, melanomacrophage centres; NS, Northern Site.

[1] Asterisk represent significance P <0.05 as analysed by one-way ANOVA with Tukey's pairwise comparisons. Significance not shown for wild fish with the health classes.

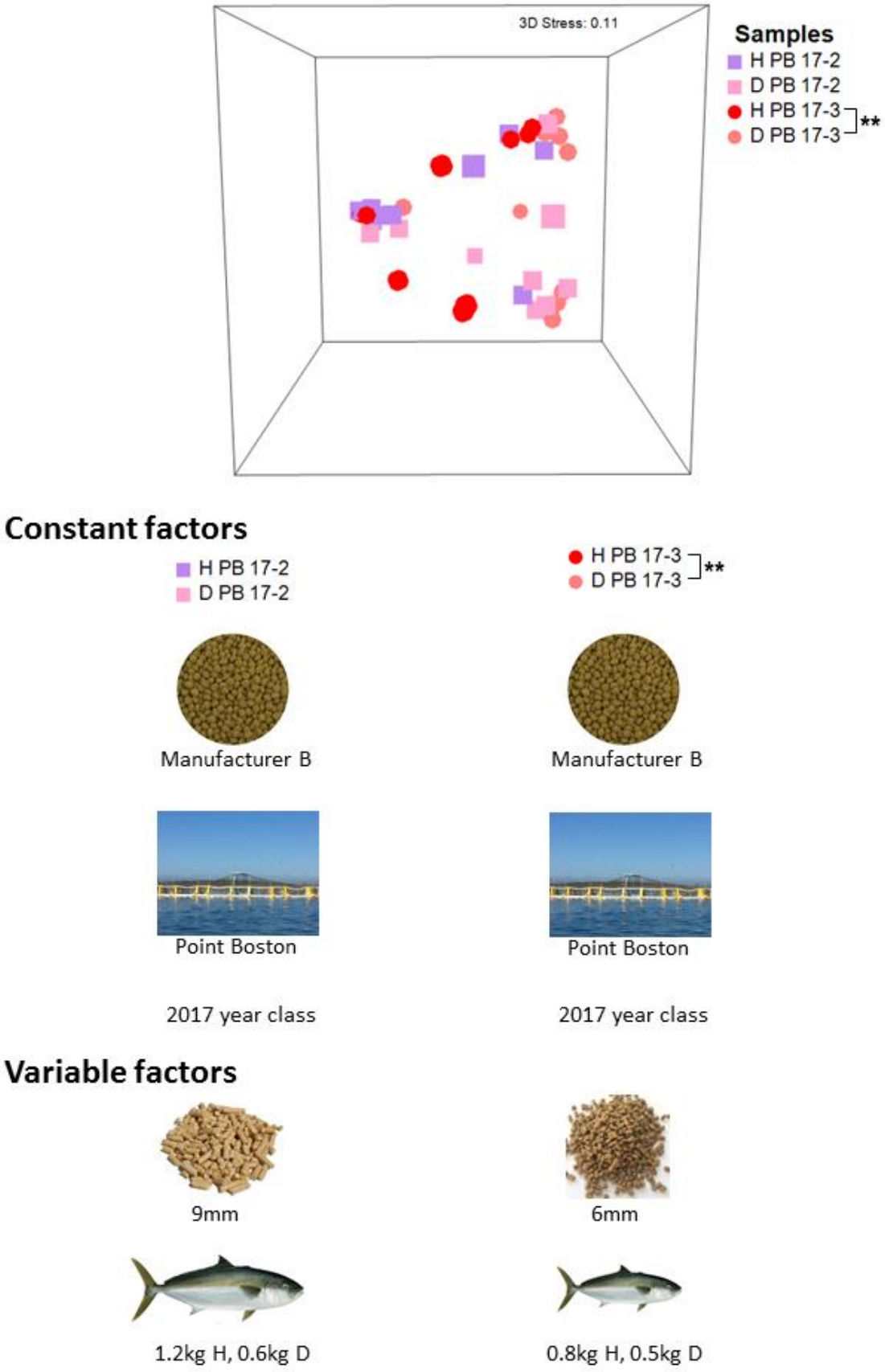


Figure 3.3.1.3.21. Difference between the global bacterial community structure of data subset 3 'same feed manufacturer, site and year class'as analysed by non-metric multidimensional scaling (nMDS), with the constant and variable factors shown.[1]

Abbreviations: D, Disease; H, Healthy; PB, Point Boston.

[1] Asterisk represent significance $P < 0.05$ as analysed by one-way PERMANOVA pairwise test

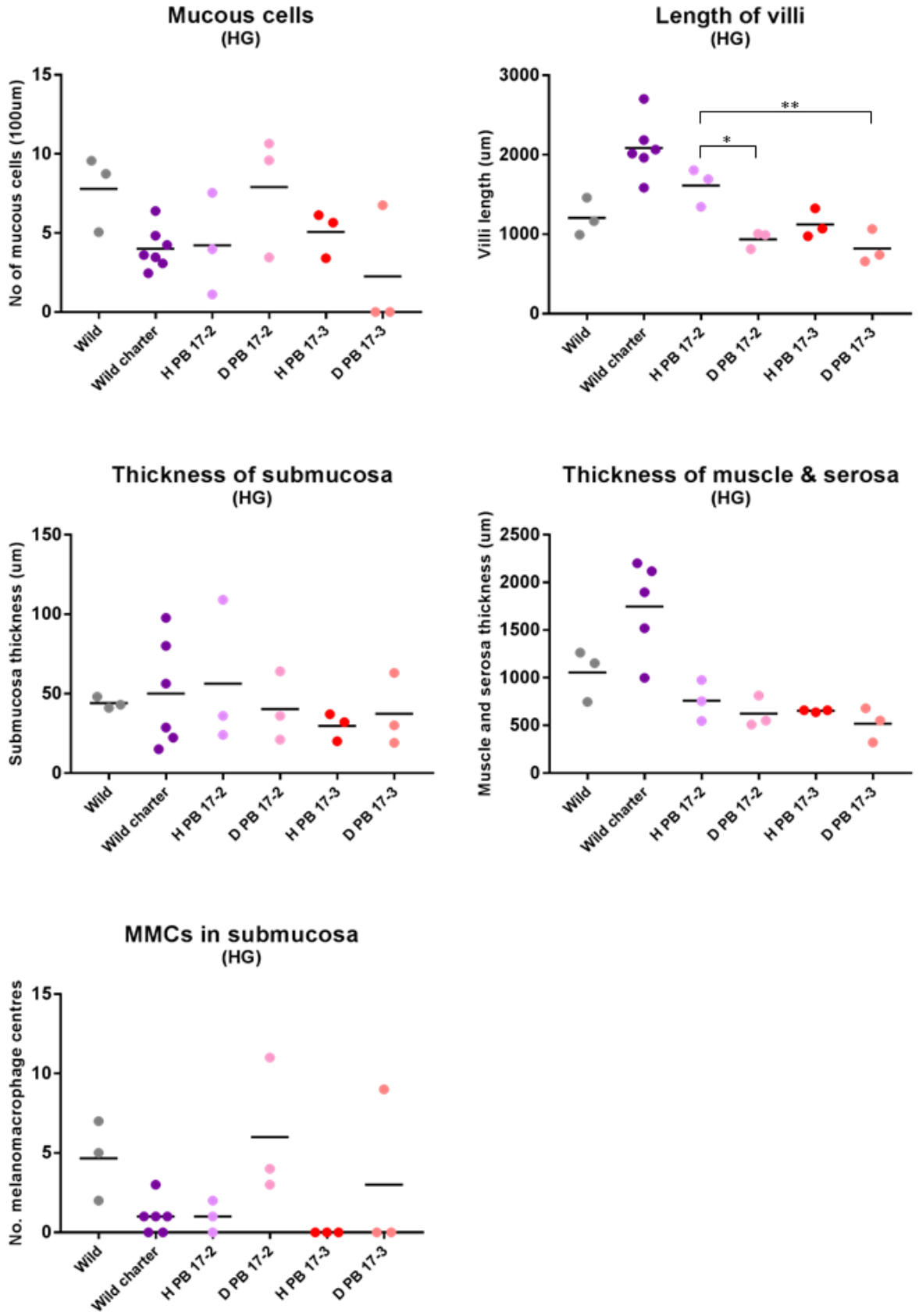


Figure 3.3.1.3.22. Histological parameters evaluated from hindgut tissue of wild and healthy vs disease fish corresponding to data subset 3 'same feed manufacturer, site and year class'.[1]
Abbreviations: D, Disease; H, Healthy; HG, hindgut; MMCs, melanomacrophage centres; PB, Point Boston.
[1] Asterisk represent significance $P < 0.05$ as analysed by one-way ANOVA with Tukey's pairwise comparisons. Significance not shown for wild fish with the health classes.

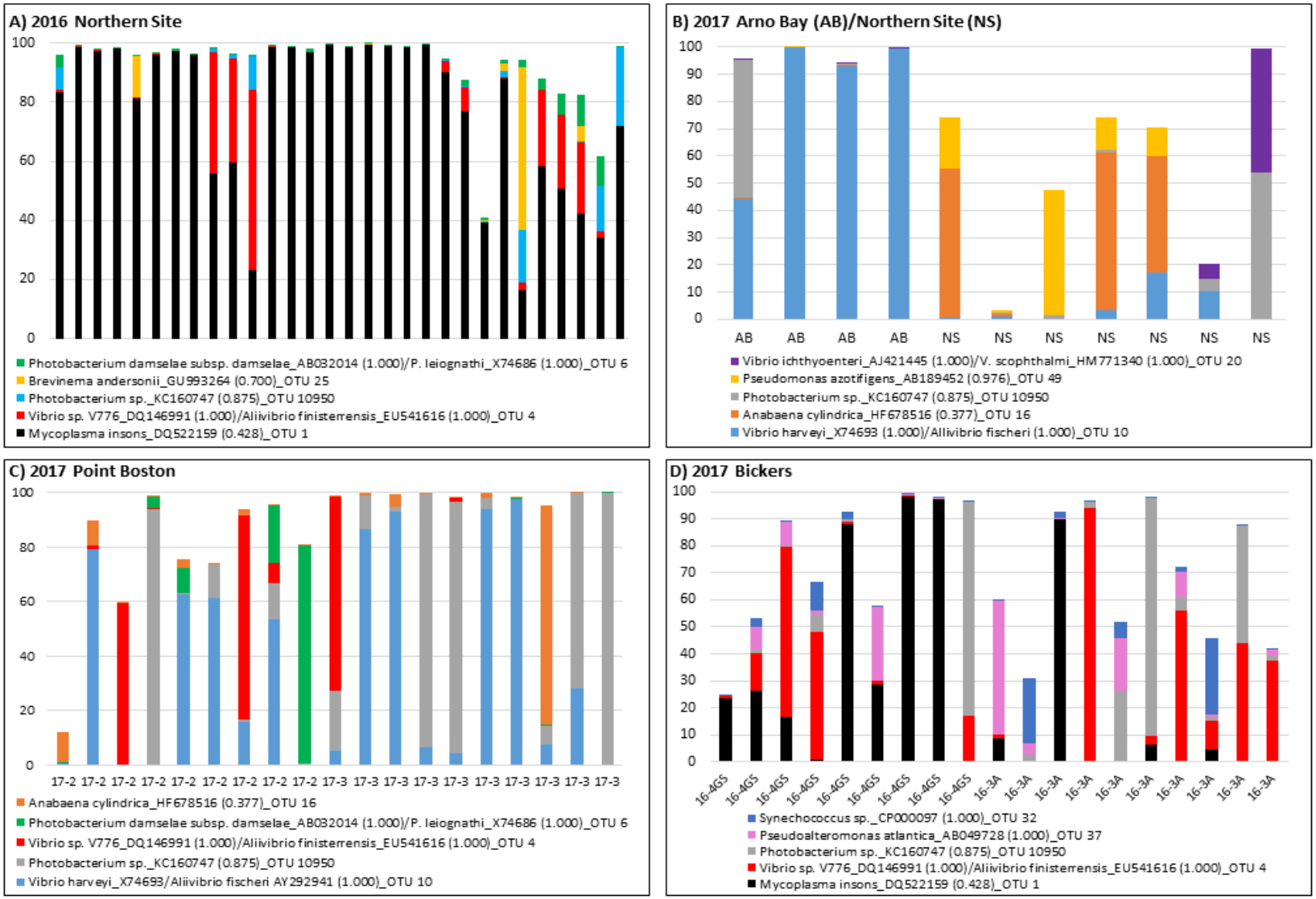


Figure 3.3.1.3.23. Top 5 taxa that contribute to the whole gut dataset for disease individuals from A) 2016 Northern Site samples, B) 2017 Arno Bay/Northern Site samples, C) 2017 Point Boston samples and D) 2017 Bickers samples.

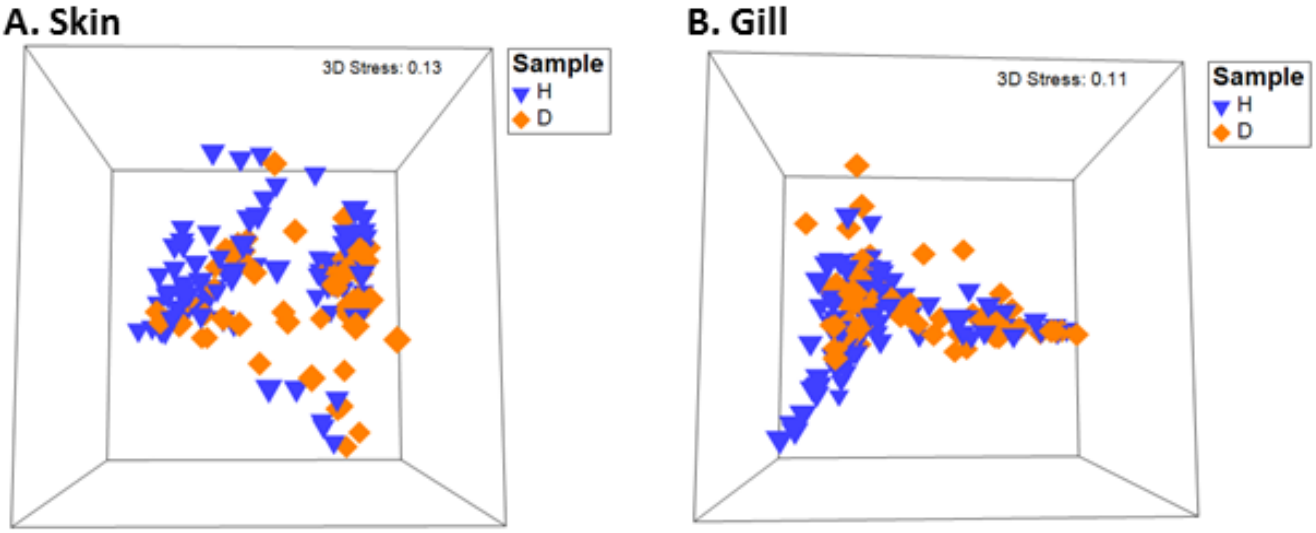
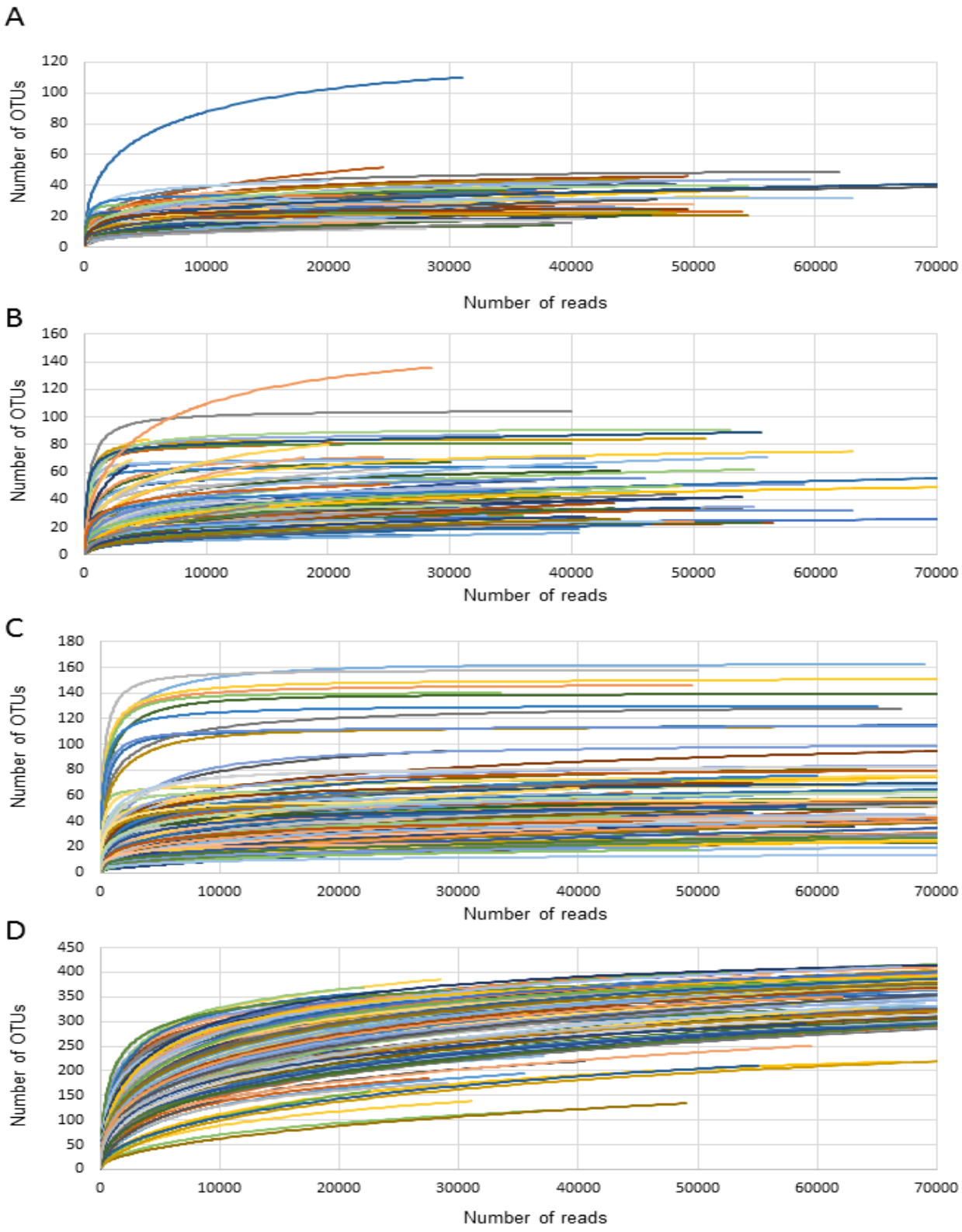
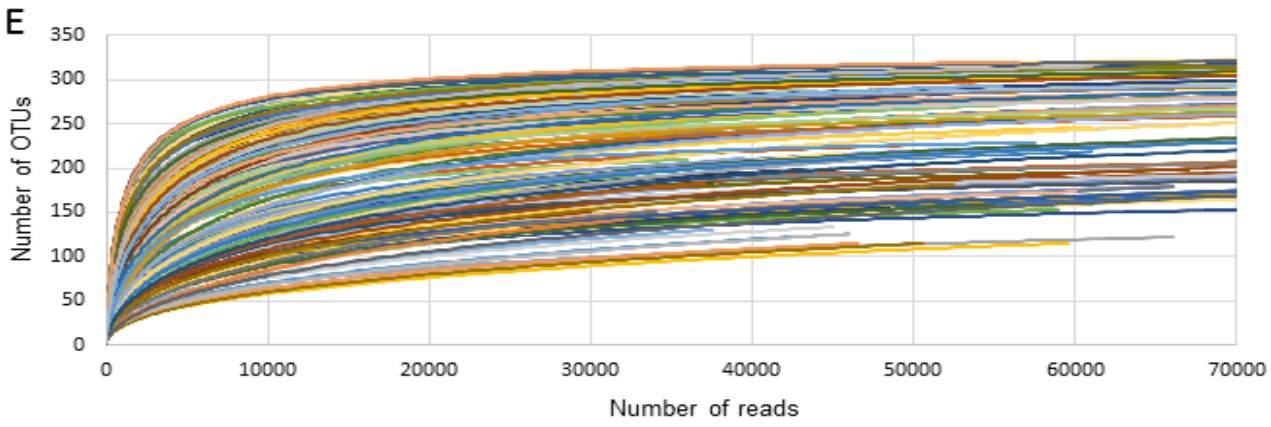


Figure 3.3.1.3.24. Difference between the global bacterial community structure of healthy and disease skin (A) and gill (B) swab samples from the 2016 and 2017 extension datasets as analysed by non-metric multidimensional scaling (nMDS).
Abbreviations: D, disease; H, healthy.



Appendix 1. Rarefaction curves portraying the number of resolved OTUs against sequencing depth of each sample from component A) 2016 health vs disease, B) gastrointestinal health (2016 health vs disease vs coccidiosis), and health vs disease extension (2016 and 2017 samples) C) gut samples, D) skin samples and E) gill samples.


E
350
300
250
200
150
100
50
0
Number of OTUs
0
10000
20000
30000
40000
50000
60000
70000
Number of reads

3.3.1.4. Manuscript - Establishing a role for microbiome manipulation in the restructuring of these communities in poor-performing Yellowtail Kingfish (Seriola lalandi) with the prospect for improving health outcomes.

Sarah R. Catalano [a], Andrew P.A. Oxley [a]

[a] South Australian Research and Development Institute, South Australia Aquatic Sciences Centre, West Beach, SA 5024, Australia.

This manuscript may be referenced as: Catalano, S.R. and Oxley, A.P.A. (2019). 3.3.1.4. Manuscript – Establishing a role for microbiome manipulation in the restructuring of these communities in poor-performing Yellowtail Kingfish (*Seriola lalandi*) with the prospect for improving health outcomes (Output 4c). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.706-759.

Abstract

In lieu of the earlier findings from 2016 and 2017 that prominent microbiome alterations (dysbioses) occur within Yellowtail Kingfish (*Seriola lalandi*; YTK) during conditions of gut enteritis and coccidiosis (as characterised by a loss of species diversity and dominance by potentially opportunistic pathogens), the question was raised as to whether the gut microbiome of poor-performing YTK could be modulated and subsequently manipulated to improve health outcomes. Specifically, whether the gut microbiome from optimal performing ('healthy') fish could be used as a prophylactic therapy for poor performing ('unhealthy') fish when transferred to the gut. For this, poor-performing fish were first identified and selected on-farm, and then transported back to the SARDI pool-farm facility, where they were allowed to acclimatise. Following this period, optimally performing ('healthy') fish were selected on-farm as microbiome 'donors', whereby faecal material was collected by stripping a total of 102 fish to obtain 110 mL of raw fecal inoculum. To improve the likelihood of obtaining a more varied microbiome to that of the poor-performing fish, 'healthy' donor fish were selected from a different site, and were of a larger size class and fed a different diet. The stripped faecal material from these animals was pooled and immediately transported back to the SARDI pool-farm, where it was then administered fresh to the poor-performing ('recipient') fish by either delivery into the surrounding seawater or via gavage. To improve the likelihood of the microbiome (or select constituents) to establish within the poor-performing fish, replicate tanks comprising fish treated with and without antibiotics were evaluated. Control tanks comprising fish not administered the faecal inoculum (with and without antibiotics) were also investigated. Samples from the gut (hindgut scrapings) and the skin (swabs) were collected from all treatments through time (three days after antibiotics were administered then two, eight and 15 days post inoculum). In addition, samples from a total of 10 on-farm pre-trial fish along with the inoculum were also collected and assessed for comparative analyses. Analysis of the active and inactive microbiome (bacterial community) constituents of the donor inoculum were assessed by extraction of the RNA and DNA, revealing the occurrence of a single dominant organism within the active fractions that was most closely related to *Vibrio* sp. V776/*Allivibrio finisterrensis*. The less active components of the inoculum (as assessed by analysis of the DNA) were more varied and likely contributed to some notable differences within the treatments. Specifically, at two days post inoculum there was an increase in taxonomic diversity and species evenness within the gut samples of fish where antibiotics and gavage inoculum were administered, and was attributed to an increase in the bacterial phyla and decrease in the abundance of potential opportunistic pathogens. This result suggests that the gavage inoculum treatment coupled with antibiotic therapy displaces potentially opportunistic species through the promotion of diversity (and possibly functionality) that could lead to improved health

outcomes. Differences in the global bacterial community structure of the skin samples from antibiotic treated fish administered the inoculum within the seawater were also observed at two days post inoculum, highlighting that both the gut and skin microbiome can be manipulated. While changes in the global bacterial communities of the gut samples from antibiotic treated fish receiving either the water or gavage inoculum were also observed eight days post administration, a reversion back to the 'original' state was recorded at 15 days. Conversely, sustained changes in the skin microbiome were apparent at both eight and 15 days, indicating prolonged effects from this treatment. From comparisons of fish where no inoculum was given (with and without antibiotics), it was also possible to establish a catalogue of taxa that were effected by antibiotic treatment. Most notable was that in the gut (likely due to the oral administration of the antibiotics) and was associated with a loss of *Cyanobacteria*, *Proteobacteria* and *Spirochaetae*, though had limited effect on *Tenericutes*. At the species level, a decrease in abundance of OTUs most closely related to *Brevinema andersonii* and *Vibrio* sp. V776/*Allivibrio finisterrensis* were observed from gut scraping samples for fish treated with antibiotics. In accordance with the above finding, antibiotic treatment also had little effect on the *Tenericutes* associated OTU most closely related to *Mycoplasma insons*. This taxon was instead enriched in the antibiotic treatment samples, highlighting that this organism may be an opportunistic pathogen that is able to dominate the gut community when other taxa are eliminated. *Mycoplasma insons* has previously been recorded as a dominant constituent in gut enteritis disease samples collected on-farm (see Manuscript 3.3.1.3), and presents an important consideration if treatment with antibiotics for YTK disease is to be used on-farm in the future. Specifically, our results show that if the combination and dosage of antibiotics used in this trial was applied to treat YTK with underlying gut enteritis disease on-farm, the issue may be exacerbated by allowing the proliferation of other potentially opportunistic species.

Introduction

With the development and utilisation of next-generation sequencing, our understanding of the structure, diversity and function of the gut microbiota has increased in current years (Mcilroy et al., 2018). This understanding has then led to the exploration of alternative therapies for treating disease conditions in humans and animals (including fish) that are focused on improving gut microbiome health, including the administration of probiotics (live microorganisms that confer a health benefit to the host) and prebiotics (a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gut microbiota, thus conferring benefits to host health) (Dittman et al., 2017; Marchesi et al., 2016). Furthermore, recent attention has been concentrated on faecal microbiota transplantation (FMT), whereby the gastrointestinal microbiota from a healthy donor is transferred via infusion of a faecal sample into the diseased individual (Gough et al., 2011; Anderson et al., 2012).

The goal of FMT is to restore host health by increasing diversity and function of the gut microbiota (Lee et al., 2018). As demonstrated for humans, FMT can allow for treatment of an individual's disease more efficiently and in a targeted fashion, providing a form of personalised healthcare which then eliminates the need for conventional drug treatments, including steroids, immunosuppressants and biological therapies, which often can have significant adverse side effects (Anderson et al., 2012; Marchesi et al., 2016), although one major concern for this approach is the potential risk of transmitting infectious diseases (Alonso and Guarner, 2013). The faecal microbiota can also be banked and reinstated at a later date, potentially after treatment that alters the structure and/or function of the gut microbiome (Mcilroy et al., 2018). FMT has the capacity to be delivered by multiple routes (e.g. enemas, oral capsules or via nasogastric tubes or endoscopy or colonoscopy) (Lee et al., 2018). Factors such as age, donor selection, post-transplantation process management, environment and the diseases the recipients and donors carry must all be taken into consideration in FMT (Coskunpinar et al., 2018). Nonetheless FMT represents a relatively cheap, low-tech operation that has minimal risk of rejection and breaks the cycle of repeated antibiotic usage (Coskunpinar et al., 2018). In humans, FMT has been explored and used to successfully manage conditions such as inflammatory bowel disease (Anderson et al., 2012), irritable colon syndrome (Coskunpinar et al., 2018), and antibiotic-associated diarrhoea due to *Clostridium difficile* infection (Borody et al., 2013). There is also future interest in the use of FMT

for autoimmune diseases along with other diverse conditions such as autism, acne, chronic fatigue syndrome, obesity and for symptomatic relief in Parkinson's disease (Anathaswamy, 2011; Borody et al., 2013; Coskunpinar et al., 2018; Lee et al., 2018).

While numerous studies have catalogued the gut bacterial communities associated with fish species and explored the factors that contribute to the observed assemblages, including environment, diet, host genetics and host health (for recent reviews see Llewellyn et al., 2014; Ghanbari et al., 2015; Egerton et al., 2018), the prospect of manipulating the gut microbiome to improve health in fish via FMT is largely unknown. Despite this, it has been shown that the regulation and increase in the life span of a short-lived species (namely African turquoise killifish, *Nothobranchius furzeri*) may be achieved by recolonising the gut of middle-aged individuals with bacteria from young donors (Smith et al., 2017). To the best of our knowledge, however, no studies have examined the utility of this approach for re-establishing deficient microbiomes of diseased fish from healthy individuals.

As demonstrated from human studies, low bacterial diversity has been associated with numerous disease conditions, including inflammatory bowel disease, psoriatic arthritis, type 1 diabetes, atopic eczema, coeliac disease, obesity and type 2 diabetes (Valdes et al., 2018). Diversity is therefore suggested to be a good indicator of a 'healthy gut' (Valdes et al., 2018). From sampling populations of healthy and disease (gut enteritis and coccidiosis) Yellowtail Kingfish (*Seriola lalandi*; YTK) on-farm in 2016 and 2017, a common feature of reduced bacterial diversity with dominance by a single taxon was observed in the disease samples. In lieu of these findings, the question was then raised as to whether the gut microbiota of poor-performing YTK can be manipulated in a similar manner to what has been done with humans by using a FMT-based approach in an attempt to improve health outcomes by restoring diversity (and likely functionality). To assess this question, a manipulation trial was developed, with six treatments examined in duplicate to assess the microbiome (bacterial community assemblages) from the hindgut and the skin surfaces of YTK to explore the inner and outer mucosal responses to microbiome manipulation.

Aim

The aim of this trial was to assess whether the microbiome (bacterial community assemblages) of poor-performing YTK could be manipulated to potentially improve health outcomes. The experiment was designed with six treatments in duplicate sampled through time in order to address three key questions, namely 1) whether antibiotic therapy itself elicits a change in the skin and gut microbiome; 2) if differences in the route of administration of the 'healthy donor' inoculum influences the resultant gut and skin microbiome of poor-performing fish treated with or without antibiotics; and 3) whether changes in the gut and skin microbiome composition of the poor-performing fish occurs and is sustained, or reverts back to the original state with time.

Methods

Experimental design

A sea-cage at Point Boston, South Australia (SA) with a cohort of poor-performing fish displaying signs of enteritis was identified by Clean Seas (CS) veterinarian Dr James Fensham and health team staff. Following cage harvest, 100 fish were selected at random and measured (cm) and weighed (kg) at the CS processing facility in Port Adelaide, SA (data not shown). As enteritis fish are characterised by a reduction in weight, the lower 20% range of measured weights (equating to $\leq$ 1.6 kg) was determined as the cut-off point for fish selection for the on-farm pre-trial sampling and microbiome manipulation trial. Ten fish with an average weight of ~1.2 kg were sampled pre-trial on-farm from the Point Boston sea-cage on the 30th May 2018, with a 1 L seawater sample from this sea-cage site also collected and processed in parallel. Fish from this sea-cage were on a 9 mm diameter pellet (Feed B). A total of 207 fish of the desired weight range were then selected and loaded into a tanker for transport from Port Lincoln to West Beach (SA), where they were subsequently unloaded into the pool-farm facility at the

South Australian Research and Development Institute (SARDI) Aquatic Sciences Centre on the 31st May 2018. Fish were held in the pool-farm system for ~4 weeks to acclimatise, during which time routine health checks and treatments were performed (e.g. for flukes), before the trial was stocked on the 24th June 2018. Twelve fish were stocked per 5000 L tank, with six treatments in duplicate (12 tanks used in total). Average fish weight at stocking was 1.68 kg. Treatments 1 and 2 were water inoculum with and without antibiotics respectively; Treatments 3 and 4 were gavage inoculum with and without antibiotics respectively; and Treatments 5 and 6 were controls (no inoculum) with and without antibiotics respectively. The system was changed from recirculating to flow through before fish arrived and maintained in this manner through the duration of the trial to eliminate environmental cross-contamination between the treatment tanks.

Antibiotic therapy

A combination therapy of three antibiotics were selected and used based on advice from CS veterinarian Dr James Fensham and results from a small-scale pilot antibiotics trial undertaken with 15 YTK in April 2018 at the SARDI pool-farm facility. The antibiotic triple-therapy consisted of 200 mg/kg of oxytetracycline and 50 mg/kg of erythromycin and metronidazole dissolved in polypropylene glycol with 1.5 mL aliquots delivered by gavage (using an ~17 cm silicone tube [Gecko Optimal] inserted through the mouth into the stomach) to each ~1.5 kg fish in the antibiotic treatments. The antibiotic mix was made up fresh, with administration to the fish between 0-2 hr later on 24th June 2018. All trial fish were placed in a low dose of AQUI-S® (AQUI-S® New Zealand Ltd., Lower Hutt, New Zealand) at a concentration of 7 mg L^{-1} of seawater followed by a high dose of AQUI-S® at 14 mg L^{-1} of seawater for ~10 min before being measured, weighed and either administered antibiotics or returned directly back to the tank system if assigned to the no antibiotic treatments.

Inoculum administration and sampling time points

Three days after antibiotics were administered on the 27th June 2018 (time point [TP] 0 – TP0), baseline samples were collected to assess the effect of the antibiotics on the gut microbiome structure and composition, with two fish sampled per tank in the morning (24 fish in total; 12 with antibiotics, 12 with no antibiotics) along with a 1L water sample collected from the system in-flow to control for the influence the environment may have on the structure and composition of the gut bacterial community. On the same day, inoculum was collected on-farm in the morning and administered fresh to Treatments 1-4 in the afternoon. The inoculum was collected by stripping and pooling faecal material from 102 'healthy' YTK at sea-cage ABK1B-17. Fish in this sea-cage were fed a different diet formulation (Feed C), were located at a different site (Bickers, Port Lincoln, SA) and were much larger in size (~3.5-4.5 kg) compared to the poor-performing fish selected for the trial. A total of 110 mL of fecal material was collected, transported back to West Beach on ice and 290 mL of filtered (0.2 µm) seawater added, resulting in a final volume of 400 mL of inoculum. Note that dosage rates were based on the study by Smith et al. (2017), in which killifish were provided inoculum at a ratio of 1 donor fish/2 recipient fish of the same weight. In our study, donor fish were ~3.5 kg and recipient fish were ~ 1.7 kg, therefore for the 80 recipient fish (~1.7 kg) requiring the inoculum, faecal material from 20 donor fish (~3.5 kg) would need to be collected. As we anticipated diluting the raw faecal material 1 in 4 before administration, we subsequently multiplied the number of donor fish required to be stripped by four, resulting in a total of 80. To control for variation in the amount of faecal material that may be collected from each fish (e.g. no or small volumes may be collected from some fish while larger volumes may be collected from others), stripping from 100 donor fish was set as the minimum. Two 500 µL samples were collected from the inoculum for discerning the active and total bacterial constituents through downstream RNA and DNA extraction respectively. These samples were stored at -20 °C prior to extraction, with 1.5 mL of RNA later added to the RNA sample for preservation (Figure 3.3.1.4.1).

For the water inoculum Treatments 1 and 2 (four tanks), the water level in each tank was dropped to 1500L, the tanks cleaned to remove any fecal material from the recipient fish and 60 mL of the inoculum added. Fish were then held in the 1500L of water with no exchange for 3 hr. Following this, tanks

were filled back up to 5000L without flushing (so fish would remain in the inoculum for longer), with 40 fish in total exposed to the water inoculum sample (four tanks, 10 fish per tank, two treatments) (Figure 3.3.1.4.1).

For the gavage inoculum Treatments 3 and 4 (four tanks), fish were netted out of the tanks and consecutively placed into a low then high dose of AQUI-S® (7 and 14 mg L^{-1}) for ~10 min at each concentration before being administered with 3 mL of inoculum via gavage, as conducted using a ~17 cm silicone tube (Gecko Optimal) inserted through the mouth and into the stomach. Fish were then placed in a net and returned back to their respective tanks. Forty YTK in total were administered the inoculum via gavage (four tanks, 10 fish from each, two treatments) (Figure 3.3.1.4.1).

Fish were then sampled at three time points: TP1, two days post inoculum (29th June), TP2, eight days post inoculum (5th July) and TP3, 15 days post inoculum (12th July), with a total trial duration of 19 days. At each time point, 24 fish in total were sampled, with two fish sampled per tank and four fish per treatment (Figure 3.3.1.4.1). At the final time point (TP3), a 1L water sample was also collected from the system in-flow and processed in parallel to control for the influence the environment may have on the structure and composition of the gut and skin bacterial communities. In addition, four 'spare' fish that had been given antibiotics and administered the inoculum via gavage were sampled five days post inoculum. These 'spare' fish had been kept as a safeguard in case mortalities arose after antibiotic treatment and gavage inoculum, allowing for fish to be re-stocked if needed. However, while no fish were lost throughout the duration of the trial, they were sampled as an additional intermittent time point, TP1a (Figure 3.3.1.4.1).

Water quality analyses

Throughout the trial, water quality parameters were monitored and recorded daily to ensure YTK were maintained at appropriate levels. This included total gas pressure (TGP) using a TGP meter (OxyGuard International A/S, Birkerød, Denmark), dissolved oxygen (DO) (mg L^{-1} and % saturation) using a DO meter (OxyGuard International A/S, Birkerød, Denmark), water temperature using a thermometer, pH using a meter (Oakton pHtestr 20; Oakton Instruments, Vernon Hills, IL, USA), ammonia using an Aquarium test kit (Ammonia NH_3/NH_{4+} test kit, Aquarium Pharmaceuticals, Chalfont, Pennysylvania, USA), carbon dioxide (CO_2) using a CO_2 meter (OxyGuard International A/S, Birkerød, Denmark) and salinity (g L^{-1}) using a portable salinity refractometer (model RF20, Extech Instruments, Nashua, NH, USA). Fish were fed the same formulated feed as on-farm (Feed B) once daily in the morning to apparent satiation, with feed with-held on sampling days. Tanks were cleaned as required.

Fish sampling

Fish were collected from sea-cages (on-farm pre-trial sampling) and tanks (microbiome manipulation trial) using a dip net with the skin, posterior from the pectoral fin above and below the lateral line, promptly swabbed using a sterile FLOQSwab (Copan Flock Technologies). Swabs were immediately placed in 200 µl of RNAlater™ (Ambion) and stored at 4°C for 1-2 days before being stored for up to a month at -20°C prior to RNA extraction. Each fish was then euthanised, weighed (g) and measured (fork length, cm). The body cavity was opened and the entire GI tract removed. The hindgut was then separated from the fore- and midgut using a sterile scalpel blade and placed on a clean surface. Using a clean pair of forceps and sterile scalpel, an incision was made along the length of the hindgut to expose the inner surface, and then a single scraping was taken with a sterile glass slide to collect the gut contents/mucosa. Scrapings were immediately placed into 50 mL falcon tubes containing stabilising buffer (RNAlater™, Ambion), labelled and stored at 4°C for 1-2 days before being stored for up to a month at -20°C prior to RNA extraction. Gloves, aluminium foil and scalpel blades were discarded and forceps cleaned with ethanol after sampling each fish to avoid cross contamination. Histology samples from the hindgut and skin were also collected from each fish and stored in 10% seawater formalin at room temperature for future reference material (though not analysed here).

RNA extraction for gut microbiome samples

RNA was extracted on ice from stabilised samples according to the methods detailed in Szafranska et al. (2014). In brief, for the hindgut scraping samples, the stabilising buffer was removed from each sample and 1 mL of cold (4 °C) RLT buffer supplemented with 1% β-mercaptoethanol was added and transferred to lysing matrix B tubes (MP Biomedicals). Samples were disrupted via bead-beating using the FastPrep-24™ 5G instrument (MP Biomedicals) at an intensity of 5.5 for 45 s, placed on ice for 3 min then disrupted a second time as described above prior to centrifugation at 14,000 × *g* for 10 min at 4°C. The supernatant was transferred to 1.5 mL RNase-free Biopur centrifuge tubes (Eppendorf) and the RNeasy minikit (Qiagen) was used to extract the RNA according to manufacturer's instructions. RNA was eluted in 30 µL of RNase free water, passed through the spin column twice to concentrate each sample and quantified using a NanoDrop 2000 spectrophotometer. To remove any source of potential contaminating gDNA, a routine DNase treatment was performed for all samples using the Turbo DNA-free™ kit (Life Technologies) following the manufacturer's instructions. Skin swab samples were extracted using the same method as the gut samples, except in the first step, the swab tip was taken out of the stabilising buffer and placed in a lysing matrix B tube (MP Biomedicals) containing 1 mL of cold (4 °C) RLT buffer supplemented with 1% β-mercaptoethanol before disruption via bead-beating. All samples were precipitated with ethanol using standard procedures, reconstituted in 30 µL of RNase free water and the RNA re-quantified using the NanoDrop. Samples were stored at -80 °C prior to use in down-stream procedures.

DNA extraction for environmental samples and inoculum

One litre aliquots of water was collected in a sterile Schott bottle from the pre-trial sea-cage location (Point Boston) and system in-flow at the start (TP0) and end (TP3) of the trial. Each bottle was labelled with the site/tank details and stored at 4°C prior to filtration and DNA extraction. Each seawater sample was filtered onto separate sterile 0.22 µM filters (Nalgene®) prior to DNA extraction using the FastDNA™ Spin Kit for Soil (MP Biomedicals) following the manufacturer's instructions. In brief, the filter paper was placed in a lysing matrix E tube with sodium phosphate and MT buffer and cells were lysed via bead-beating using the FastPrep-24™ 5G instrument (MP Biomedicals) at an intensity of 5.5 for 45 s. Samples were subsequently centrifuged for 10 min at 14,000 × *g* and the supernatant transferred to 1.5 mL DNA LoBind tubes (Eppendorf). Following the addition of a protein precipitation solution, the samples were mixed and centrifuged to pellet the precipitate before the supernatant was transferred to a clean 15 mL centrifuge tube supplemented with Binding Matrix solution. The DNA was captured on SPIN filter tubes and washed, re-eluted in 100 µL of DES and quantified using a NanoDrop 2000 spectrophotometer followed by precipitation with ethanol using standard procedures. The pelleted DNA was reconstituted in 30 µL of RNase free water and re-quantified using the NanoDrop. The (DNA) inoculum sample was extracted using the same method as for the seawater sample, except an aliquot of the inoculum was added to the lysing matrix E tube with sodium phosphate and MT buffer in the first step, instead of filter paper. Samples were stored at 4 °C prior to use in down-stream procedures.

cDNA synthesis, PCR amplification, AMPure bead-purification and Picogreen quantification

For the (RNA) inoculum sample, skin swabs and gut scrapings, the RNA extracts were converted to cDNA to assess for the active (and likely resident) bacterial constituents using the Superscript™ III First Strand Synthesis System (Life Technologies) following the manufacturer's instructions and stored at -20°C prior to PCR amplification. The V1-V2 hypervariable region of the 16S rRNA gene was amplified for all samples (DNA and cDNA samples) as described by Camarinha-Silva et al. (2014); though included a pre-enrichment of the V1-V2 target region by conducting a 20 cycle PCR reaction with primers 27F and 338R as described by Chaves-Moreno et al. (2015). Specifically, 2 µL of cDNA and 5 µL of each environmental DNA extract was used as template in the first round of PCR, with 1 µL aliquots from the first round of PCR used as template in a second 15 cycle PCR reaction to append the forward barcode and reverse adapter sequences complementary to the Illumina platform specific

adaptors. One microlitre aliquots of the second PCR reaction were subsequently used as a template in a third 10 cycle PCR to append the Illumina multiplexing sequencing and index primers. PCR amplicons were visualised via agarose gel electrophoresis and products of the expected size (~438 bp) were purified using Agencourt AMPure XP beads (Beckman Coulter). Samples were quantified in duplicate using the Quant-iT™ Picogreen® dsDNA kit (Life Technologies) following the manufacturer's instructions. Approximately 100 samples were pooled for each library in equimolar ratios and sequenced on the MiSeq platform (Illumina, San Diego, CA) using 250 nucleotide (nt) paired-end sequencing chemistry through the Australian Genome Research Facility (AGRF). As a sequencing control, amplicons generated from a single bacterial species (*Lactobacillus reuteri*) were included within each Illumina index within each of the libraries. The final list of samples that generated good-quality microbiomic libraries for this component of work are presented in Table 3.3.1.4.1.

Bioinformatics analysis

In total, 12,986,344 million sequence reads were derived from 222 samples (of the 226 that were collected). Four samples failed to amplify enough material to produce good-quality NGS libraries. This was accounted for in the experimental approach by allowing for the ample replication of fish. Sequence reads were paired using PEAR (version 0.9.5) (Zhang et al., 2014), where primers were identified and removed. Paired-end reads were quality filtered, with removal of low-quality reads, full-length duplicate sequences (after being counted) and singleton sequences using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010), USEARCH (version 8.0.1623) (Edgar, 2010; Edgar et al., 2011) and UPARSE software (Edgar, 2013). Reads were mapped to Operational Taxonomic Units (OTUs) using a minimum identity of 97%, removing putative chimeras using the RDP-gold database as a reference (Cole et al., 2014). These OTUs were further filtered as conducted previously (Zhang et al., 2016) where only those that contributed to >0.01% of the host-associated dataset (gut samples only) or >0.01% of the environmental water samples were used (see Table 3.3.1.4.2 for a summary of OTUs remaining post-filtering). Rarefaction curves were used to inspect (retrospectively) sampling depth for each gut sample (Appendix 1). Further interrogation of the resultant OTUs was conducted using the Seqmatch function of the RDP database (Wang et al., 2007) as well as SILVA (Quast et al., 2013), whereby lineages based on the SILVA taxonomy and best hits from RDP were assigned to each OTU alongside the corresponding RDP sequence similarity value (SeqMatch, S_ab score). The S_ab score represents the number of unique 7-base oligomers shared between an OTU and a known sequence contained in the RDP database divided by the lowest number of unique oligos in either of the two sequences. A S_ab score of 1.000 represents an identical match to the nearest database sequence, with values closer to 1.000 providing greater confidence in the identification OTU sequence.

Statistical analysis

In order to explore for patterns across the global bacterial communities, a data matrix comprising the percent standardised abundances of OTUs was used to construct a sample-similarity matrix using the Bray-Curtis algorithm (Bray and Curtis, 1957), where samples were then ordinated using non-metric multidimensional scaling (nMDS) with 50 random restarts (Clarke et al., 2001). Significant differences between *a priori* pre-defined groups of samples (e.g. environmental water samples vs gut scraping samples) were evaluated using permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations, allowing for type III (partial) sums of squares, fixed effects sum to zero for mixed terms, and exact p-values generated using unrestricted permutation of raw data (Anderson, 2001). Groups of samples were considered significantly different at $P < 0.05$. Pairwise tests in PERMANOVA were used to determine which *a priori* pre-defined categories (e.g. no inoculum vs water inoculum vs gavage inoculum) were significantly different. The multivariate analyses (nMDS and PCO plots), relative percent abundance of bacterial phyla and rarefaction curves were performed and calculated using PRIMER (v.7.0.11), PRIMER-E, Plymouth Marine Laboratory, UK (Clarke et al., 2001).

Conventional measures of species diversity, richness and evenness were calculated using algorithms for total OTUs (S), Pielou's evenness (J'), Shannon diversity (H') and Simpson (1-λ), while taxonomic diversity was calculated using algorithms for taxonomic distinctness: average taxonomic distinctness (avTD - delta+) and variation in taxonomic distinctness (varTD - lambda+) using PRIMER (v.7.0.11) (Clarke et al., 2001). These univariate indicators of diversity (S, J', H', 1-λ, avTD, varTD) were plotted in Prism v. 7.01 (Graphpad Software Inc.). For further presentation of data, relative abundance plots of the top 15 most abundant gut and skin OTUs, and top OTUs in the gut and skin no antibiotics group compared to the antibiotics group were constructed in Excel. To obtain the identification of the closest cultured species for each of the most abundant OTUs, the corresponding sequence was blasted against the RDP isolate database only. A similarity score in parenthesis is presented for each OTU in these plot.

Results

This experimental trial examined whether the microbiome could be manipulated in poor-performing fish. In particular, three key questions were addressed: 1) is there a change in the skin and gill microbiome due to antibiotic therapy (treatments with and without antibiotics); 2) do differences in the route of administration of the 'healthy' inoculum influence the resultant gut and skin microbiome of the poor-performing fish treated with or without antibiotics (gavage vs water inoculum treatments with and without antibiotics; and 3) whether changes in the gut and skin microbiome composition of the poor-performing fish occurs and is sustained, or reverts back to the original state with time (four sampling time points).

General observations

Global community structure

In the nMDS plot of all the samples irrespective of trial treatment type, there was a clear separation between the global community structure of the three environmental (tank/seawater) samples, on-farm pre-trial skin samples, trial skin samples through time and gut samples (including inoculum, on-farm pre-trial and trial samples through time) (Figure 3.3.1.4.2 A).

For the gut samples only (irrespective of trial treatment type), there was overlap in the nMDS plot and no significant difference between the trial samples collected through time (Figure 3.3.1.4.2 B, Table 3.3.1.4.3). Differences were observed between the on-farm pre-trial samples compared to TP2 (P = 0.0063), TP3 (P = 0.0111) and inoculum (P = 0.0009) samples (Table 3.3.1.4.3). Although some of the on-farm, TP0 and TP1 gut samples grouped towards the inoculum cluster, there was a significant difference between the on-farm pre-trial and trial samples compared to the inoculum (Figure 3.3.1.4.2 B, Table 3.3.1.4.3).

For the skin samples only (irrespective of trial treatment type), four distinct clusters were observed and included on-farm pre-trial, trial, inoculum and environmental tank/sea water samples (Figure 3.3.1.4.2 C). Within the trial samples clusters, a shift from left to right from TP0 through to TP3 was observed (Figure 3.3.1.4.2 C). These patterns were confirmed by PERMANOVA, with a significant difference between on-farm pre-trial samples and trial samples through time, within the trial samples through time and between the inoculum and pre-trial and trial samples through time (Figure 3.3.1.4.2 C, Table 3.3.1.4.4).

1. Antibiotics vs no antibiotics

Global community structure

For the gut dataset, the on-farm pre-trial and no antibiotic groups had greatest individual heterogeneity with a wide spread of the sample points, whereas the antibiotic samples clustered together in the nMDS

plot, apart from two samples (Figure 3.3.1.4.3 A). These two samples may be outliers, indicating an inadequate delivery of the antibiotics (e.g. due to the tubing not reaching into the stomach, loss during administration or from regurgitation once the fish were returned to the tanks). From pairwise comparisons, a significant difference was recorded between the on-farm pre-trial and TP0 samples when antibiotics were administered (P = 0.0183), but not for the no antibiotic group (P = 0.0859) (Table 3.3.1.4.5 A). There was also a significant difference in the global community structure in samples from fish administered antibiotics compared to those not given antibiotics (P = 0.0071, Table 3.3.1.4.5 A). Collectively, these results indicate that the gut global bacterial community structure did not change between on-farm and after the fish were transported to the pool-farm facility (despite having been kept in this system for one month prior to the start of the trial), although administrating antibiotics did change the global gut community dynamics.

For the skin dataset, clear separation between the on-farm pre-trial and trial samples was observed, along with a slight shift in the clustering of the antibiotic compared to the no antibiotic treatment group (Figure 3.3.1.4.3 B). These observations were confirmed by PERMANOVA, with a significant difference between all three groups (Table 3.3.1.4.5 B). Therefore, although antibiotics have some effect, changes arising in the skin from moving fish from sea-cages to tanks appears to be more profound.

Bacterial phyla

For the gut samples, specific bacterial phyla were observed to be notably reduced in abundance in the antibiotic treatment groups compared to the on-farm pre-trial and no antibiotic groups. Specific taxa that decreased in abundance included *Cyanobacteria*, *Proteobacteria* and *Spirochaetae*. Only representatives from phyla *Tenericutes* persisted in the samples from the antibiotic treatment groups. Differences were also observed between the on-farm pre-trial and no antibiotic trial groups, with a greater relative abundance of *Spirochaetae* in the tank-based (no antibiotic) samples. Note that *Spirochaetea* have previously been documented as the dominant phyla observed in gut scraping samples of YTK kept in the pool-farm facility as part of the formulated feeds vs natural diets and lipid/emulsifier trials (see Manuscript 3.3.1.2). The RNA inoculum sample (comprising active constituents) was dominated by *Proteobacteria,* whereas the DNA inoculum sample (comprising active and inactive constituents) comprised less *Proteobacteria* and greater representation from *Cyanobacteria, Chloroflexi* and *Firmicutes* (Figure 3.3.1.4.4 B).

For the skin samples, the main difference between the on-farm pre-trial and trial samples at the bacterial phyla level was the replacement of *Cyanobacteria* with *Patescibacteria* and *Latescibacteria*. Lower relative abundance of *Bacteroidetes* but higher relative abundance of *Proteobacteria* was also observed in the trial samples compared to on-farm pre-trial samples (Figure 3.3.1.4.4 B). No clear differences between bacterial phyla composition or relative abundance was observed between the no antibiotics compared to the antibiotics sample groups (Figure 3.3.1.4.4 B).

Top OTUs

For the gut scraping samples, representation from multiple taxa, including *Mycoplasma insons* (OTU 1, similarity [S_ab] score 0.420), *Neorickettsia helminthoeca* (OTU 8, S_ab score 0.472), *Vibrio* sp. V776 (OTU2, S_ab score 1.000), *Ehrlichia* sp. trout isolate (OTU 7, S_ab score 0.821) and *Pseudoalteromonas* species (OTU 14, S_ab score 1.000; and OTU 5, S_ab score 1.000) was recorded in the on-farm pre-trial samples (Figure 3.3.1.4.5). OTU 1, with closest sequence similarity to *Mycoplasma insons* (S_ab score 0.420), was also observed in the trial no antibiotic samples, along with increased relative abundance of *Brevinema andersonii* (OTU 3, S_ab score 0.644), although other taxa, including *Pseudoalteromonas* species (OTU 14, S_ab score 1.000; and OTU 5, S_ab score 1.000), were decreased in abundance (Figure 3.3.1.4.5). In the antibiotic trial group, samples were dominated by *Mycoplasma insons* (OTU 1, S_ab score 0.420) (Figure 3.3.1.4.5).

Antibiotics appeared to have the greatest effect on the abundance of two key taxa in the gut scraping samples (namely *Brevinema andersonii* [OTU 3, S_ab score 0.644] and *Vibrio* sp. V776/*Allivibrio finisterrensis* [OTU 2, S_ab score 1.000]) which decreased in abundance and were two of the three top contributors in the no antibiotics trial group at time point 0 (Figure 3.3.1.4.6). However enrichment of *Mycoplasma insons* (OTU 1, S_ab score 0.420) and *Cetobacterium somerae* (OTU 36, S_ab score 1.000) was recorded in the antibiotic trial samples compared to no antibiotics at this time point (Figure 3.3.1.4.6).

For the skin swab samples, clear differences were observed between the on-farm pre-trial and trial samples for the top 15 bacterial taxa. On-farm pre-trial samples were primarily dominated by *Polaribacter* species, including *P. dokdonensis* (OTU 21, S_ab score 0.591), *P. marinivivus* (OTU 18, S_ab score 0.756) and *Polaribacter* sp. (OTU 68, S_ab score 0.567), as well as *Synechococcus* sp. (OTU 27, S_ab score 0.947) (Figure 3.3.1.4.7). The overall taxonomic diversity was greater in the trial samples, with replacement of the *Polaribacter* species by a range of other organisms, including *Candidatus Aquirestis calciphila* (OTU 10, S_ab score 0.459), *Lewinella persicus* (OTU 19, S_ab score 0.754), *Vibrio tasmaniensis* (OTU 29, S_ab score 1.000) and *Oleispira antarctica* (OTU 13, S_ab score 0.952) (Figure 3.3.1.4.7). Additional taxa beyond the top 15 were also observed to contribute to the relative abundance in both the on-farm pre-trial and trial samples.

Out of the top 15 skin taxa recorded from the no antibiotic samples, 11 were decreased in abundance in the antibiotic trial group, while three were enriched (namely *Vibrio tasmaniensis* [OTU 29, S_ab score 1.000], *Pseudoalteromonas* sp. [OTU 5, S_ab score 1.000] and *Colwellia* sp. [OTU 10350, S_ab score 0.991]) and one showed no change in abundance (namely *Sulfitobacter mediterraneus* [OTU 1508, S_ab score 0.953]) (Figure 3.3.1.4.8).

2 and 3. Effect of route of inoculum administration (gavage inoculum vs water inoculum) and changes through time (four sampling time points)

Global community structure

For the gut scraping samples at the first time point, TP1 (two days post inoculum, five days after antibiotic administration), there was a significant difference between antibiotics and no antibiotics samples for groups where no inoculum was administered (P = 0.0271, Table 3.3.1.4.6 A) (Figure 3.3.1.4.9 A). This indicates changes in the global community structure of the gut microbiome due to antibiotic therapy is maintained for at least 5 days after administration. However, when inoculum was also administered (either into the water or via gavage), there was no significant difference between the antibiotics and no antibiotics groups (P = 0.0872 and P = 0.0555, respectively, Table 3.3.1.4.6 A) (Figure 3.3.1.4.9 A). There was also no significant difference in the gut global bacterial community structure for the antibiotics group for samples from fish where no inoculum was given compared to water inoculum, as well as no inoculum compared to gavage inoculum (P = 0.390 and P = 0.1738, respectively, Table 3.3.1.4.6 A). The same was true for the no antibiotics + no inoculum group compared to both the antibiotics + water inoculum (P = 0.0558), and antibiotics + gavage inoculum samples (P = 0.0558) (Table 3.3.1.4.6 A) (Figure 3.3.1.4.9 A). This indicates that for both antibiotics and no antibiotics groups at this first time point (TP1, two days post inoculum), there is no change in the gut global bacterial community structure with the addition of inoculum either via gavage or into the water. Nonetheless, a significant difference was recorded between the no antibiotics + no inoculum group compared to the TP1a (five days post inoculum) antibiotics + gavage inoculum samples (P = 0.0308, Table 3.3.1.4.6 A). However the TP1a antibiotics + gavage inoculum samples were significantly different to the RNA inoculum samples (P = 0.0282, Table 3.3.1.4.6 A) (Figure 3.3.1.4.9 A). Therefore, for this treatment (i.e. antibiotics + gavage inoculum, five days post inoculum), a change in the global community structure was apparent and is different to what was observed in the RNA inoculum sample. No significant differences were recorded between the water and gavage inoculum samples, both with antibiotics (P = 0.2758) and without antibiotics (P = 0.5108) (Table 3.3.1.4.6 A, Figure 3.3.1.4.9 A). Therefore, the gut community structure of gavage and water inoculum samples remained the same after TP1 (two days post inoculum). A significant difference was recorded between the RNA inoculum

samples and the water and gavage inoculum groups with and without antibiotics (Table 3.3.1.4.6 A, Figure 3.3.1.4.9 A). Therefore the global community structure of the inoculum treatment groups (gavage and water with and without antibiotics) at TP1 was different to that observed in the RNA inoculum.

For the gut scraping samples at TP2 (eight days post inoculum, 11 days after antibiotics administration), there was no significant difference between the antibiotics and no antibiotics samples for groups where no inoculum was administered (P = 0.1693, Table 3.3.1.4.6 B) (Figure 3.3.1.4.9 B). This indicates the global community structure of the gut microbiome reverts back to its 'original' state between 5 and 11 days after antibiotic administration. Interestingly, a significant difference was recorded between antibiotics + gavage inoculum compared to no antibiotics + gavage inoculum (P = 0.0320), as well as between no antibiotics + no inoculum compared to antibiotics + water inoculum (P = 0.0259) and antibiotics + gavage inoculum (P = 0.0265) (Table 3.3.1.4.6 B, Figure 3.3.1.4.9 B). The former indicates antibiotics may still be having an effect 11 days post administration, while the latter suggests that at TP2, there is a change in the global gut community structure with the addition of inoculum into the water and via gavage for fish given antibiotics compared to those where no antibiotics or inoculum were administered. However, as observed at TP1, a significant difference was recorded between the RNA inoculum samples and the water and gavage inoculum groups with and without antibiotics (Table 3.3.1.4.6 B, Figure 3.3.1.4.9 B). Therefore, the global community structure of the inoculum treatment groups (gavage and water with and without antibiotics) at TP2 was different to that observed in the RNA inoculum.

For the gut scraping samples at TP3 (15 days post inoculum, 18 days after antibiotics administration), there was no significant difference between antibiotic and no antibiotic samples for groups where no inoculum was administered (P = 0.1668, Table 3.3.1.4.6 C) (Figure 3.3.1.4.9 C). This follows what was observed for the TP1 and TP2 samples, specifically that the global bacterial community structure reverts back to its 'original' state between 5 and 11 days after antibiotic administration and maintains this reversion up until 18 days after antibiotic administration. All other comparisons between the water and gavage inoculum groups with and without antibiotics were not significantly different, highlighting that after time (15 days post inoculum), the microbiome reverts back to its 'original' state with any differences observed at TP1 and TP2 not maintained at TP3 (Table 3.3.1.4.6 C, Figure 3.3.1.4.9 C). Furthermore, as observed at TP1 and TP2, a significant difference was still recorded between the RNA inoculum samples and the water and gavage inoculum groups with and without antibiotics at TP3 (Table 3.3.1.4.6 C, Figure 3.3.1.4.9 C). Therefore, the global community structure of the inoculum treatment groups (gavage and water with and without antibiotics) at TP3 was different to that observed in the RNA inoculum.

Due to such dissimilarities between the inoculum and skin samples, which confounded the nMDS analyses, Principal Co-ordinates (PCO) analysis was instead calculated with results plotted to evaluate differences in the global community structure of the skin and inoculum samples at TP1 (2 days post inoculum), TP1a (5 days post inoculum), TP2 (8 days post inoculum) and TP3 (15 days post inoculum) (Figure 3.3.1.4.10). At all three time points (TP1a with TP1), there was clear separation between the on-farm pre-trial samples compared to the tank trial samples, irrespective of treatment type (i.e. no inoculum, water inoculum or gavage inoculum) (Figure 3.3.1.4.7). As the skin is in constant contact with the surrounding environment, it was expected that fish in the different cultivation systems (e.g. 'natural' offshore conditions compared to a 'modulated' environment onshore) would exhibit different microbiome structures. As expected, having come from the gut, the inoculum sample separated away from and was significantly different to all of the skin swab samples (Figure 3.3.1.4.10, Table 3.3.1.4.7). At TP1 and TP2, there was no significant difference between the antibiotics and no antibiotics samples for groups where no inoculum was administered (Table 3.3.1.4.7 A, B), however, a significant difference was observed at TP3 (Table 3.3.1.4.7 C). For TP1, the only significant difference recorded between the treatment types was for the no inoculum compared to the water inoculum group when antibiotics were administered (P = 0.0281, Table 3.3.1.4.7 A). This indicates the combination of antibiotic therapy and administration of inoculum into the surrounding environment of the fish has a rapid effect on the resultant skin bacterial profile (by TP1, 2 days post inoculum). A greater number of significant differences in the skin global community structure between treatment types was observed at

the later sampling time points of 8 days (TP2) and 15 days (TP3) post inoculum (Table 3.3.1.4.7). In particular, at TP2 and TP3, a significant difference was also recorded between the antibiotics and no antibiotics groups for both water and gavage inoculum, as well as between the no inoculum and water inoculum or no inoculum and gavage inoculum groups with and without antibiotics (Table 3.3.1.4.7 B, C). While a significant difference was recorded between the water inoculum and gavage inoculum groups with and without antibiotics at TP2 (Table 3.3.1.4.7 B), at TP3 these groups were not significantly different (Table 3.3.1.4.7 C).

Bacterial phyla

For the gut samples, the on-farm pre-trial poor-performing fish (sampled one month before TP1) were represented by either phyla *Cyanobacteria* (one fish), *Proteobacteria* (four fish) or *Tenericutes* (four fish) (Figure 3.3.1.4.11). After one month in the pool-farm facility with no antibiotic therapy or inoculum treatment, there was almost a complete loss of *Cyanobacteria* and *Proteobacteria*, with replacement by *Spirochaetes*, although abundance of *Tenericutes* was maintained over this period (Figure 3.3.1.4.11 A).

At TP1, for the group administered antibiotics, almost complete dominance by *Tenericutes* was observed irrespective of treatment type (i.e. no inoculum, water inoculum or gavage inoculum), although greatest phyla diversity was recorded in the gavage inoculum fish. In particular, representation by *Tenericutes*, *Proteobacteria*, *Cyanobacteria*, *Chloroflexi*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria* was recorded in two of the four fish from the gavage inoculum cohort at this time point (Figure 3.3.1.4.11 A). For the treatment types (water or gavage inoculum) without antibiotics, *Spirochaetes* and *Tenericutes* were observed as the dominant phyla in three out of the four water inoculum and one out of the four gavage inoculum fish. The remaining water inoculum fish were dominated by *Proteobacteria*, with the remaining gavage inoculum fish dominated by *Proteobacteria* (two fish) or *Spirochaetes* (one fish). A decrease in the abundance of *Tenericutes* was observed in the gavage compared to water inoculum samples (Figure 3.3.1.4.11 A).

At TP2, dominance by *Tenericutes* was maintained in the group administered antibiotics irrespective of treatment type, with loss of phyla diversity that was observed at TP1 in the gavage inoculum fish (Figure 3.3.1.4.11 B). For the group without antibiotics, the contribution of *Proteobacteria* in the water and gavage inoculum samples that was observed at TP1 was reduced, with instead greater phyla dominance by *Tenericutes* alongside *Spirochaetes*. *Firmicutes* and *Bacteroidetes* also observed in one no inoculum and one water inoculum sample (Figure 3.3.1.4.11 B).

At TP3, dominance by *Tenericutes* was maintained in the group administered antibiotics irrespective of treatment type, although additional phyla, including *Proteobacteria*, *Bacteroidetes* and *Firmicutes* was observed in one water inoculum and two gavage inoculum fish (Figure 3.3.1.4.11 C). For the no antibiotics group, *Tenericutes* and *Spirochaetes* remained as dominant phyla as observed at TP2 irrespective of treatment type, with *Firmicutes* and *Bacteroidetes* again recorded as minor constituents in two water inoculum samples (Figure 3.3.1.4.11 C).

The DNA inoculum (global constituents) was characterised by *Proteobacteria* and *Chloroflexi* as the dominant phyla and *Cyanobacteria*, *Firmicutes* and *Actinobacteria* as minor contributors (Figure 3.3.1.4.11). Conversely, the RNA inoculum sample (representing the active constituents), was dominated by the phylum *Proteobacteria* (Figure 3.3.1.4.11). Conserved taxonomy at the lower class (*Gammaproteobacteria*), order (*Vibrionales*), family (*Vibrionaceae*) and genus (*Allivibrio*) levels was also observed (Figure 3.3.1.4.13). This was due to the dominance of a single bacterial species with closest similarity to *Vibrio* sp. V776/*Allivibrio finisterrensis* (OTU 2, S_ab score 1.000), with a relative abundance of up to 97%.

For the skin samples, representation by *Proteobacteria*, *Bacteroidetes*, *Cyanobacteria* and *Firmicutes* was observed in the pre-trial on-farm fish (Figure 3.3.1.4.12). After one month in the pool-farm facility with no antibiotic therapy or inoculum treatment, *Bacteroidetes* and *Cyanobacteria* were decreased in abundance, whereas *Proteobacteria* was increased. Minor representation by additional phyla, including

Actinobacteria, *Firmicutes*, *Patescibacteria* and *Epsilonbacteraeota* was also observed (Figure 3.3.1.4.12 A). At TP1, a similar phyla profile of dominance by *Proteobacteria* and representation by *Bacteroidetes* was observed for all treatment types (i.e. no inoculum, water inoculum or gavage inoculum) with and without antibiotics, along with minor representation by *Cyanobacteria*, *Actinobacteria*, *Firmicutes*, *Patescibacteria* and *Epsilonbacteraeota* (Figure 3.3.1.4.12 A). This pattern was maintained through time, with a similar phyla profile observed at TP2 and TP3 (Figure 3.3.1.4.12 B, C).

Top 15 OTUs

The on-farm pre-trial gut samples were primarily dominated by OTU 1 with closest sequence similarity to *Mycoplasma insons* (S_ab score 0.420), although *Vibrio* sp. V776/*Allivibrio finisterrensis* (OTU 2, S_ab score 1.000) and *Neorickettsia helminthoeca* (OTU 8, S_ab score 0.472) were also recorded as dominant constituents. Representation by additional taxa, with closest sequence similarity to *Ehrlichia* sp. 'trout isolate' (OTU 7, S_ab score 0.821) and *Pseudoalteromonas* species (*P. haloplanktis*, OTU 14, S_ab score 1.000 and *Pseudoalteromonas* sp., OTU 5, S_ab score 1.000) were also recorded in some individuals. After one month in the pool-farm facility with no antibiotic therapy or inoculum treatment, the occurrence of *Brevinema andersonii* (OTU 3, S_ab score 0.644) was recorded alongside *M. insons* (OTU 1, S_ab 0.420) (Figure 3.3.1.4.14).

At TP1, for the group administered antibiotics, almost complete dominance by *M. insons* (OTU 1, S_ab 0.420) was observed irrespective of treatment type (i.e. no inoculum, water inoculum or gavage inoculum), although this organism was notably decreased in abundance in two of the gavage inoculum samples, which also had representation by additional taxa beyond the top 15 (Figure 3.3.1.4.14). Furthermore, certain taxa that had been recorded in the on-farm pre-trial fish were not observed in the TP1 antibiotics group irrespective of treatment type, including *Vibrio* sp. V776/*A. finisterrensis* (OTU 2, S_ab score 1.000), *Ehrlichia* sp. 'trout isolate' (OTU 7, S_ab score 0.821) and *Pseudoalteromonas* species (*P. haloplanktis*, OTU 14, S_ab score 1.000 and *Pseudoalteromonas* sp., OTU 5, S_ab score 1.000) (Figure 3.3.1.4.14). For the no antibiotics group, representation by three taxa was recorded for the water and gavage inoculum samples, including *M. insons* (OTU 1, S_ab 0.420) and *Vibrio* sp. V776/*A. finisterrensis* (OTU 2, S_ab score 1.000), as also documented from the on-farm pre-trial fish, and *B. andersonii* (OTU 3, S_ab score 0.644), as also documented from the no antibiotics + no inoculum group (Figure 3.3.1.4.14).

Similarly to TP1, the antibiotics group at TP2 was dominated by *M. insons* (OTU 1, S_ab 0.420) irrespective of treatment type, with >95% relative abundance recorded in some samples from the no inoculum, water inoculum and gavage inoculum groups (Figure 3.3.1.4.14). For the no antibiotics group, loss of *Vibrio* sp. V776/*A. finisterrensis* (OTU 2, S_ab score 1.000) was observed in the water and gavage inoculum samples, with dominance by either *M. insons* (OTU 1, S_ab score 0.420) or *B. andersonii* (OTU 3, S_ab score 0.644). No distinct differences between the no inoculum, water inoculum and gavage inoculum samples were observed (Figure 3.3.1.4.14).

Again, similar to TP1 and TP2, the antibiotics group at TP3 was dominated by *M. insons* (OTU 1, S_ab 0.420) irrespective of treatment type, with >95% relative abundance recorded in some samples from the no inoculum, water inoculum and gavage inoculum groups (Figure 3.3.1.4.14). There was also representation from additional taxa in one water inoculum and one gavage inoculum sample (Figure 3.3.1.4.14). For the no antibiotics group, loss of *Vibrio* sp. V776/*A. finisterrensis* (OTU 2, S_ab score 1.000) was maintained irrespective of treatment type, along with dominance by either *M. insons* (OTU 1, S_ab score 0.420) or *B. andersonii* (OTU 3, S_ab score 0.644) (Figure 3.3.1.4.14). OTU 20, with similarity to *Treponema pallidum* (S_ab score 0.474) also recorded from one no inoculum + no antibiotics sample (Figure 3.3.1.4.14).

For the skin samples, contribution by taxa beyond the top 15 was observed for all the samples (including on-farm pre-trial, no inoculum, water inoculum and gavage inoculum with and without antibiotics through time), indicating greater taxonomic diversity in the skin compared to the gut microbiome (Figure 3.3.1.4.15). Shifts in relative abundance of key taxa was also observed between groups. While

the on-farm pre-trial samples were primarily represented by two *Polaribacter* species (*P. dokdonensis*, OTU 21, S_ab score 0.591 and *P. marinivivus*, OTU 18, S_ab score 0.756), after one month in the pool-farm facility with no antibiotic therapy or inoculum treatment, these two organisms were notably decreased in abundance, where instead greater overall and diversity was observed (Figure 3.3.1.4.15).

At TP1, similarities in the bacterial taxa profile were observed between the antibiotics and no antibiotics groups irrespective of treatment type, including representation from *Colwellia* sp. (OTU 10350, S_ab score 0.991), *Pseudoalteromonas* species (*Pseudoalteromonas* sp., OTU 5, S_ab score 1.000 and *P. haloplanktis*, OTU 14, S_ab score 1.000), *Psychrobacter* sp. (OTU 49, S_ab score 1.000), *Lewinella persicus* (OTU 19, S_ab score 0.754) and *Halioglobus japonicus* (OTU 28, S_ab score 0.683) (Figure 3.3.1.4.15).

At TP2, the occurrence of *Photobacterium* sp. (OTU 15, S_ab score 0.526) from the no inoculum + antibiotics group and *Psychromonas profunda* (OTU 37, S_ab score 0.824) from the water and gavage inoculum groups with antibiotics were also observed in addition to the taxa recorded at TP1, along with a decrease in abundance of *Pseudoalteromonas* sp. (OTU 5, S_ab score 1.000) (Figure 3.3.1.4.15). For the no antibiotics group, in addition to the taxa recorded at TP1, *Photobacterium* sp. (OTU 15, S_ab score 0.526) was recorded across all treatment types, along with a decrease in abundance of *Pseudoalteromonas* sp. (OTU 5, S_ab score 1.000) and *P. haloplanktis* (OTU 14, S_ab score 1.000) and an increase in abundance of *Aestuariibacter* sp. (OTU 40, S_ab 0.692) in the water inoculum samples at TP2 compared to TP1 (Figure 3.3.1.4.15). Taxa profiles of the gavage inoculum samples at TP1 and TP2 were similar (Figure 3.3.1.4.15).

At TP3, similarities were again evident between the treatment types with and without antibiotics and compared to TP1 and TP2, although the relative abundance of *Photobacterium* sp. (OTU 15, S_ab score 0.526) increased in the no inoculum with or without antibiotics groups, and water and gavage inoculum samples without antibiotics at TP3 compared to TP2 (Figure 3.3.1.4.15). In addition, *Aestuariibacter* sp. (OTU 40, S_ab score 0.692) was decreased in abundance in all samples compared to TP2, with an increase in abundance of *Pseudoalteromonas* sp. (OTU 2672, S_ab score 0.918) and *Litoribacillus peritrichatus* (OTU 53, S_ab score 0.912) (Figure 3.3.1.4.15).

In summary, the key bacterial taxa that were maintained through time for all skin swab trial samples (no inoculum, water inoculum and gavage inoculum with and without antibiotics) included *Colwellia* sp. (OTU 10350, S_ab score 0.991), *Psychrobacter* sp. (OTU 49, S_ab score 1.000) and *Lewinella persicus* (OTU 19, S_ab score 0.754). Additional taxa were then recorded at each time point, including *Pseudoalteromonas haloplanktis* (OTU 14, S_ab score 1.000) and *Oleispira antarctica* (OTU 13, S_ab score 0.683) at TP1; *Photobacterium* sp. (OTU 15, S_ab score 0.526), *Psychromonas profunda* (OTU 37, S_ab score 0.824) and *Aestuariibacter* sp. (OTU 40, S_ab score 0.692) at TP2; and *Photobacterium* sp. (OTU 15, S_ab score 0.526), *Psychromonas profunda* (OTU 37, S_ab score 0.824), *Pseudoalteromonas* sp. (OTU 2672, S_ab score 0.918) and *Litoribacillus peritrichatus* (OTU 53, S_ab score 0.912) at TP3 (Figure 3.3.1.4.15).

Diversity indices

For the gut samples at TP1, an increase in total species richness, evenness (Pielou's) and diversity (Shannon, Simpson and delta+) was observed for the water and gavage inoculum samples with antibiotics compared to the no inoculum control, however, this trend was not as apparent for the no antibiotics treatment groups compared to the control (Figure 3.3.1.4.16).

At TP2, while an increase in total species richness between the no inoculum control group compared to the gavage inoculum group with antibiotics was observed, evenness (Pielou's) and diversity (Shannon and Simpson) were lower. For the no antibiotics group, total species richness, evenness (Pielou's) and diversity (Shannon and Simpson) were also lower in the gavage inoculum compared to no inoculum control group (Figure 3.3.1.4.17).

At TP3, total species richness was greatest in the no inoculum control groups with and without antibiotics compared to the water and gavage inoculum groups with and without antibiotics, with the

opposite trend of greater species evenness (Pielou's) and diversity (Shannon and Simpson) observed for the water and gavage inoculum groups with and without antibiotics compared to the no inoculum control groups with and without antibiotics (Figure 3.3.1.4.18).

For the skin swab samples, total species richness, evenness (Pielou's, lambda+) and diversity (Shannon, Simpson and delta+) was similar between all trial samples at each time point (Figures 3.3.1.4.19, 3.3.1.4.20 and 3.3.1.4.21), although values for these diversity indices were higher in the skin swab samples compared to the gut samples. Compared to the on-farm pre-trial skin swab samples, total species richness, evenness (Pielou's) and diversity (Shannon and Simpson) was greater in the tank trial skin swab samples irrespective of treatment type or time point (Figures 3.3.1.4.19, 3.3.1.4.20 and 3.3.1.4.21).

Discussion

This trial aimed to determine if the microbiome of poor-performing fish could be modulated to improve health outcomes. To elucidate this, a sea-cage at Point Boston containing a population of poor-performing fish fed Feed B was identified, with fish selected from this sea-cage for the trial based on weight (specifically fish with weights in the lowest 20% range were targeted, equating to ≤1.6 kg). After a settling in period of one month in the onshore tank based system (SARDI pool-farm facility), the trial commenced, with six treatments evaluated in duplicate. In particular, Treatments 1 and 2 were water inoculum with and without antibiotics respectively; Treatments 3 and 4 were gavage inoculum with and without antibiotics respectively; and Treatments 5 and 6 were controls (no inoculum) with and without antibiotics respectively. The system was changed from recirculating to flow through before fish arrived and maintained in this manner through the duration of the trial to eliminate environmental cross-contamination between the treatment tanks. Hindgut scrapings and skin swab samples were collected from the on-farm pre-trial fish, at TP0 three days after antibiotics were administered and three subsequent time points post inoculum (e.g. TP1, two days post inoculum; TP2, eight days post inoculum; and TP3, 15 days post inoculum). The inoculum was collected from stripping 102 'healthy' YTK of a larger size class (~3.5-4.5 kg), on a different diet formulation (Feed C) and from a different sea-cage site (Bickers) on-farm, with the inoculum delivered fresh to the treatment fish on the same day it was collected. The treatments were designed to address three key questions, specifically 1) whether antibiotic therapy itself elicits a change in the skin and gut microbiome (treatments with and without antibiotics); 2) if differences in the route of administration of the 'healthy donor' inoculum influences the resultant gut and skin microbiome of the poor-performing fish treated with or without antibiotics (gavage vs water inoculum treatments with and without antibiotics); 3) whether changes in the gut and skin microbiome composition of the poor-performing fish occurs and is sustained, or reverts back to the original state with time (four sampling time points).

Compositional comparisons were also evaluated in relation to those of the surrounding seawater (collected on-farm [Point Boston] and from the pool-farm facility system in-flow at the start [TP0] and end [TP3] of the trial) to determine if environmentally-independent gut and skin community assemblages are selected for in the gut and skin respectively. Consistent with previous observations (see Manuscripts 3.3.1.1, 3.3.1.2 and 3.3.1.3), the global bacterial community composition between the environmental seawater samples and the gut samples was markedly different, again supporting the notion that YTK are able to regulate and maintain their own environmentally-independent bacterial communities in the gut. In addition, differences were also observed between the global community structure of the environmental seawater samples compared to the skin samples, highlighting that although YTK are in direct contact with their external environment, they are still able to regulate and maintain their own host-specific bacterial assemblages in the skin. This is consistent with the findings of Legrand et al. (2018) for healthy YTK sampled on-farm in southern Australian waters, along with what has been reported for other teleosts (Wang et al., 2010; Chiarello et al., 2015; Schmidt et al., 2015).

RNA and DNA inoculum samples

While a healthy gut microbiome is characterised by a diverse network of metabolically interacting microbial members (Abbeele et al., 2013), an imbalance or critical disturbance in the normal community members and loss of diversity is typical of a dysbiotic state (Alonso and Guarner, 2013; Montalban-Arques et al., 2015). Therefore by targeting a specific sea-cage of fish on-farm that had been identified by CS staff as 'healthy', and were from a different farming site and of a different size class, it was anticipated that the gut microbiome of these 'healthy donor' fish and the poor-performing fish selected for in the trial would be different. This was confirmed by a clear separation in the nMDS plot and significant difference between the inoculum samples and the on-farm pre-trial samples. However, it was also assumed that the gut microbiome composition of the 'healthy donor' fish would be diverse, with an array of bacterial phyla and taxa contributing to the total relative abundance, as was observed from the 'healthy' fish collected on-farm as part of the 2016 health vs disease activity (see Manuscript 3.3.1.3). While bacterial phyla diversity, including representation from the *Proteobacteria*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes* and *Actinobacteria*, was observed in the DNA inoculum (representing the global constituents), the RNA inoculum (representing the active constituents) was dominated by a single phylum (*Proteobacteria*), class (*Gammaproteobacteria*), order (*Vibrionales*), family (*Vibrionaceae*) and genus (*Allivibrio*). At the taxa level, it was revealed that OTU 2, with similarity to *Vibrio* sp. V776/*Allivibrio finisterrensis* (similarity [S_ab] score 1.000), was the organism responsible for this loss of diversity, with a relative abundance of ~97% in the RNA inoculum sample. Displacement of the microbiome and dominance by a single taxon, which subsequently reduces functionality, are characteristics of an imbalanced, dysbiotic state, and may highlight that the 'healthy donor' fish were suffering from an underlying disease condition. Nonetheless, *Vibrio* sp. V776/*Allivibrio finisterrensis* had not been identified as the sole dominant constituent in enteritis disease samples collected from the 2016 and 2017 health vs disease surveys on-farm, although it was recorded as the second most abundant taxa after *Mycoplasma insons* in the 2016 Northern Site and 2017 Bickers site disease fish (see Manuscript 3.3.1.3). This dominance and potential dysbiotic state of the 'healthy donor' fish may therefore confound the results of the microbiome manipulation trial and is taken into consideration when reporting on the findings below.

Effect of antibiotic therapy on the gut and skin microbiome of YTK

In humans, antibiotics can interrupt the healthy balance of the gut microbiome, leading to loss of microbial competitors for some opportunistic bacteria, thereby increasing their relative abundance as well as affecting gut microbiome composition and diversity (Francino, 2016). However, nothing is known concerning the effects of antibiotics on the gut microbiome and histopathology of YTK, therefore a small-scale antibiotics trial was conducted in April 2018 at the SARDI pool-farm facility, with gross and histopathological examination of each fish, and total plate counts of aerobic and anaerobic culture from the hindgut, assessed (Veterinary Diagnostics Laboratory, University of Adelaide, Roseworthy Campus). Based on the results (using a combination therapy of three antibiotics; 200mg/kg oxytetracycline and 50mg/kg erythromycin and metronidazole), a reduction in both aerobic and anaerobic bacterial plate counts two days post antibiotics administration without compromising overall fish pathology and health was observed. Consequently, this antibiotic combination and dosage was then used in the microbiome manipulation trial. To coincide with the knock-down of bacterial taxa that observed in the small-scale trial after two days, but which then began to re-establish after four days, the inoculum was administered to the poor-performing fish three days after antibiotic therapy. As no microbiome samples were analysed from the small-scale antibiotics trial, the specific bacterial phyla and taxa that this combination therapy was effective against was unknown. From the microbiome manipulation trial, hindgut scraping and skin swab samples were collected from the on-farm pre-trial fish and compared to the baseline tank trial samples collected one month later that had either been treated with antibiotics three days prior or given no therapy for the month of holding in the pool-farm facility.

For the gut samples, while there was no significant difference in the global community structure of the on-farm pre-trial samples and the no antibiotics samples (collected one month later), the group treated with antibiotics was significantly different to both. In particular, the combination and dosage of antibiotics used in the trial was effective against taxa from three main bacterial phyla, including *Cyanobacteria*, *Proteobacteria* and *Spirochaetae*, although the therapy had limited effect on *Tenericutes*. Specifically, reduced abundance of *Brevinema andersonii* (OTU 3, S_ab score 0.644) and *Vibrio* sp. V776/*Allivibrio finisterrensis* (OTU 2, S_ab score 1.000) was observed, although *Mycoplasma insons* (OTU 1, S_ab score 0.420), and to a lesser extent, *Cetobacterium somerae* (OTU 36, S_ab score 1.000), increased in abundance. *Mycoplasma insons* has previously been recorded as a dominant taxa in disease YTK sampled from the 2016 and 2017 health vs disease surveys on-farm (see Manuscript 3.3.1.3), and it's enrichment observed here indicates the antibiotics therapy used in the trial has no effect on this organism, and that it is likely an opportunistic pathogen that is able to proliferate and dominate once competition by other taxa is eliminated. This is not unexpected, as mycoplasma are typically parasitic species that are characterised by their uniquely small genomes and lack of a cell wall, where they are able to evade the host's immunological responses by infecting the cellular membranes (Razin et al., 1998). For this reason, infections arising from mycoplasma are thus often difficult to treat with antibiotics, as was confirmed here. Wang et al. (2019) also observed increased relative abundance of potentially pathogenic taxa, including *Plesiomonas*, *Aeromonas*, *Shewanella* and *Pseudomonas* species, in Channel Catfish (*Ictalurus punctatus*) fed with medicated feed supplemented with florfenicol. Our results present an important finding for YTK farm management, as if antibiotics are to be used in the future to treat disease cohorts in an effort to improve health outcomes, then the combination used here is proven to have no effect on *M. insons*, one of the most dominant taxa, and may even exacerbate the disease issue but allowing this organism to establish complete dominance in the population. As a weak sequence identity to this species was observed (S_ab score 0.420), it is likely that this OTU represents a related taxon within the broader family *Mycoplasmataceae* (or Order *Mycoplasmatales*), highlighting that further work is required to determine its definitive identification and associated pathogenicity.

Although the antibiotics were delivered via gavage into the stomach of the fish, differences in the community structure and composition of the outer-surface skin microbiome were evident between the no antibiotics and antibiotics groups. In particular, out of the top 15 taxa in the no antibiotics group, 11 were decreased in abundance in the antibiotics group (e.g. among others, *Candidatus Aquirestis calciphila*, OTU 10, S_ab score 0.459; *Lewinella persicus*, OTU 19, S_ab score 0.754; and *Fluviicola taffensis*, OTU 13, S_ab score 0.952), while three were enriched (e.g. *Vibrio tasmaniensis*, OTU 29, S_ab score 1.000; *Pseudoalteromonas* sp, OTU 5, S_ab score 1.000; and *Colwellia* sp., OTU 10350, S_ab score 0.991) and one showed no change in abundance (e.g. *Sulfitobacter mediterraneus*, OTU 1508, S_ab 0.953). Legrand et al. (2018) also showed changes in the outer-surface skin and gill microbiome of YTK that are reflective of an underlying gut-associated enteritis disease, highlighting that gut health status is an important factor which defines skin and gill bacterial assemblages. Note that additional taxa beyond the top 15 were observed to contribute to the relative abundance in both the on-farm pre-trial and trial skin swab samples at all time points, indicating that there is greater taxa diversity in the skin compared to the gut microbiome of YTK.

Unlike the observations made for the gut microbiome, with no change in community composition between the on-farm pre-trial fish and those sampled one month later in the pool-farm facility, differences in the skin microbiome composition were observed. As the outer-most skin surface, unlike the gut, is in constant contact with the external environment, it is expected that a change from the 'natural' on-farm seawater environment to a 'modulated' tank-based seawater environment will elicit changes in the resultant skin microbiome. In particular, the main changes observed were a higher relative abundance of *Bacteroidetes* followed by *Proteobacteria*, with minor representation by *Cyanobacteria* in the on-farm pre-trial fish compared to replacement of *Cyanobacteria* with *Patescibacteria* and *Latescibacteria*, and lower relative abundance of *Bacteroidetes* but higher relative abundance of *Proteobacteria* in the trial samples. Interestingly, the abundances of phyla observed for the on-farm pre-trial fish were similar to that reported from YTK exhibiting signs of early and late-stage gut enteritis, whereas the abundances in the trial samples, particularly for the antibiotics group, were similar to that reported from healthy and wild YTK (Legrand et al., 2018). This suggests that antibiotic

therapy delivered to the gut may be exerting a positive influence and improvement in health for the resultant skin microbiome. This is further supported by the down-regulation in the antibiotic treated group of the top OTU in the no antibiotics trial group, *Candidatus Aquirestis calciphila* (OTU 10, S_ab score 0.459), an organism that has previously been documented as one of the significantly abundant OTUs in the skin of early enteritis YTK (Legrand et al., 2018).

Inoculum administration (water vs gavage) through time

For the gut samples, a significant difference was recorded between all treatment types (e.g. no inoculum, water inoculum and gavage inoculum) at all three sampling time points (e.g. two, eight and 15 days post inoculum) with and without antibiotics compared to the RNA inoculum sample. This broadly indicates that a shift towards a gut microbiome composition that was observed in the inoculum did not occur for the poor-performing fish at any time point with any treatment type with or without antibiotics. However, as indicated earlier, dominance by a single taxon in the RNA inoculum may confound these results. Nonetheless, other interesting findings were still uncovered.

Firstly, the occurrence of *Brevinema andersonii* (OTU 3, S_ab score 0.644), which was documented from the on-farm pre-trial fish, however through time, was found to increase in abundance in the no antibiotics group irrespective of treatment type. This organism has previously been documented as the dominant taxa in two other SARDI pool-farm facility nutritional trials; formulated feeds vs natural diets, and low and high lipid levels with and without emulsifiers (see Manuscript 3.3.1.2). Although seasonality, year class and dietary formulations varied between these three trials, the size of the fish sampled at the completion of each trial overlaps, namely being 1.4-1.8 kg. It therefore appears that the conditions governing this pool-farm facility environment coupled with this specific size class of 1.4-1.8 kg YTK enriches for *B. andersonii* (OTU 3, S_ab score 0.644). This is important to note for future trials where microbiome samples may be collected, as although the two previous nutritional trials ran for approximately three months, this organism was also seen to dominant after only one month and could confound the results of future studies of this size class if fish are held in this system for any time longer than one month.

Secondly, for two fish individuals at TP1 that were given antibiotics and the inoculum via gavage, greater phyla and taxa diversity, down-regulation of the potential opportunistic pathogen *M. insons* (OTU 1, S_ab score 0.420), and greater evenness (Pielou's) and diversity (Shannon and Simpson) was observed. In particular, at the phyla level there was decreased abundance of the dominant *Tenericutes* coupled with representation from additional phyla including *Proteobacteria*, *Cyanobacteria*, *Chloroflexi*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria*. At the OTU level, *M. insons* (OTU 1, S_ab score 0.420) was almost reduced to 0% relative abundance in one of the two fish, with decreased abundance to less than 40% in the second fish (compared to >95% relative abundance in fish given antibiotics but with no inoculum or water inoculum). Additional taxa beyond the top 15 were also observed to contribute to the total relative abundance in these two samples. This finding is encouraging and collectively supports an improvement in gut health with greater functionality for these fish given antibiotics and the inoculum via gavage at two days post inoculum. This does warrant further investigation into the ability to manipulate the microbiome of poor-performing fish to improve health outcomes, as although this positive effect was short-lived (not observed in antibiotics + gavage inoculum fish at TP2 [eight days] or TP3 [15 days] post inoculum), a refined trial could be undertaken in the future, focusing on this treatment type, but repeating the dose of the inoculum between two and eight days after administration, and potentially weekly thereafter. To ensure diversity in the inoculum used, strains of known-beneficial taxa (e.g. *Bacillus* spp., lactic acid bacteria [*Carnobacterium* sp., *Lactobacillus* sp., *Lactococcus* sp., *Streptococcus* sp., *Weissella* sp.], *Pseudomonas* sp. [*P. flurescens*] and *Vibrio* sp. [*V. alginolyticus*, *V. salmonicida*-like]; Schulze et al., 2006; Pérez et al., 2010; Marchesi et al., 2016) could be included, along with those taxa that have previously been observed to be down-regulated in disease samples (e.g. *Geobacillus stearothermophilus*/*G. thermoparaffinivorans* [OTU 45, S_ab score 1.000], *Bacillus smithii* [OTU 83, S_ab score 1.000] and *Bacillus* sp./*Geobacillus stearothermophilus* [OTU 234, S_ab score 0.977], see Manuscript 3.3.1.3 for further details). For

application on-farm, introduction of the inoculum on-feed could be investigated in the refined trial, which would also allow for easier continual repeat dosing of the fish with the inoculum.

Lastly, at TP2, significant differences in the global community structure were observed between the no antibiotics + no inoculum group compared to the water and gavage inoculum samples with antibiotics. Although the community structure of the water and gavage inoculum samples was still different to that observed from the RNA inoculum, this result does support the notion that the gut microbiome can be manipulated, with significant differences observed eight days post inoculum for water and gavage treatments alongside antibiotic therapy. Note that at 15 days post inoculum, there was no significant difference between any of the treatment types with or without antibiotics, indicating that the microbiome can change between two to eight days post inoculum, but then reverts back to the original state between eight and 15 days post inoculum. This provides further valuable information that will assist in the design of additional, refined trials, firstly indicating that a second dosage of inoculum would be needed between eight to 15 days after the first dosage to prevent a reversion back to the original state, secondly that antibiotic therapy appears to be required in order to modulate the microbiome and lastly, that administering inoculum into the surrounding environment can also elicit a change in the gut microbiome global community structure, with higher dosages potentially providing an earlier and/or longer maintained response.

As was observed for the gut samples, a significant difference was recorded between all treatment types (e.g. no inoculum, water inoculum and gavage inoculum) at all three sampling time points (e.g. two, eight and 15 days post inoculum) with and without antibiotics compared to the RNA inoculum sample for the skin swab dataset. This is as expected, with the RNA inoculum representing a gut microbiome assemblage and the skin swab samples representing skin microbiome assemblages. Interestingly, at TP1 (two days post inoculum), a significant difference in the global community structure was observed between the no antibiotics + no inoculum group compared to the antibiotics + water inoculum group. This highlights that both the gut and skin microbiome be manipulated, even though outcomes were different to what was observed in the RNA inoculum, and that antibiotic therapy coupled with the inoculum is required to elicit the change. Given that the outer skin surface is in constant contact with the surrounding environment, changes were expected to be observed for the water inoculum treatments, however the time frame was rapider than predicted, with this difference observed two days post inoculum. Unlike what was observed for the gut microbiome, through time a reversion back to the original state was not recorded for the skin samples, with additional significant differences between treatments documented at TP2 and TP3, including for the no antibiotics group and gavage inoculum samples. The latter supports what was indicated earlier and reported by Legrand et al. (2018), in that changes directed at the gut (e.g. gavage inoculum) also have an effect on the outer skin surface microbiome composition.

Conclusion and Recommendation

The microbiome manipulation trial showed that the gut and skin microbiome (bacterial assemblages) of YTK can be modulated by antibiotic therapy coupled with gavage inoculation, which can contribute to increased diversity and evenness of microbe communities and a decrease in potentially opportunistic pathogens. This was likely due to the enrichment of the community by the donor inoculum. While such increases in diversity likely support improved health through the displacement of pathogens and the potential occurrence of more diverse function, further work is required to elucidate this through the use of more advanced omics-based techniques (a topic being explored as part of the student activites on this project, see Section 4). A prolonged effect of the therapy was evident in the skin, while only temporary effects were observed in the gut, highlighting that studies should include repeat doses and/or higher concentrations of the inoculum to sustain beneficial outcomes. Trialing administration of inoculum on-feed is also recommended, which would facilitate repeat dosing and is applicable on-farm. Including more varied microbiomes or individual strains that have known therapeutic potential or that were observed to be depleted in diseased individuals (see Manuscript 3.3.1.3) is suggested. As an additional component of this work, we also provided a catalogue of bacterial taxa from the skin and gut that were negatively affected by the single dose of 200mg/kg oxytetracycline and 50mg/kg

erythromycin and metronidazole. While a broad variety of taxa were influenced, this treatment appeared to have limited effect on an organism with closest sequence identity to *Mycoplasma insons*, which was observed as a dominant constituent in YTK with an underlying gut enteritis (see Manuscript 3.3.1.3). Therefore if antibiotic therapy was to be investigated for farm management to treat gut enteritis in YTK, the combination and dosage of antibiotics used in this trial may exacerbate the issue by allowing the proliferation of potentially opportunistic pathogens.

Findings

This component of the work found that antibiotic therapy caused a decrease in abundance of a number of key taxa in the gut and skin, though at this dose had no effect on a single organism with closest similarity to *Mycoplasma insons*. This organism was observed in diseased YTK on-farm, and its enrichment in fish treated with antibiotics indicates its potential as an opportunistic pathogen that can evade treatment. Investigating its relevance when antibiotics are used in management of disease in YTK, would be beneficial for fish health. Although the global bacterial community structure of all experimental treatments through time was significantly different to that observed from the active (RNA) components of the 'healthy' inoculum, which was unexpectedly dominated by a single taxon, the gut and skin microbiome of poor-performing fish can be manipulated. The combination of antibiotics and gavage delivery of the inoculum resulted in increased bacterial diversity and greater species evenness, and a decrease in the abundance of potentially opportunistic pathogens in the gut samples two days post inoculum, highlighting favourable health outcomes. While at eight days post inoculum significant differences in the global bacterial community structure in the gut from fish receiving the inoculum via delivery into the seawater and by gavage were observed, no differences were apparent at day 15. Instead, there appeared to be reversion to the original community structure, indicating that subsequent inoculum dosages are likely required in order to maintain changes. Differences in the global community structure of the skin microbiome was also recorded at two days post inoculum for the water gavage treatment with antibiotics, as well as for eight and 15 days post inoculum for both the water and gavage treatments (with and without antibiotics), highlighting the sensitivity and prolonged effects of these treatments on the skin microbiome.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit program, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds. The authors would also like to acknowledge the support of the South Australian Research and Development Institute (SARDI) for provision of the pool-farm and Molecular Sciences Laboratory facilities at West Beach, South Australia. Thanks to Dr Jay Dent and Dr Erin Bubner of Clean Seas, and Clean Seas health team staff for coordinating and assisting with on-farm sample collections and delivery of fish to the SARDI pool-farm facility. Thanks to Dr Matt Bansemer, Paul Skordas, Thibault Legrand and Leigh Kuerschner for assistance with fish stocking, sampling and husbandry, and Dr Melissa Wos-Oxley of Flinders University for supporting and guiding the statistical analyses. Thanks also to Dr Stephen Pyecroft, Cheryl Day and Ken Lee (Veterinary Diagnostic Lab, University of Adelaide, Roseworthy Campus) for performing the pathological and microbiological analyses of the fish from the small-scale antibiotics trial.

References

Abbeele, P.V., Verstraete, W., Aidy, S.E., Geirnaert, A., Wiele, T.V., 2013. Prebiotics, faecal transplants and microbial network units to stimulate biodiversity of the human gut microbiome. Microbial Biotechnology 6, 335-340.

Alonso, V.R., Guarner, F., 2013. Linking the gut microbiota to human health. British Journal of Nutrition 109, S21-S26.

Anathaswamy, A., 2011. Fecal transplant eases symptoms of Parkinson's. The New Scientist 209, 8-9.

Anderson, J.L., Edney, R.J., Whelan, K., 2012. Systematic review: faecal microbiota transplantation in the management of inflammatory bowel disease. Alimentary Pharmacology and Therapeutics 36, 503-516.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology 26, 32-46.

Borody, T.J., Brandt, L.J., Paramsothy, S., 2013. Therapeutic faecal microbiota transplantation: current status and future developments. Current Opinion Gastroenterology 30, 97-105.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. Ecological monographs 27(4), 325-349.

Camarinha-Silva, A., Jáuregui, R., Chaves-Moreno, D., Oxley, A.P.A., Schaumburg, F., Becker, K., Wos-Oxley, M.L., Pieper, D.H., 2014. Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. Environmental Microbiology 16(9), 2939-2952.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7(5), 335-336.

Chaves-Moreno, D., Plumeier, I., Kahl, S., Krismer, B., Peschel, A., Oxley, A.P.A., Jauregui, R., Pieper, D.H., 2015. The microbial community structure of the cotton rat nose. Environmental Microbiology Reports 7(6), 929-935.

Chiarello, M., Villéger, S., Bouvier, C., Bettarel, Y., Bouvier, T., 2015. High diversity of skin-associated bacterial communities of marine fishes is promoted by their high variability among body parts, individuals and species. FEMS Microbiology and Ecology 91.

Clarke, K.R., Warwick, R.M.R.M., Laboratory, P.M., 2001. Change in marine communities: an approach to statistical analysis and interpretation: Plymouth, U.K.: PRIMER-E Ltd.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Research 42 (Database issue), D633-D642.

Coskunpinar, E., Islamzade, F., Yilmaz, E.P., Ors, C.H., Adak, H., Yesiltepe, A., Turksoylu, S., Dural, A.C., 2018. The importance of fecal transplantation in personalized medicine. Bezmialem Science 6, 305-311.

Dittmann, K.K., Rasmusen, B.B., Castex, M., Gram, L., Bentzon-Tilla, M., 2017. The aquaculture microbiome at the centre of business creation. Microbial Biotechnology 10, 1279-1282.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19), 2460-2461.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nature Methods 10, 996-998.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27(16), 2194-2200.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., Ross, R.P., 2018. The gut microbiota of marine fish. Frontiers in Microbiology 9, 1-17.

Francino, M., 2016. Antibiotics and the human gut microbiome: dysbioses and accumulation of resistances. Frontiers in Microbiology 6, 1543.

Ghanbari, M., Kneifel, W., Domig, K.J. 2015. A new view of the fish gut microbiome: Advances from next-generation sequencing. Aquaculture 448, 464-475.

Gough, E., Shaikh, H., Manges, A.R., 2011. Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent Clostridium difficile infection. Clinical Infection and Disease 53, 994-1002.

Lee, P., Yacyshyn, B.R., Yacyshyn, M.B., 2018. Gut microbiota and obesity: an opportunity to alter obesity through faecal microbiota transplant (FMT). Diabetes, Obesity and Metabolism, 1-12.

Legrand, T.P.R.A., Catalano, S.R., Wos-Oxley, M.L., Stephens, F., Landos, M., Bansemer, M.S., Stone, D.A.J., Qin, J.G., Oxley, A.P.A., 2018. The inner workings of the outer surface: skin and gill microbiota as indicators of changing gut health in Yellowtail Kingfish. Frontiers in Microbiology 8, 2664.

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H., Derome, N., 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Frontiers in Microbiology 5, 1-17.

Marchesi, J.R., Adams, D.H., Fava, F., Hermes, G.D.A., Hirschfield, G.M., Hold, G., Quarishi, M.N., Kinross, J., Smidt, H., Tuohy, K.M., Thomas, L.V., Zoetendal, E.G., Hart, A., 2016. The gut microbiota and host health: a new clinical frontier. Gut 65, 330-339.

Mcilroy, J., Segal, J.P., Mullish, B.H., Quraishi, M.N., Gasbarrini, A., Cammarota, G., Ianiro, G., 2018. Current and future targets for faecal microbiota transplantation. Human Microbiome Journal (In Press).

Montalban-Arques, A., De Schryver, P., Bossier, P., Gorkiewicz, G., Mulero, V., Gatlin, D.M., Galindo-Villegas, J., 2015. Selective manipulation of the gut microbiota improves immune status in vertebrates. Frontiers in Immunology 6, 1-14.

Pérez, T., Balcázar, J.L., Ruiz-Zarzueka, I., Halaihel, N., Vendrell, D., de Blas, I., Múzquiz, J.L., 2010. Host-microbiota interactions within the fish intestinal ecosystem. Mucosal Immunity 3(4), 355-60.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41, D590-D596.

Razin, S., Yogev, D., Naot, Y., 1998. Molecular biology and pathogenicity of *Mycoplasmas*. Microbiology and Molecular Biology Reviews 62, 1094-1156.

Schmidt, V.T., Smith, K.F., Melvin, D.W., Amaral-Zettler, L.A., 2015. Community assembly of a euryhaline fish microbiome during salinity acclimation. Molecular Ecology 24, 2537-2550.

Schulze, A.D., Alabi, A.O., Tattersall-Sheldrake, A.R., Miller, K.M., 2006. Bacterial diversity in a marine hatchery: balance between pathogenic and potentially probiotic bacterial strains. Aquaculture 256, 50-73.

Smith, P., Willemsen, D., Popkes, M., Metge, F., Gandiwa, E., Reichard, M., Valenzano, D.R., 2017. Regulation of life span by the gut microbiota in the short-lived African turquoise killifish. eLife 6, e27014.

Szafranska, A.K., Oxley, A.P.A., Chaves-Moreno, D., Horst, S.A., Roßlenbrolch, S., Peters, G., Goldmann, O., Rohde, M., Sinha, B., Pleper, D.H., Löffler, B., Jauregul, R., Wos-Oxley, M.L., Medina, E., 2014. High-resolution transcriptomic analysis of the adaptive responses of *Staphylococcus aureus* during acute and chronic phases of osteomyelitis. mBio 5(6), e01775-14.

Valdes, A.M., Walter, J., Segal, E., Spector, T.D., 2018. Role of the gut microbiota in nutrition and health. BMJ 361, 36-44.

Wang, E., Yuan, Z., Wang, K., Gao, D., Liu, Z., Liles, M.R., 2019. Consumption of florfenicol-medicated feed alters the composition of the channel catfish intestinal microbiota including enriching the relative abundance of opportunistic pathogens. Aquaculture 501, 111-118.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology 73(16), 5261-5267.

Wang, W., Zhou, Z., He, S., Liu, Y., Cao, Y., Shi, P., Yao, B., Ringo, E., 2010. Identification of the adherent microbiota on gills and skins of poly-cultured gibel carp (*Carassius auratus gibelio*) and bluntnose black bream (*Megalorama mablycephala* Yih). Aquaculture Research, 1-12.

Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30(5), 614-20.

Zhang, M., Sun, Y., Liu, Y., Qiao, F., Chen, L., Liu, W-T, Du, Z., Li, E., 2016. Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference. Aquaculture 454, 72.

Table 3.3.1.4.1. Sample information pertaining to the microbiome manipulation trial.

Sample type	Gut/ skin swab	Fork length (cm)	Weight (g)	Location	Site	Cage/Tank ID	Date sample collected	library size	# bacterial OTUs
water	-	-	-	Port Lincoln	Point Boston	ABK1-18	30/05/2018	46941	389
water	-	-	-	SARDI	Pool-farm facility	Tank 8	27/06/2018	78347	231
water	-	-	-	SARDI	Pool-farm facility	Tank 8	12/07/2018	48634	407
RNA inoculum	-	-	-	Port Lincoln	Bickers	ABK1B-17	27/06/2018	50644	36
RNA inoculum	-	-	-	Port Lincoln	Bickers	ABK1B-17	27/06/2018	86268	36
RNA inoculum	-	-	-	Port Lincoln	Bickers	ABK1B-17	27/06/2018	75364	51
RNA inoculum	-	-	-	Port Lincoln	Bickers	ABK1B-17	27/06/2018	97894	60
DNA inoculum	-	-	-	Port Lincoln	Bickers	ABK1B-17	27/06/2018	43163	68
DNA inoculum	-	-	-	Port Lincoln	Bickers	ABK1B-17	27/06/2018	54661	113
on-farm pre-trial	HG	48	1160	Port Lincoln	Point Boston	ABK1-18	30/05/2018	53339	24
on-farm pre-trial	HG	34.5	550	Port Lincoln	Point Boston	ABK1-18	30/05/2018	122380	101
on-farm pre-trial	HG	44.5	1340	Port Lincoln	Point Boston	ABK1-18	30/05/2018	67313	48
on-farm pre-trial	HG	45	930	Port Lincoln	Point Boston	ABK1-18	30/05/2018	69844	118
on-farm pre-trial	HG	48.5	1500	Port Lincoln	Point Boston	ABK1-18	30/05/2018	112037	109
on-farm pre-trial	HG	49	1400	Port Lincoln	Point Boston	ABK1-18	30/05/2018	77317	58
on-farm pre-trial	HG	46	1130	Port Lincoln	Point Boston	ABK1-18	30/05/2018	106953	89
on-farm pre-trial	HG	48	1340	Port Lincoln	Point Boston	ABK1-18	30/05/2018	115620	97
on-farm pre-trial	HG	48	1340	Port Lincoln	Point Boston	ABK1-18	30/05/2018	96326	66
TP0									
1 - A+WI	HG	50	1825	SARDI	Pool-farm facility	Tank 1	27/06/2018	69460	130
1 - A+WI	HG	49.5	1705	SARDI	Pool-farm facility	Tank 1	27/06/2018	74452	151
1 - A+WI	HG	47	1502	SARDI	Pool-farm facility	Tank 2	27/06/2018	79826	91
1 - A+WI	HG	46.5	1509	SARDI	Pool-farm facility	Tank 2	27/06/2018	81407	130
2 - NoA+WI	HG	48.5	1692	SARDI	Pool-farm facility	Tank 5	27/06/2018	71259	110
2 - NoA+WI	HG	50.5	1804	SARDI	Pool-farm facility	Tank 5	27/06/2018	81096	93
2 - NoA+WI	HG	49.5	1819	SARDI	Pool-farm facility	Tank 6	27/06/2018	84202	116
2 - NoA+WI	HG	48.5	1632	SARDI	Pool-farm facility	Tank 6	27/06/2018	113687	70
6 – NoA+NoI	HG	46.5	1334	SARDI	Pool-farm facility	Tank 9	27/06/2018	65932	74
6 – NoA+NoI	HG	49.5	1833	SARDI	Pool-farm facility	Tank 9	27/06/2018	95591	85
6 – NoA+NoI	HG	48.5	1866	SARDI	Pool-farm facility	Tank 10	27/06/2018	110854	51
6 – NoA+NoI	HG	50	1861	SARDI	Pool-farm facility	Tank 10	27/06/2018	77718	62
5 – A+NoI	HG	48	1410	SARDI	Pool-farm facility	Tank 11	27/06/2018	113643	40
5 – A+NoI	HG	50.5	1823	SARDI	Pool-farm facility	Tank 12	27/06/2018	50371	96
5 – A+NoI	HG	49.5	1716	SARDI	Pool-farm facility	Tank 12	27/06/2018	46725	64
3 – A+GI	HG	49	1702	SARDI	Pool-farm facility	Tank 3	27/06/2018	47533	39
3 – A+GI	HG	47.5	1615	SARDI	Pool-farm facility	Tank 3	27/06/2018	39190	77
4 – NoA+GI	HG	47	1600	SARDI	Pool-farm facility	Tank 7	27/06/2018	36612	37
4 – NoA+GI	HG	48	1710	SARDI	Pool-farm facility	Tank 7	27/06/2018	62502	74
3 – A+GI	HG	49.5	1771	SARDI	Pool-farm facility	Tank 4	27/06/2018	43808	53
3 – A+GI	HG	49.5	1571	SARDI	Pool-farm facility	Tank 4	27/06/2018	62554	151
4 – NoA+GI	HG	51	1700	SARDI	Pool-farm facility	Tank 8	27/06/2018	76779	149
4 – NoA+GI	HG	48	1709	SARDI	Pool-farm facility	Tank 8	27/06/2018	47817	63
TP1									
1 - A+WI	HG	48.5	1642	SARDI	Pool-farm facility	Tank 1	29/06/2018	54962	261
1 - A+WI	HG	50	1746	SARDI	Pool-farm facility	Tank 2	29/06/2018	67728	60
1 - A+WI	HG	49	1806	SARDI	Pool-farm facility	Tank 2	29/06/2018	50389	53
3 – A+GI	HG	46	1496	SARDI	Pool-farm facility	Tank 3	29/06/2018	62517	61
3 – A+GI	HG	48.5	1581	SARDI	Pool-farm facility	Tank 3	29/06/2018	57432	62
3 – A+GI	HG	45.5	1383	SARDI	Pool-farm facility	Tank 4	29/06/2018	25275	197
3 – A+GI	HG	49	1667	SARDI	Pool-farm facility	Tank 4	29/06/2018	34610	118
2 - NoA+WI	HG	49	1680	SARDI	Pool-farm facility	Tank 5	29/06/2018	57874	69
2 - NoA+WI	HG	48.5	1626	SARDI	Pool-farm facility	Tank 5	29/06/2018	30900	127
2 - NoA+WI	HG	47	1539	SARDI	Pool-farm facility	Tank 6	29/06/2018	56295	128

Sample type	Gut/ skin swab	Fork length (cm)	Weight (g)	Location	Site	Cage/Tank ID	Date sample collected	library size	# bacterial OTUs
2 - NoA+WI	HG	48	1502	SARDI	Pool-farm facility	Tank 6	29/06/2018	65656	52
4 – NoA+GI	HG	48.5	1719	SARDI	Pool-farm facility	Tank 7	29/06/2018	120725	59
4 – NoA+GI	HG	50.5	1668	SARDI	Pool-farm facility	Tank 7	29/06/2018	63769	21
4 – NoA+GI	HG	48.5	1570	SARDI	Pool-farm facility	Tank 8	29/06/2018	69442	67
4 – NoA+GI	HG	49.5	1626	SARDI	Pool-farm facility	Tank 8	29/06/2018	90239	73
6 – NoA+NoI	HG	49	1618	SARDI	Pool-farm facility	Tank 9	29/06/2018	105348	36
6 – NoA+NoI	HG	48	1679	SARDI	Pool-farm facility	Tank 9	29/06/2018	72700	92
6 – NoA+NoI	HG	49.5	1826	SARDI	Pool-farm facility	Tank 10	29/06/2018	92349	82
6 – NoA+NoI	HG	51	1864	SARDI	Pool-farm facility	Tank 10	29/06/2018	68602	136
5 – A+NoI	HG	47.5	1487	SARDI	Pool-farm facility	Tank 11	29/06/2018	114212	55
5 – A+NoI	HG	48	1609	SARDI	Pool-farm facility	Tank 11	29/06/2018	68437	113
5 – A+NoI	HG	46.5	1483	SARDI	Pool-farm facility	Tank 12	29/06/2018	74076	50
5 – A+NoI	HG	49.5	1809	SARDI	Pool-farm facility	Tank 12	29/06/2018	109898	128
TP1a									
3 – A+GI	HG	51	1927	SARDI	Pool-farm facility	Tank 14	02/07/2018	46551	82
3 – A+GI	HG	49	1833	SARDI	Pool-farm facility	Tank 14	02/07/2018	70135	61
3 – A+GI	HG	50	1862	SARDI	Pool-farm facility	Tank 14	02/07/2018	67997	72
3 – A+GI	HG	50	1853	SARDI	Pool-farm facility	Tank 14	02/07/2018	57347	41
TP2									
1 - A+WI	HG	48.5	1681	SARDI	Pool-farm facility	Tank 1	05/07/2018	81049	148
1 - A+WI	HG	50	1834	SARDI	Pool-farm facility	Tank 1	05/07/2018	64438	68
1 - A+WI	HG	47	1576	SARDI	Pool-farm facility	Tank 2	05/07/2018	100378	94
1 - A+WI	HG	45	1267	SARDI	Pool-farm facility	Tank 2	05/07/2018	102944	74
3 – A+GI	HG	47.5	1661	SARDI	Pool-farm facility	Tank 3	05/07/2018	66015	134
3 – A+GI	HG	50.5	1807	SARDI	Pool-farm facility	Tank 3	05/07/2018	73466	138
3 – A+GI	HG	47	1561	SARDI	Pool-farm facility	Tank 4	05/07/2018	69030	94
3 – A+GI	HG	48.5	1711	SARDI	Pool-farm facility	Tank 4	05/07/2018	72785	130
2 - NoA+WI	HG	49.5	1797	SARDI	Pool-farm facility	Tank 5	05/07/2018	75958	68
2 - NoA+WI	HG	46	1606	SARDI	Pool-farm facility	Tank 5	05/07/2018	60902	117
2 - NoA+WI	HG	47.5	1543	SARDI	Pool-farm facility	Tank 6	05/07/2018	50418	76
2 - NoA+WI	HG	44.5	1347	SARDI	Pool-farm facility	Tank 6	05/07/2018	128537	44
4 – NoA+GI	HG	49.5	1691	SARDI	Pool-farm facility	Tank 7	05/07/2018	80328	55
4 – NoA+GI	HG	50	1729	SARDI	Pool-farm facility	Tank 7	05/07/2018	56406	90
4 – NoA+GI	HG	52	1821	SARDI	Pool-farm facility	Tank 8	05/07/2018	36248	58
4 – NoA+GI	HG	49.5	1726	SARDI	Pool-farm facility	Tank 8	05/07/2018	94602	54
6 – NoA+NoI	HG	48.5	1682	SARDI	Pool-farm facility	Tank 9	05/07/2018	57484	81
6 – NoA+NoI	HG	49	1625	SARDI	Pool-farm facility	Tank 9	05/07/2018	73420	83
6 – NoA+NoI	HG	51	1991	SARDI	Pool-farm facility	Tank 10	05/07/2018	58291	141
6 – NoA+NoI	HG	52.5	1964	SARDI	Pool-farm facility	Tank 10	05/07/2018	73961	86
5 – A+NoI	HG	48.5	1746	SARDI	Pool-farm facility	Tank 11	05/07/2018	58026	144
5 – A+NoI	HG	45.5	1477	SARDI	Pool-farm facility	Tank 11	05/07/2018	56455	65
5 – A+NoI	HG	49	1680	SARDI	Pool-farm facility	Tank 12	05/07/2018	61716	132
TP3									
1 - A+WI	HG	48.5	1799	SARDI	Pool-farm facility	Tank 1	12/07/2018	85740	106
1 - A+WI	HG	49.5	1913	SARDI	Pool-farm facility	Tank 1	12/07/2018	111447	86
1 - A+WI	HG	49.5	1712	SARDI	Pool-farm facility	Tank 2	12/07/2018	102081	117
3 – A+GI	HG	49.5	1712	SARDI	Pool-farm facility	Tank 3	12/07/2018	130385	57
3 – A+GI	HG	48	1574	SARDI	Pool-farm facility	Tank 3	12/07/2018	98639	95
3 – A+GI	HG	49.5	1845	SARDI	Pool-farm facility	Tank 4	12/07/2018	95489	81
3 – A+GI	HG	49	1674	SARDI	Pool-farm facility	Tank 4	12/07/2018	62347	116
2 - NoA+WI	HG	45	1250	SARDI	Pool-farm facility	Tank 5	12/07/2018	44846	115
2 - NoA+WI	HG	50	1787	SARDI	Pool-farm facility	Tank 5	12/07/2018	87215	171
2 - NoA+WI	HG	49	1565	SARDI	Pool-farm facility	Tank 6	12/07/2018	81803	117
2 - NoA+WI	HG	48.5	1731	SARDI	Pool-farm facility	Tank 6	12/07/2018	91611	69

Sample type	Gut/ skin swab	Fork length (cm)	Weight (g)	Location	Site	Cage/Tank ID	Date sample collected	library size	# bacterial OTUs
4 – NoA+GI	HG	50	1858	SARDI	Pool-farm facility	Tank 7	12/07/2018	72240	77
4 – NoA+GI	HG	49.5	1670	SARDI	Pool-farm facility	Tank 7	12/07/2018	73062	58
4 – NoA+GI	HG	51.5	2067	SARDI	Pool-farm facility	Tank 8	12/07/2018	58427	69
4 – NoA+GI	HG	48	1682	SARDI	Pool-farm facility	Tank 8	12/07/2018	85571	101
6 – NoA+NoI	HG	50	1903	SARDI	Pool-farm facility	Tank 9	12/07/2018	106112	133
6 – NoA+NoI	HG	50	1717	SARDI	Pool-farm facility	Tank 9	12/07/2018	120377	151
6 – NoA+NoI	HG	52	1876	SARDI	Pool-farm facility	Tank 10	12/07/2018	67417	142
6 – NoA+NoI	HG	51.5	2042	SARDI	Pool-farm facility	Tank 10	12/07/2018	46113	30
5 – A+NoI	HG	47.5	1602	SARDI	Pool-farm facility	Tank 11	12/07/2018	71103	162
5 – A+NoI	HG	49	1788	SARDI	Pool-farm facility	Tank 12	12/07/2018	106085	102
5 – A+NoI	HG	47	1522	SARDI	Pool-farm facility	Tank 12	12/07/2018	60537	148
on-farm pre-trial	Sk	48	1160	Port Lincoln	Point Boston	ABK1-18	30/05/2018	91676	408
on-farm pre-trial	Sk	34.5	550	Port Lincoln	Point Boston	ABK1-18	30/05/2018	91680	338
on-farm pre-trial	Sk	44.5	1340	Port Lincoln	Point Boston	ABK1-18	30/05/2018	48635	363
on-farm pre-trial	Sk	48.5	1500	Port Lincoln	Point Boston	ABK1-18	30/05/2018	84816	451
on-farm pre-trial	Sk	49	1400	Port Lincoln	Point Boston	ABK1-18	30/05/2018	56807	353
on-farm pre-trial	Sk	46	1130	Port Lincoln	Point Boston	ABK1-18	30/05/2018	23579	223
on-farm pre-trial	Sk	48	1340	Port Lincoln	Point Boston	ABK1-18	30/05/2018	48661	433
on-farm pre-trial	Sk	51	1430	Port Lincoln	Point Boston	ABK1-18	30/05/2018	49067	343
on-farm pre-trial	Sk	48	1340	Port Lincoln	Point Boston	ABK1-18	30/05/2018	63760	462
TP0									
1 - A+WI	Sk	50	1825	SARDI	Pool-farm facility	Tank 1	27/06/2018	39396	554
1 - A+WI	Sk	49.5	1705	SARDI	Pool-farm facility	Tank 1	27/06/2018	40030	562
1 - A+WI	Sk	47	1502	SARDI	Pool-farm facility	Tank 2	27/06/2018	59120	586
1 - A+WI	Sk	46.5	1509	SARDI	Pool-farm facility	Tank 2	27/06/2018	64149	560
2 - NoA+WI	Sk	48.5	1692	SARDI	Pool-farm facility	Tank 5	27/06/2018	37518	498
2 - NoA+WI	Sk	50.5	1804	SARDI	Pool-farm facility	Tank 5	27/06/2018	51975	479
2 - NoA+WI	Sk	49.5	1819	SARDI	Pool-farm facility	Tank 6	27/06/2018	71591	574
2 - NoA+WI	Sk	48.5	1632	SARDI	Pool-farm facility	Tank 6	27/06/2018	32537	548
6 – NoA+NoI	Sk	46.5	1334	SARDI	Pool-farm facility	Tank 9	27/06/2018	32307	555
6 – NoA+NoI	Sk	49.5	1833	SARDI	Pool-farm facility	Tank 9	27/06/2018	20910	489
6 – NoA+NoI	Sk	48.5	1866	SARDI	Pool-farm facility	Tank 10	27/06/2018	33279	506
6 – NoA+NoI	Sk	50	1861	SARDI	Pool-farm facility	Tank 10	27/06/2018	48609	495
5 – A+NoI	Sk	44.5	1301	SARDI	Pool-farm facility	Tank 11	27/06/2018	37254	531
5 – A+NoI	Sk	48	1410	SARDI	Pool-farm facility	Tank 11	27/06/2018	22962	495
5 – A+NoI	Sk	50.5	1823	SARDI	Pool-farm facility	Tank 12	27/06/2018	26029	530
5 – A+NoI	Sk	49.5	1716	SARDI	Pool-farm facility	Tank 12	27/06/2018	23545	483
3 – A+GI	Sk	49	1702	SARDI	Pool-farm facility	Tank 3	27/06/2018	24477	517
3 – A+GI	Sk	47.5	1615	SARDI	Pool-farm facility	Tank 3	27/06/2018	35775	535
4 – NoA+GI	Sk	47	1600	SARDI	Pool-farm facility	Tank 7	27/06/2018	27440	560
4 – NoA+GI	Sk	48	1710	SARDI	Pool-farm facility	Tank 7	27/06/2018	23538	554
3 – A+GI	Sk	49.5	1771	SARDI	Pool-farm facility	Tank 4	27/06/2018	15293	420
3 – A+GI	Sk	49.5	1571	SARDI	Pool-farm facility	Tank 4	27/06/2018	24848	449
4 – NoA+GI	Sk	51	1700	SARDI	Pool-farm facility	Tank 8	27/06/2018	25562	486
4 – NoA+GI	Sk	48	1709	SARDI	Pool-farm facility	Tank 8	27/06/2018	36218	505
TP1									
1 - A+WI	Sk	48.5	1642	SARDI	Pool-farm facility	Tank 1	29/06/2018	23010	472
1 - A+WI	Sk	48.5	1639	SARDI	Pool-farm facility	Tank 1	29/06/2018	28443	341
1 - A+WI	Sk	50	1746	SARDI	Pool-farm facility	Tank 2	29/06/2018	26214	522
1 - A+WI	Sk	49	1806	SARDI	Pool-farm facility	Tank 2	29/06/2018	24573	490
3 – A+GI	Sk	46	1496	SARDI	Pool-farm facility	Tank 3	29/06/2018	20382	530
3 – A+GI	Sk	48.5	1581	SARDI	Pool-farm facility	Tank 3	29/06/2018	26711	532
3 – A+GI	Sk	45.5	1383	SARDI	Pool-farm facility	Tank 4	29/06/2018	24723	503
3 – A+GI	Sk	49	1667	SARDI	Pool-farm facility	Tank 4	29/06/2018	59747	585

Sample type	Gut/ skin swab	Fork length (cm)	Weight (g)	Location	Site	Cage/Tank ID	Date sample collected	library size	# bacterial OTUs
2 - NoA+WI	Sk	49	1680	SARDI	Pool-farm facility	Tank 5	29/06/2018	76445	608
2 - NoA+WI	Sk	48.5	1626	SARDI	Pool-farm facility	Tank 5	29/06/2018	38966	569
2 - NoA+WI	Sk	47	1539	SARDI	Pool-farm facility	Tank 6	29/06/2018	64166	623
2 - NoA+WI	Sk	48	1502	SARDI	Pool-farm facility	Tank 6	29/06/2018	49486	556
4 – NoA+GI	Sk	48.5	1719	SARDI	Pool-farm facility	Tank 7	29/06/2018	19762	442
4 – NoA+GI	Sk	50.5	1668	SARDI	Pool-farm facility	Tank 7	29/06/2018	25930	582
4 – NoA+GI	Sk	48.5	1570	SARDI	Pool-farm facility	Tank 8	29/06/2018	37821	498
4 – NoA+GI	Sk	49.5	1626	SARDI	Pool-farm facility	Tank 8	29/06/2018	60333	567
6 – NoA+NoI	Sk	49	1618	SARDI	Pool-farm facility	Tank 9	29/06/2018	55458	608
6 – NoA+NoI	Sk	48	1679	SARDI	Pool-farm facility	Tank 9	29/06/2018	62196	578
6 – NoA+NoI	Sk	49.5	1826	SARDI	Pool-farm facility	Tank 10	29/06/2018	54947	613
6 – NoA+NoI	Sk	51	1864	SARDI	Pool-farm facility	Tank 10	29/06/2018	117147	591
5 – A+NoI	Sk	47.5	1487	SARDI	Pool-farm facility	Tank 11	29/06/2018	32943	375
5 – A+NoI	Sk	48	1609	SARDI	Pool-farm facility	Tank 11	29/06/2018	62425	549
5 – A+NoI	Sk	46.5	1483	SARDI	Pool-farm facility	Tank 12	29/06/2018	68188	558
5 – A+NoI	Sk	49.5	1809	SARDI	Pool-farm facility	Tank 12	29/06/2018	19289	506
TP1a									
3 – A+GI	Sk	51	1927	SARDI	Pool-farm facility	Tank 14	02/07/2018	21366	387
3 – A+GI	Sk	49	1833	SARDI	Pool-farm facility	Tank 14	02/07/2018	32849	401
3 – A+GI	Sk	50	1862	SARDI	Pool-farm facility	Tank 14	02/07/2018	45529	462
3 – A+GI	Sk	50	1853	SARDI	Pool-farm facility	Tank 14	02/07/2018	49827	542
TP2									
1 - A+WI	Sk	48.5	1681	SARDI	Pool-farm facility	Tank 1	05/07/2018	30193	361
1 - A+WI	Sk	50	1834	SARDI	Pool-farm facility	Tank 1	05/07/2018	30761	408
1 - A+WI	Sk	47	1576	SARDI	Pool-farm facility	Tank 2	05/07/2018	32629	466
1 - A+WI	Sk	45	1267	SARDI	Pool-farm facility	Tank 2	05/07/2018	36992	511
3 – A+GI	Sk	47.5	1661	SARDI	Pool-farm facility	Tank 3	05/07/2018	36523	467
3 – A+GI	Sk	50.5	1807	SARDI	Pool-farm facility	Tank 3	05/07/2018	40754	479
3 – A+GI	Sk	47	1561	SARDI	Pool-farm facility	Tank 4	05/07/2018	26268	502
3 – A+GI	Sk	48.5	1711	SARDI	Pool-farm facility	Tank 4	05/07/2018	39000	460
2 - NoA+WI	Sk	49.5	1797	SARDI	Pool-farm facility	Tank 5	05/07/2018	36795	473
2 - NoA+WI	Sk	46	1606	SARDI	Pool-farm facility	Tank 5	05/07/2018	77037	543
2 - NoA+WI	Sk	47.5	1543	SARDI	Pool-farm facility	Tank 6	05/07/2018	54193	472
2 - NoA+WI	Sk	44.5	1347	SARDI	Pool-farm facility	Tank 6	05/07/2018	41792	518
4 – NoA+GI	Sk	49.5	1691	SARDI	Pool-farm facility	Tank 7	05/07/2018	35586	504
4 – NoA+GI	Sk	50	1729	SARDI	Pool-farm facility	Tank 7	05/07/2018	56263	528
4 – NoA+GI	Sk	52	1821	SARDI	Pool-farm facility	Tank 8	05/07/2018	63761	531
4 – NoA+GI	Sk	49.5	1726	SARDI	Pool-farm facility	Tank 8	05/07/2018	27472	443
6 – NoA+NoI	Sk	48.5	1682	SARDI	Pool-farm facility	Tank 9	05/07/2018	36114	449
6 – NoA+NoI	Sk	49	1625	SARDI	Pool-farm facility	Tank 9	05/07/2018	27875	500
6 – NoA+NoI	Sk	51	1991	SARDI	Pool-farm facility	Tank 10	05/07/2018	38541	470
6 – NoA+NoI	Sk	52.5	1964	SARDI	Pool-farm facility	Tank 10	05/07/2018	29674	476
5 – A+NoI	Sk	48.5	1746	SARDI	Pool-farm facility	Tank 11	05/07/2018	40532	491
5 – A+NoI	Sk	45.5	1477	SARDI	Pool-farm facility	Tank 11	05/07/2018	50140	521
5 – A+NoI	Sk	49.5	1824	SARDI	Pool-farm facility	Tank 12	05/07/2018	47713	514
5 – A+NoI	Sk	49	1680	SARDI	Pool-farm facility	Tank 12	05/07/2018	60964	540
TP3									
1 - A+WI	Sk	48.5	1799	SARDI	Pool-farm facility	Tank 1	12/07/2018	64110	464
1 - A+WI	Sk	49.5	1913	SARDI	Pool-farm facility	Tank 1	12/07/2018	75074	473
1 - A+WI	Sk	48.5	1610	SARDI	Pool-farm facility	Tank 2	12/07/2018	64142	509
1 - A+WI	Sk	49.5	1712	SARDI	Pool-farm facility	Tank 2	12/07/2018	33787	464
3 – A+GI	Sk	49.5	1712	SARDI	Pool-farm facility	Tank 3	12/07/2018	19683	390
3 – A+GI	Sk	48	1574	SARDI	Pool-farm facility	Tank 3	12/07/2018	40387	448
3 – A+GI	Sk	49.5	1845	SARDI	Pool-farm facility	Tank 4	12/07/2018	25462	448

Sample type	Gut/ skin swab	Fork length (cm)	Weight (g)	Location	Site	Cage/Tank ID	Date sample collected	library size	# bacterial OTUs
3 – A+GI	Sk	49	1674	SARDI	Pool-farm facility	Tank 4	12/07/2018	36855	477
2 - NoA+WI	Sk	45	1250	SARDI	Pool-farm facility	Tank 5	12/07/2018	31408	425
2 - NoA+WI	Sk	50	1787	SARDI	Pool-farm facility	Tank 5	12/07/2018	21927	442
2 - NoA+WI	Sk	49	1565	SARDI	Pool-farm facility	Tank 6	12/07/2018	28056	460
2 - NoA+WI	Sk	48.5	1731	SARDI	Pool-farm facility	Tank 6	12/07/2018	41935	493
4 – NoA+GI	Sk	50	1858	SARDI	Pool-farm facility	Tank 7	12/07/2018	18516	400
4 – NoA+GI	Sk	49.5	1670	SARDI	Pool-farm facility	Tank 7	12/07/2018	22290	473
4 – NoA+GI	Sk	51.5	2067	SARDI	Pool-farm facility	Tank 8	12/07/2018	42284	454
4 – NoA+GI	Sk	48	1682	SARDI	Pool-farm facility	Tank 8	12/07/2018	23796	445
6 – NoA+NoI	Sk	50	1903	SARDI	Pool-farm facility	Tank 9	12/07/2018	30863	455
6 – NoA+NoI	Sk	50	1717	SARDI	Pool-farm facility	Tank 9	12/07/2018	18896	383
6 – NoA+NoI	Sk	52	1876	SARDI	Pool-farm facility	Tank 10	12/07/2018	49848	489
6 – NoA+NoI	Sk	51.5	2042	SARDI	Pool-farm facility	Tank 10	12/07/2018	44657	457
5 – A+NoI	Sk	47.5	1602	SARDI	Pool-farm facility	Tank 11	12/07/2018	60313	493
5 – A+NoI	Sk	47.5	1587	SARDI	Pool-farm facility	Tank 11	12/07/2018	62714	510
5 – A+NoI	Sk	49	1788	SARDI	Pool-farm facility	Tank 12	12/07/2018	47390	484
5 – A+NoI	Sk	47	1522	SARDI	Pool-farm facility	Tank 12	12/07/2018	39428	447

Abbreviations: A, antibiotics; GI, gavage inoculum; NoA, no antibiotics; NoI, no inoculum; Sk, skin swab; TP, time point; WI, water inoculum.

Table 3.3.1.4.2. Summary of sequenced sample parameters for the microbiome manipulation trial.

Data-set	**No. of samples**	**Total reads**	**Median library size**	**Range**	**Bacterial OTUs post- filtering**
Microbiome manipulation	222	12,986,344	56,631	15,293-130,385	993

Table 3.3.1.4.3. One-way PERMANOVA: Pairwise test between the gut microbiome manipulation trial samples through time, gut on-farm pre-trial samples and inoculum.[1,2]

Sample group	P	Significant?
TP0_HG, TP1_HG	0.5685	No
TP0_HG, TP1a_HG	0.2515	No
TP0_HG, TP2_HG	0.2371	No
TP0_HG, TP3_HG	0.3879	No
TP1_HG, TP1a_HG	0.1101	No
TP1_HG, TP2_HG	0.0528	No
TP1_HG, TP3_HG	0.1892	No
TP1a_HG, TP2_HG	0.2105	No
TP1a_HG, TP3_HG	0.2044	No
TP2_HG, TP3_HG	0.5757	No
On-farm pre-trial_HG, TP0_HG	0.0828	No
On-farm pre-trial_HG, TP1_HG	0.0923	No
On-farm pre-trial_HG, TP1a_HG	0.0642	No
On-farm pre-trial_HG, TP2_HG	**0.0063**	Yes
On-farm pre-trial_HG, TP3_HG	**0.0111**	Yes
On-farm pre-trial_HG, inoculum	**0.0009**	Yes
TP0_HG, inoculum	**0.0001**	Yes
TP1_HG, inoculum	**0.0001**	Yes
TP1a_HG, inoculum	**0.0043**	Yes
TP2_HG, inoculum	**0.0001**	Yes
TP3_HG, inoculum	**0.0001**	Yes

[1] Significant difference denoted by $P < 0.05$, bolded if significant.
[2] TP1 - 2 days post inoculum administration and 5 days after antibiotics administration; TP1a – 5 days post inoculum and 8 days after antibiotics administration; TP2 - 8 days post inoculum, 11 days after antibiotics administration; TP3 - 15 days post inoculum, 18 days after antibiotics administration.
Abbreviations: HG, hindgut; TP, time point.

Table 3.3.1.4.4. One-way PERMANOVA: Pairwise test between the skin microbiome manipulation trial samples through time, skin on-farm pre-trial samples and inoculum.[1,2]

Sample group	P	Significant?
TP0_SK, TP1_SK	**0.0001**	Yes
TP0_SK, TP1a_SK	**0.0001**	Yes
TP0_SK, TP2_SK	**0.0001**	Yes
TP0_SK, TP3_SK	**0.0001**	Yes
TP1_SK, TP1a_SK	**0.0038**	Yes
TP1_SK, TP2_SK	**0.0001**	Yes
TP1_SK, TP3_SK	**0.0001**	Yes
TP1a_SK, TP2_SK	**0.0004**	Yes
TP1a_SK, TP3_SK	**0.0002**	Yes
TP2_SK, TP3_SK	**0.0001**	Yes
On-farm pre-trial_SK, TP0_SK	**0.0001**	Yes
On-farm pre-trial_SK, TP1_SK	**0.0001**	Yes
On-farm pre-trial_SK, TP1a_SK	**0.0014**	Yes
On-farm pre-trial_SK, TP2_SK	**0.0001**	Yes
On-farm pre-trial_SK, TP3_SK	**0.0001**	Yes
On-farm pre-trial_SK, inoculum	**0.0003**	Yes
TP0_SK, inoculum	**0.0001**	Yes
TP1_SK, inoculum	**0.0001**	Yes
TP1a_SK, inoculum	**0.0050**	Yes
TP2_SK, inoculum	**0.0001**	Yes
TP3_SK, inoculum	**0.0001**	Yes

[1] Significant difference denoted by $P < 0.05$, bolded if significant.
[2] TP1 - 2 days post inoculum administration and 5 days after antibiotics administration; TP1a – 5 days post inoculum and 8 days after antibiotics administration; TP2 - 8 days post inoculum, 11 days after antibiotics administration; TP3 - 15 days post inoculum, 18 days after antibiotics administration.
Abbreviations: Sk, skin; TP, time point.

Table 3.3.1.4.5. One-way PERMANOVA: Pairwise test between the A) gut on-farm pre-trial samples, time point 0 with and without antibiotics and inoculum, and B) skin on-farm pre-trial samples and time point 0 with and without antibiotics.[1,2]

Sample group	P	Significant?
A) Gut dataset		
On-farm pre-trial_HG, TP0_HG_A	**0.0183**	Yes
On-farm pre-trial_HG, TP0_HG_NoA	0.0859	No
TP0_HG_A, TP0_HG_NoA	**0.0071**	Yes
On-farm pre-trial_HG, RNA inoculum	**0.0017**	Yes
On-farm pre-trial_HG, DNA inoculum	**0.0182**	Yes
TP0_HG_A, RNA inoculum	**0.0004**	Yes
TP0_HG_A, DNA inoculum	**0.0126**	Yes
TP0_HG_NoA, RNA inoculum	**0.0005**	Yes
TP0_HG_NoA, DNA inoculum	**0.0139**	Yes
RNA inoculum, DNA inoculum	0.0629	No
B) Skin dataset		
On-farm pre-trial_Sk, TP0_Sk_A	**0.0001**	Yes
On-farm pre-trial_Sk, TP0_Sk_NoA	**0.0001**	Yes
TP0_Sk_A, TP0_Sk_NoA	**0.0182**	Yes

[1] Significant difference denoted by $P < 0.05$, bolded if significant.
[2] No comparisons with the inoculum for skin dataset as already shown in Figure 3.3.1.4.2 C that the gut inoculum samples are distinct from the skin swab samples.
Abbreviations: A, antibiotics; HG, hindgut; NoA, no antibiotics; Sk, skin; TP, time point.

Table 3.3.1.4.6. One-way PERMANOVA: Pairwise comparisons comparing the route of inoculum administration (no inoculum vs water vs gavage) with and without antibiotics for YTK gut scraping samples at A) time point 1, B) time point 2 and C) time point 3.[1,2]

Sample group	P	Significant?
A) TP1		
A+NoI, NoA+NoI	**0.0271**	Yes
A+WI, NoA+WI	0.0872	No
A+GI, NoA+GI	0.0555	No
A+NoI, A+WI	0.3090	No
A+NoI, A+GI	0.1738	No
A+NoI, TP1a_A+GI	0.3919	No
NoA+NoI, A+WI	0.0558	No
NoA+NoI, A+GI	0.0558	No
NoA+NoI, TP1a_A+GI	**0.0308**	Yes
A+WI, A+GI	0.2758	No
NoA+WI, NoA+GI	0.5108	No
RNA inoculum, A+WI	**0.0312**	Yes
RNA inoculum, A+GI	**0.0301**	Yes
RNA inoculum, TP1a_A+GI	**0.0282**	Yes
RNA inoculum, NoA+WI	**0.0279**	Yes
RNA inoculum, NoA+GI	**0.0276**	Yes
B) TP2		
A+NoI, NoA+NoI	0.1693	No
A+WI, NoA+WI	0.0591	No
A+GI, NoA+GI	**0.0320**	Yes
A+NoI, A+WI	0.5197	No
A+NoI, A+GI	0.1473	No
NoA+NoI, A+WI	**0.0259**	Yes
NoA+NoI, A+GI	**0.0265**	Yes
A+WI, A+GI	0.2302	No
NoA+WI, NoA+GI	0.9406	No
RNA inoculum, A+WI	**0.0251**	Yes
RNA inoculum, A+GI	**0.0309**	Yes
RNA inoculum, NoA+WI	**0.0306**	Yes
RNA inoculum, NoA+GI	**0.0311**	Yes
C) TP3		
A+NoI, NoA+NoI	0.1668	No
A+WI, NoA+WI	0.1153	No
A+GI, NoA+GI	0.1465	No
A+NoI, A+WI	0.0983	No
A+NoI, A+GI	0.3149	No
NoA+NoI, A+WI	0.4073	No
NoA+NoI, A+GI	0.6295	No
A+WI, A+GI	0.9213	No
NoA+WI, NoA+GI	0.5993	No
RNA inoculum, A+WI	**0.0280**	Yes
RNA inoculum, A+GI	**0.0292**	Yes
RNA inoculum, NoA+WI	**0.0289**	Yes
RNA inoculum, NoA+GI	**0.0320**	Yes

[1] Significant difference denoted by $P < 0.05$, bolded if significant.

[2] TP1 - 2 days post inoculum administration and 5 days after antibiotics administration; TP1a – 5 days post inoculum and 8 days after antibiotics administration; TP2 - 8 days post inoculum, 11 days after antibiotics administration; TP3 - 15 days post inoculum, 18 days after antibiotics administration.

Abbreviations: A, antibiotics; GI, gavage inoculum; NoA, no antibiotics; NoI, no inoculum; TP, time point; WI, water inoculum.

Table 3.3.1.4.7. One-way PERMANOVA: Pairwise comparisons comparing the route of inoculum administration (no inoculum vs water vs gavage) with and without antibiotics for YTK skin swab samples at A) time point 1, B) time point 2 and C) time point 3.[1,2]

Sample group	P	Significant?
A) TP1		
A+NoI, NoA+NoI	0.3674	No
A+WI, NoA+WI	0.1994	No
A+GI, NoA+GI	0.2528	No
A+NoI, A+WI	**0.0281**	Yes
A+NoI, A+GI	0.0882	No
A+NoI, TP1a_A+GI	0.0841	No
NoA+NoI, A+WI	0.0582	No
NoA+NoI, A+GI	0.1422	No
NoA+NoI, TP1a_A+GI	0.0597	No
A+WI, A+GI	0.2811	No
NoA+WI, NoA+GI	0.3131	No
RNA inoculum, A+WI	**0.0306**	Yes
RNA inoculum, A+GI	**0.0285**	Yes
RNA inoculum, TP1a_A+GI	**0.0306**	Yes
RNA inoculum, NoA+WI	**0.0282**	Yes
RNA inoculum, NoA+GI	**0.0294**	Yes
B) TP2		
A+NoI, NoA+NoI	0.0819	No
A+WI, NoA+WI	**0.0273**	Yes
A+GI, NoA+GI	**0.0269**	Yes
A+NoI, A+WI	**0.0284**	Yes
A+NoI, A+GI	**0.0247**	Yes
NoA+NoI, A+WI	**0.0281**	Yes
NoA+NoI, A+GI	**0.0277**	Yes
A+WI, A+GI	**0.0309**	Yes
NoA+WI, NoA+GI	**0.0270**	Yes
RNA inoculum, A+WI	**0.0267**	Yes
RNA inoculum, A+GI	**0.0277**	Yes
RNA inoculum, NoA+WI	**0.0312**	Yes
RNA inoculum, NoA+GI	**0.0272**	Yes
C) TP3		
A+NoI, NoA+NoI	**0.0282**	Yes
A+WI, NoA+WI	**0.0283**	Yes
A+GI, NoA+GI	**0.0351**	Yes
A+NoI, A+WI	**0.0287**	Yes
A+NoI, A+GI	**0.0265**	Yes
NoA+NoI, A+WI	**0.0297**	Yes
NoA+NoI, A+GI	**0.0283**	Yes
A+WI, A+GI	0.1418	No
NoA+WI, NoA+GI	0.3948	No
RNA inoculum, A+WI	**0.0273**	Yes
RNA inoculum, A+GI	**0.0306**	Yes
RNA inoculum, NoA+WI	**0.0278**	Yes
RNA inoculum, NoA+GI	**0.0291**	Yes

[1] Significant difference denoted by $P < 0.05$, bolded if significant.

[2] TP1 - 2 days post inoculum administration and 5 days after antibiotics administration; TP1a – 5 days post inoculum and 8 days after antibiotics administration; TP2 - 8 days post inoculum, 11 days after antibiotics administration; TP3 - 15 days post inoculum, 18 days after antibiotics administration.

Abbreviations: A, antibiotics; GI, gavage inoculum; NoA, no antibiotics; NoI, no inoculum; TP, time point; WI, water inoculum.

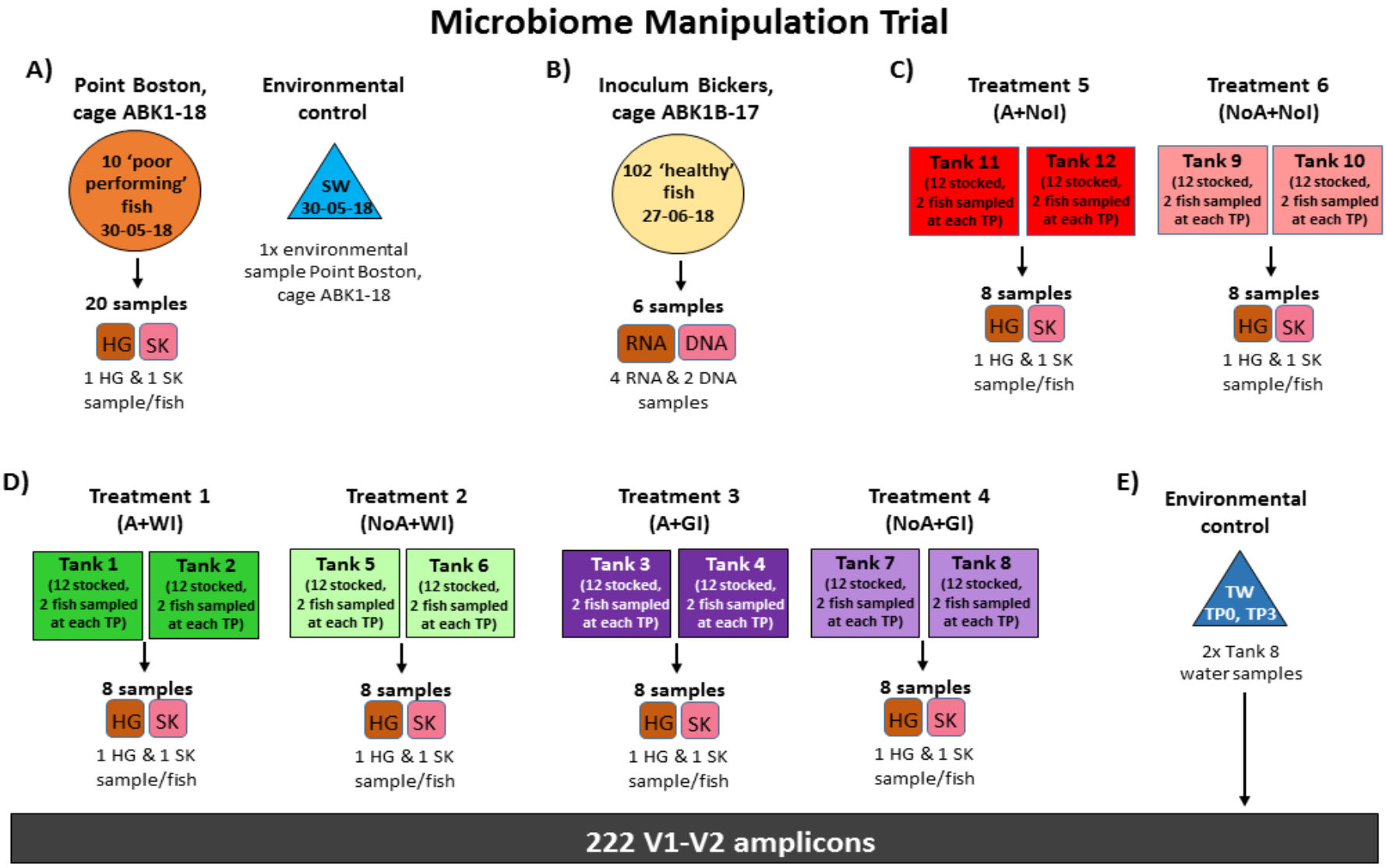


Figure 3.3.1.4.1. Experimental design for the microbiome manipulation trial including A) on-farm pre-trial sampling, B) inoculum collection and sampling, C) control trial treatments, D) experimental trial treatments and E) tank trial environmental samples.[1]

Abbreviations: A, antibiotics; GI, gavage inoculum; HG, hindgut; NoA, no antibiotics; NoI, no inoculum; SK, skin; SW, seawater; TP, time point; TW, tank water; WI, water inoculum.

[1] Twelve fish were stocked in each tank, with two fish sampled per tank at each sampling time point (TP0, TP1, TP2, TP3). Tank water samples were collected from Tank 8 at TP0 and TP3.

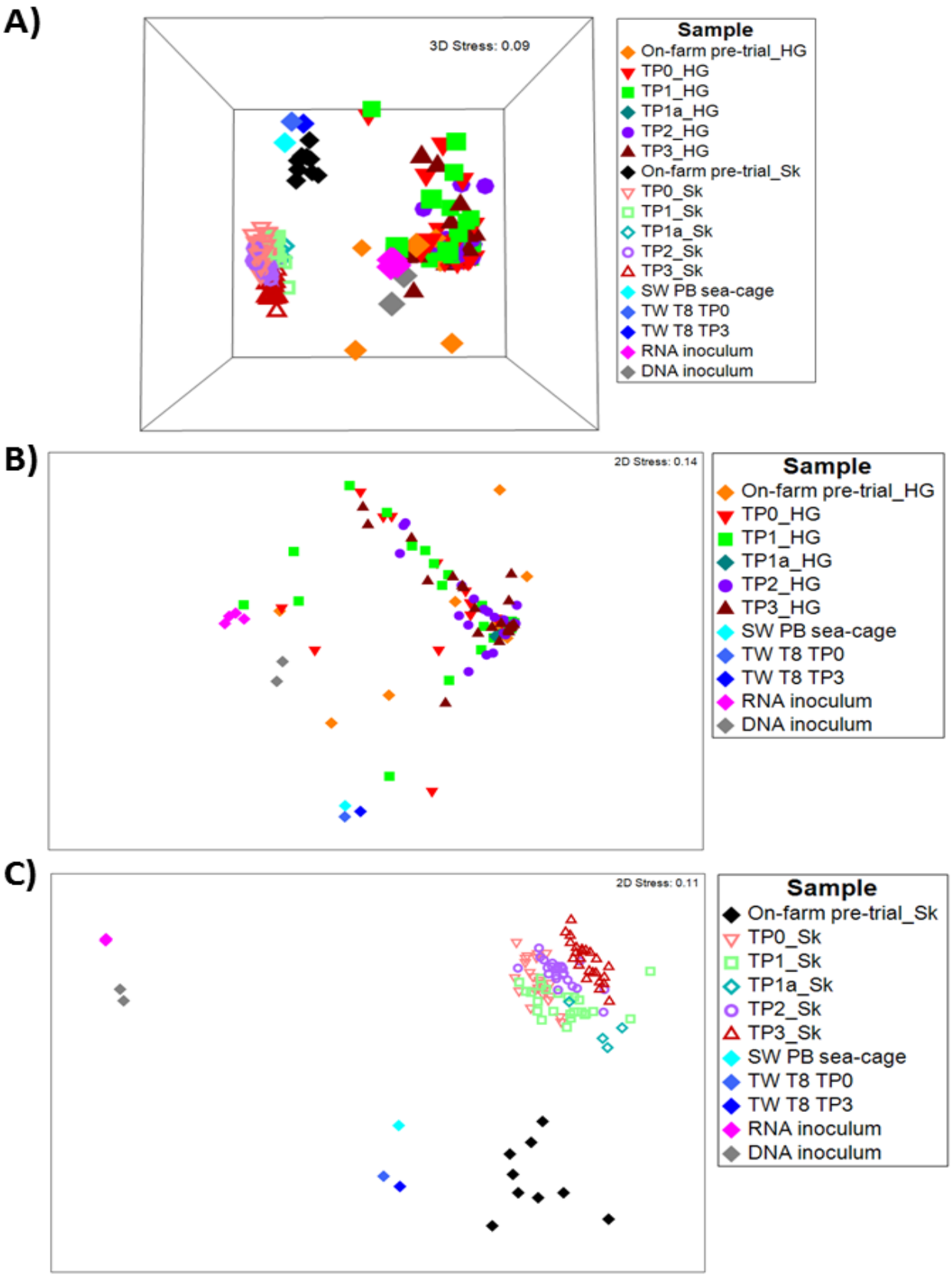


Figure 3.3.1.4.2. Differences between the global bacterial community structures through time for A) all samples, B) hindgut scraping samples only (with tank/seawater and inoculum samples) and C) skin swab samples only (with tank/seawater and inoculum samples) as analysed by non-metric multidimensional scaling (nMDS).[1]

[1] Samples not split into treatments for these plots.

Abbreviations: HG, hindgut; Sk, skin; T, tank; TP, time point; TW, tank water; SW, seawater.

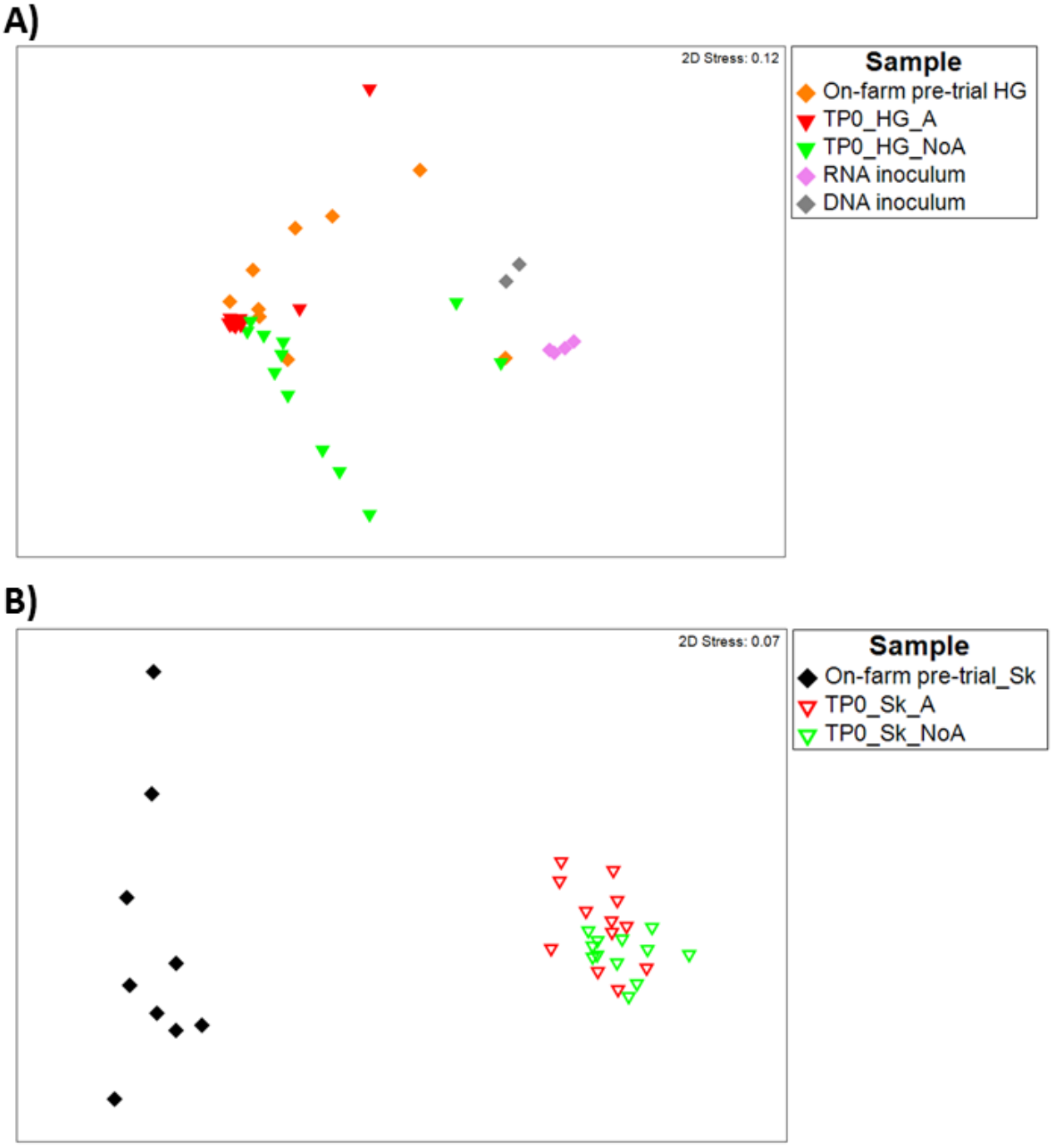


Figure 3.3.1.4.3. Differences between the global bacterial community structures for A) gut scrapings from on-farm pre-trial samples, time point 0 with and without antibiotics and inoculum samples, and B) skin swabs from on-farm pre-trial samples and time point 0 with and without antibiotics as analysed by non-metric multidimensional scaling (nMDS).[1]

[1] No comparisons with the inoculum for skin dataset as already shown in Figure 3.3.1.4.2 C that the gut inoculum samples are distinct from the skin swab samples.

Abbreviations: A, antibiotics; HG, hindgut; NoA, no antibiotics; Sk, skin; TP, time point.

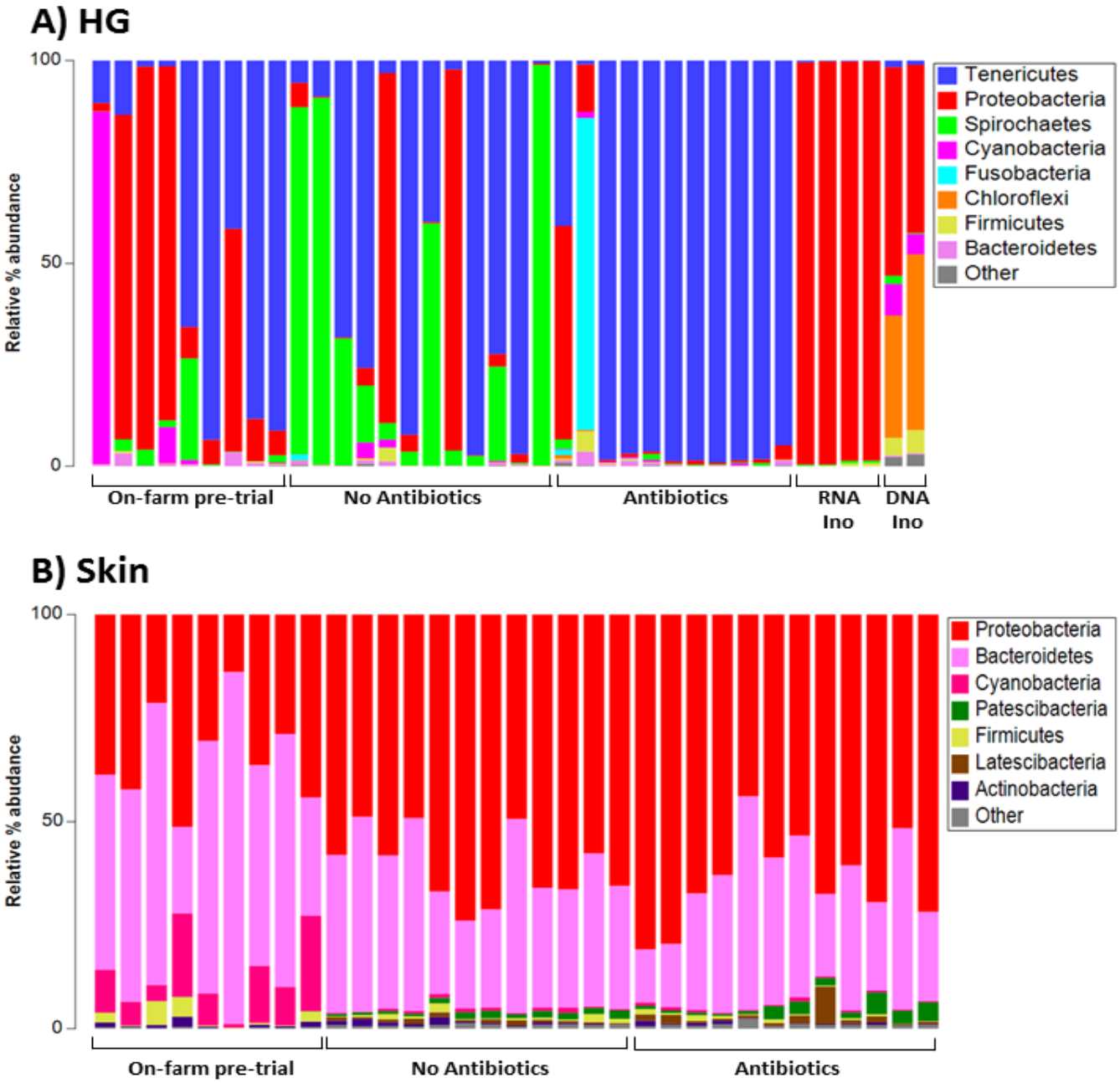
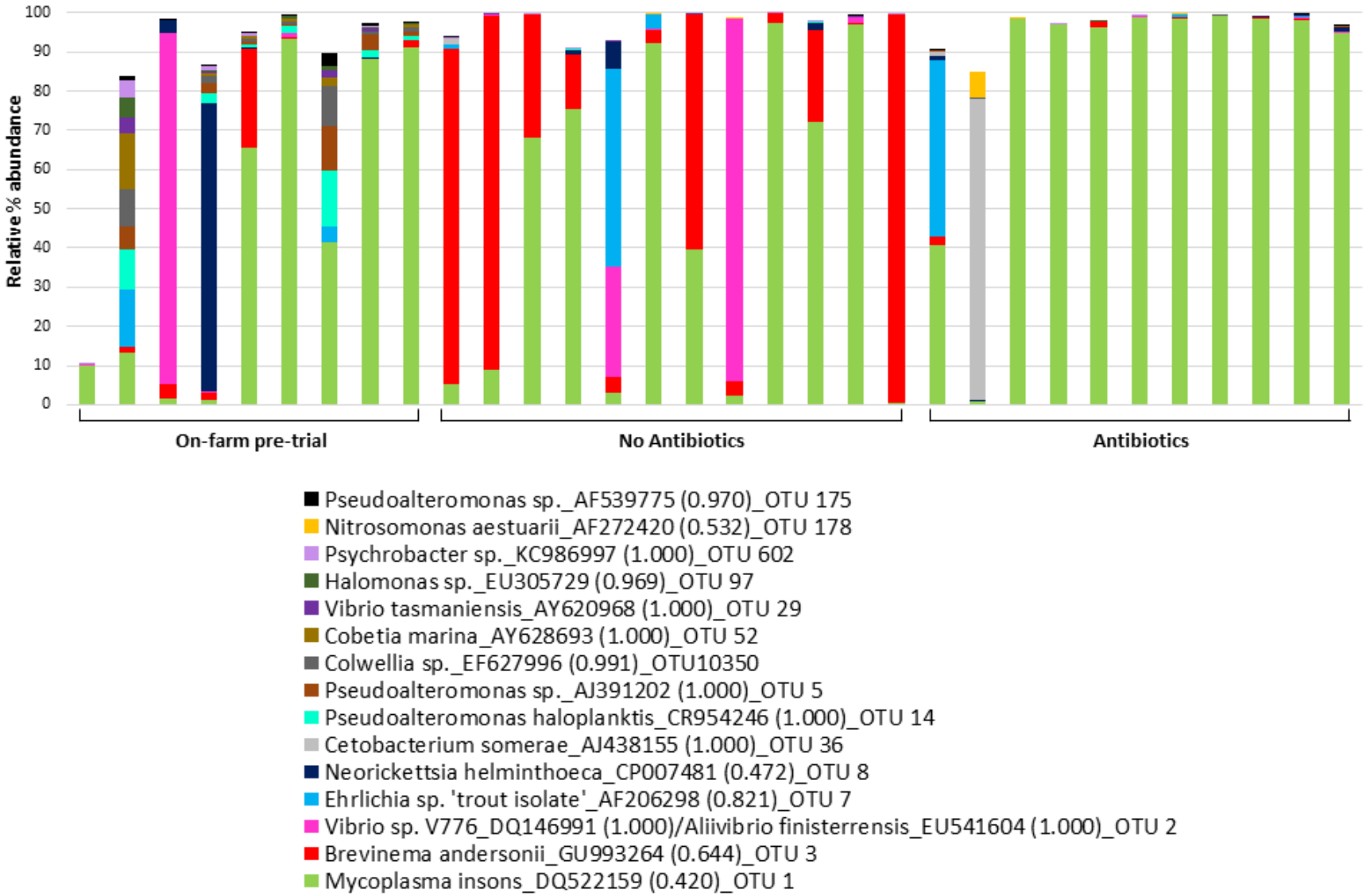


Figure 3.3.1.4.4. Relative percent abundance of bacterial phyla associated with the A) gut scrapings from on-farm pre-trial samples, time point 0 samples with antibiotics, time point 0 samples without antibiotics and inoculum samples, and B) skin swabs from on-farm pre-trial samples, time point 0 samples with antibiotics and time point 0 samples without antibiotics.
Abbreviations: HG, hindgut; Ino, inoculum.



Figure

3.3.1.4.5. Relative percent abundance of the 15 most abundant bacterial OTUs in gut scrapings from on-farm pre-trial samples, time point 0 samples with no antibiotics and time point 0 samples with antibiotics.

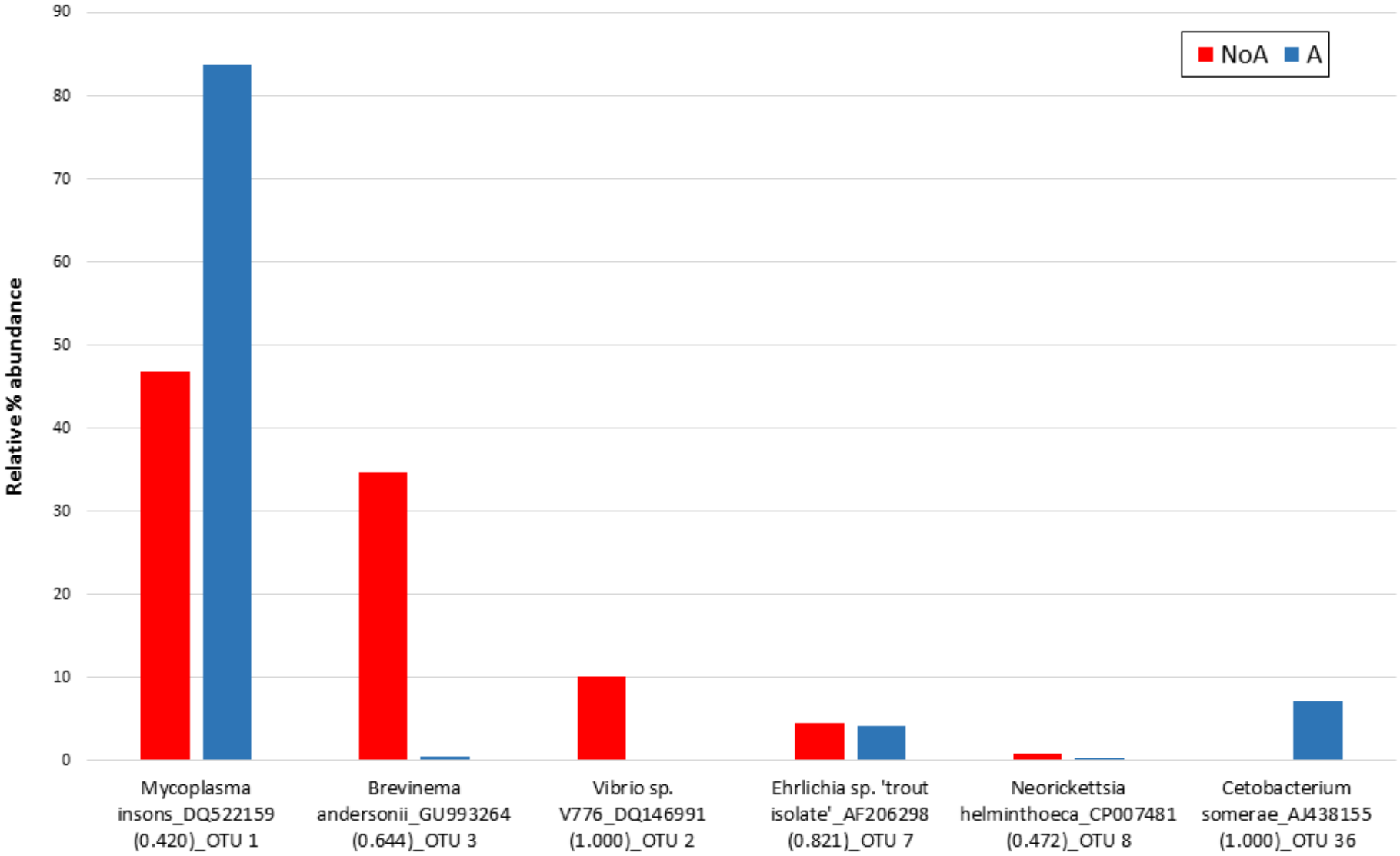
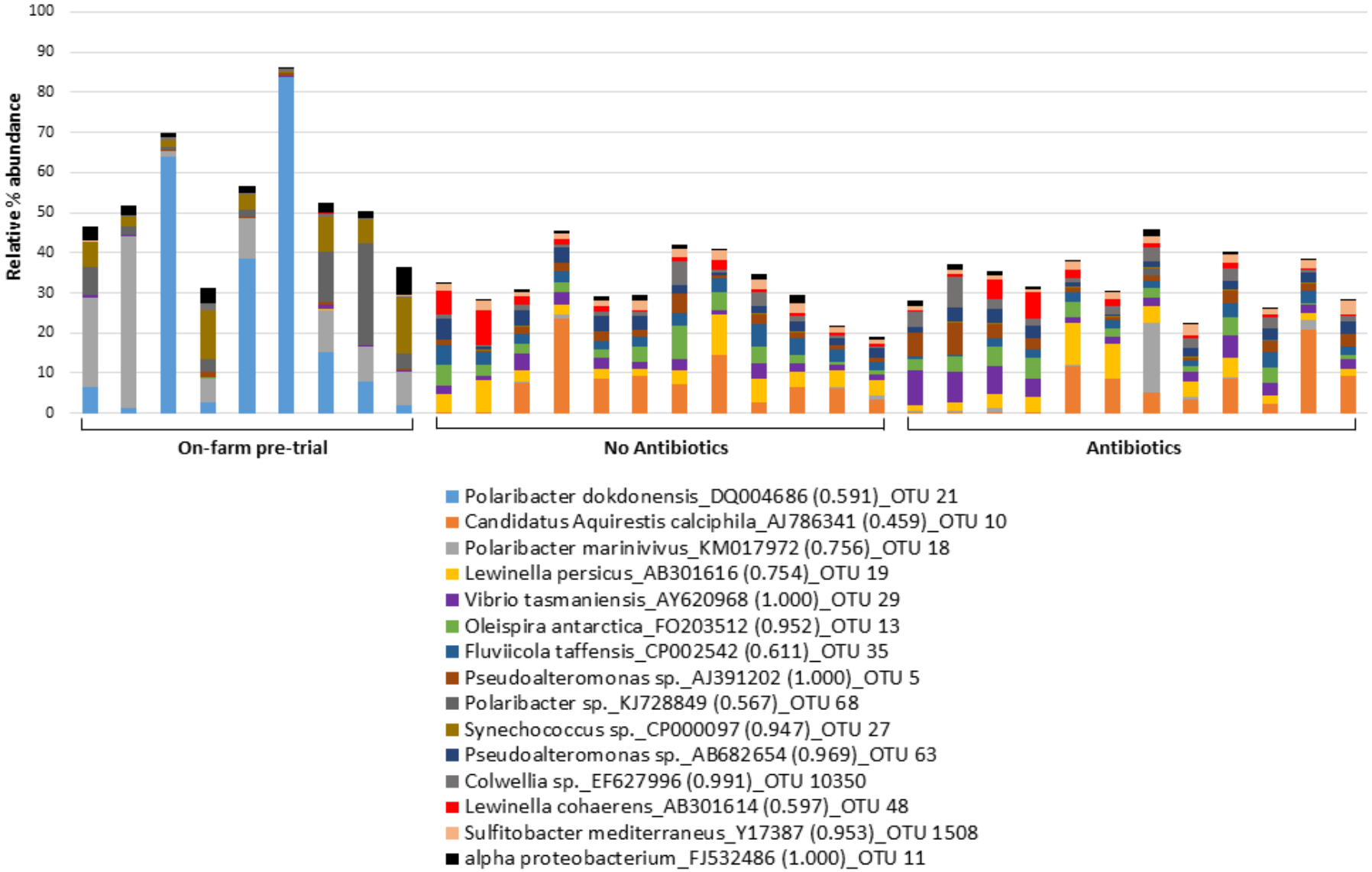


Figure 3.3.1.4.6. Relative percent abundance of the 6 most abundant bacterial OTUs in the no antibiotics time point 0 (red) gut scraping samples in comparison to the corresponding abundances for these OTUs in the antibiotics samples at time point 0 (blue).



Figure

3.3.1.4.7. Relative percent abundance of the 15 most abundant bacterial OTUs in skin swabs from on-farm pre-trial samples, time point 0 samples with no antibiotics and time point 0 samples with antibiotics

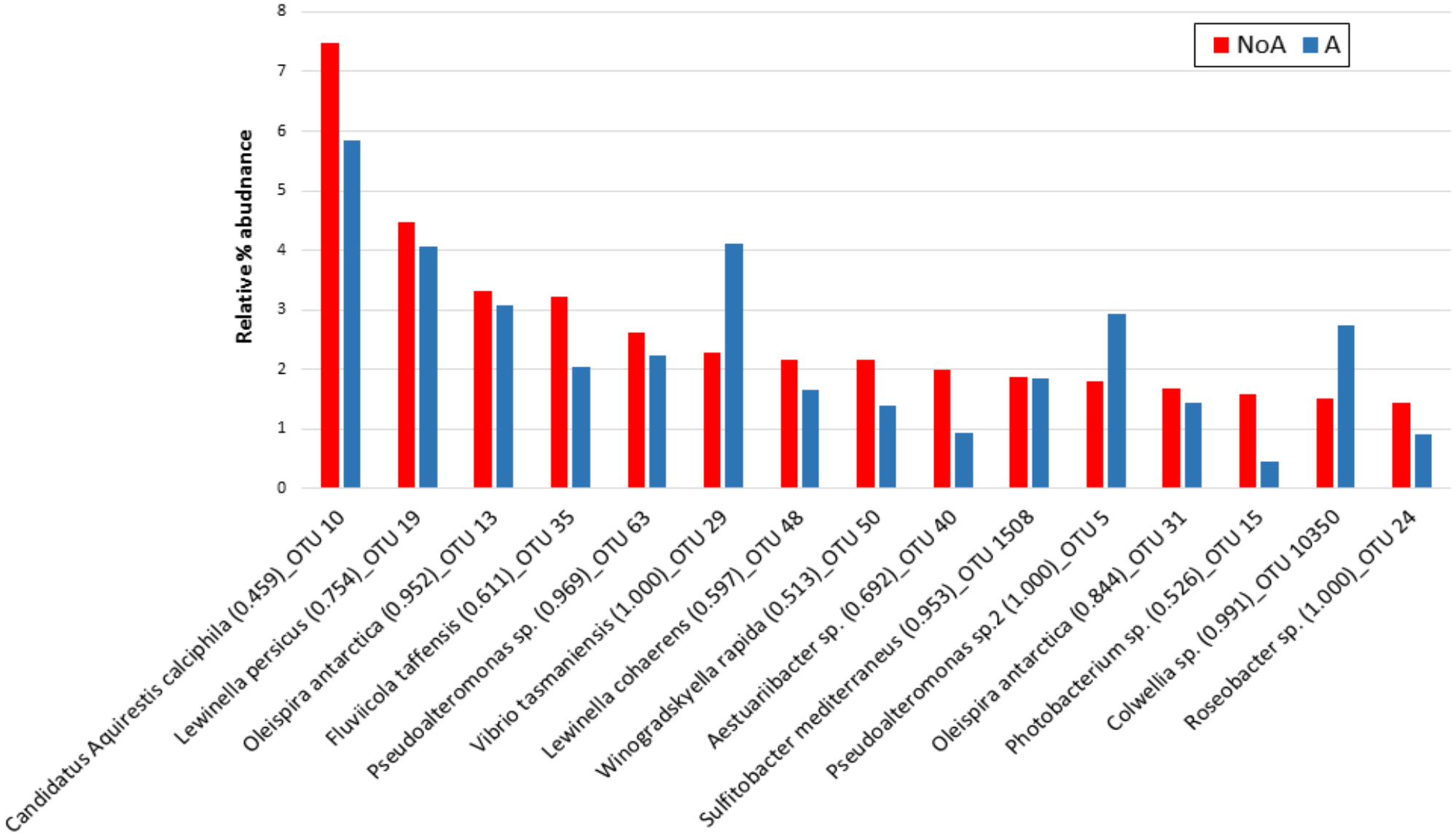


Figure 3.3.1.4.8. Relative percent abundance of the 15 most abundant bacterial OTUs in the no antibiotics skin swab samples at time point 0 (red), with the corresponding abundances for these OTUs in the antibiotics samples at time point 0 (blue).

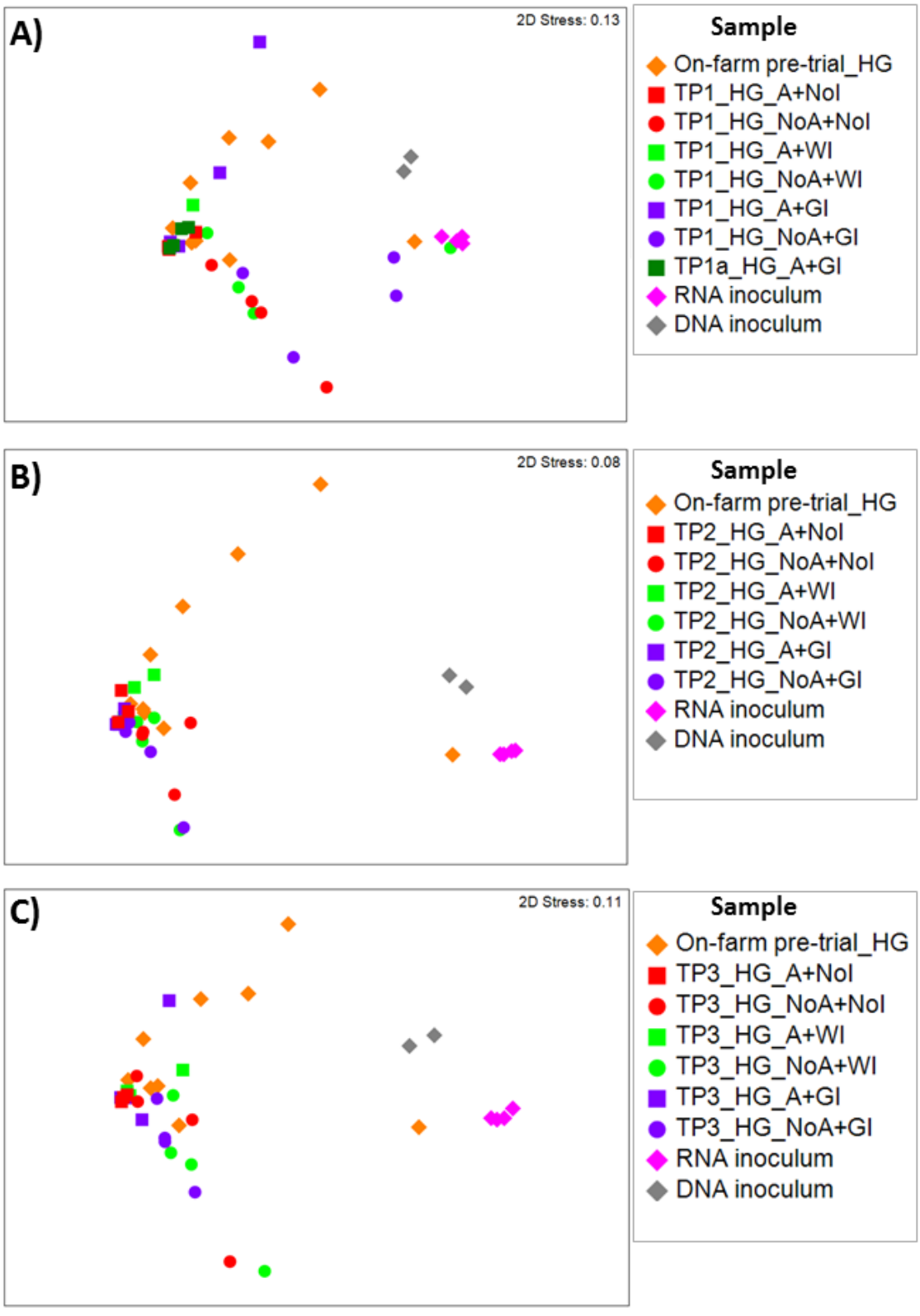


Figure 3.3.1.4.9. Difference between the global community structure for the YTK gut scrapings of no inoculum, water inoculum and gavage inoculum samples with and without antibiotics at A) TP1, B) TP2 and C) TP3 as analysed by non-metric multidimensional scaling (nMDS).[1]

Abbreviations: A, antibiotics; GI, gavage inoculum; NoA, no antibiotics; NoI, no inoculum; TP, time point; WI, water inoculum.

[1] TP1 - 2 days post inoculum administration and 5 days after antibiotics administration; TP1a – 5 days post inoculum and 8 days after antibiotics administration; TP2 - 8 days post inoculum, 11 days after antibiotics administration; TP3 - 15 days post inoculum, 18 days after antibiotics administration.

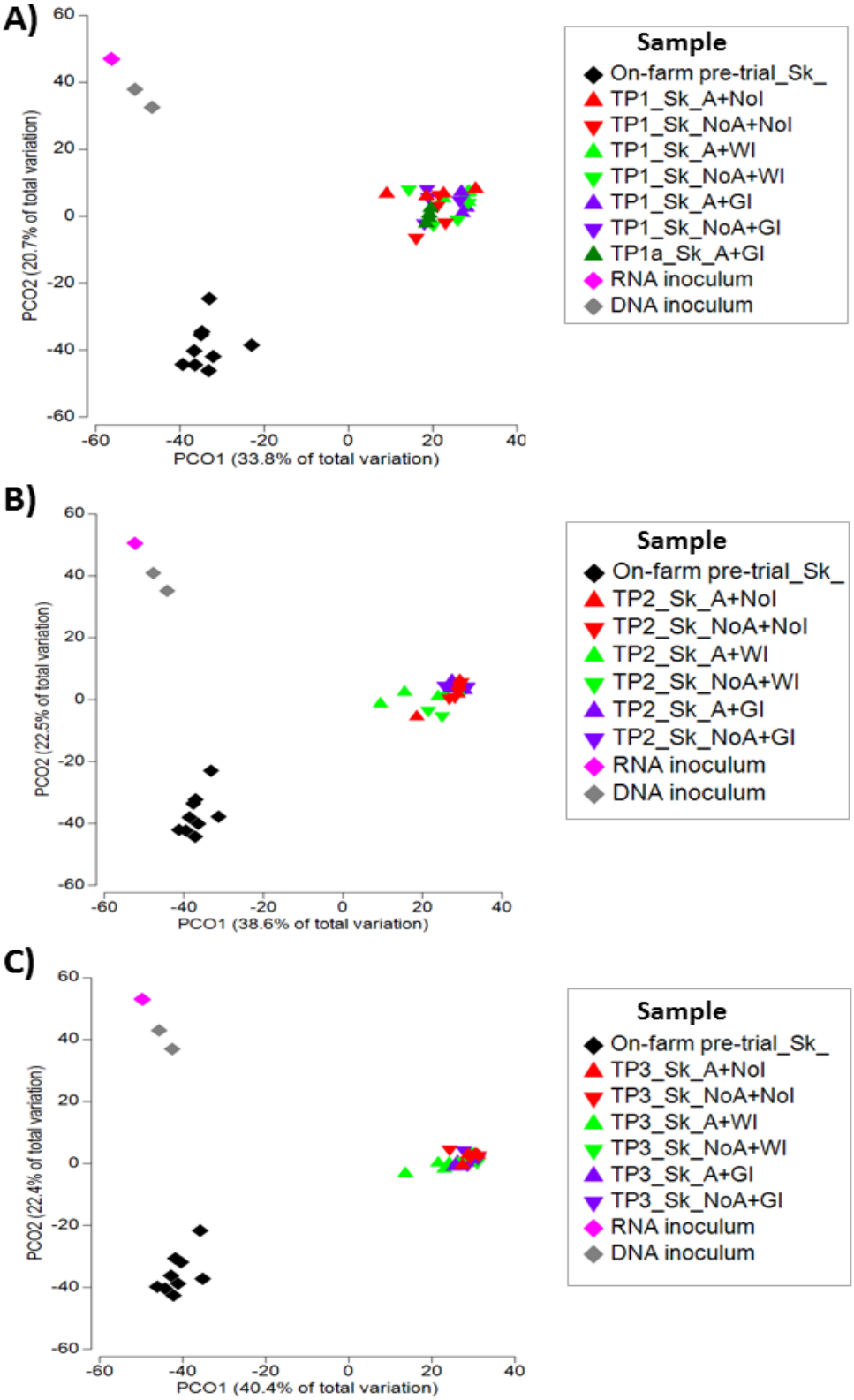


Figure 3.3.1.4.10. Difference between the global community structure for the YTK skin swabs of no inoculum, water inoculum and gavage inoculum samples with and without antibiotics at A) TP1, B) TP2 and C) TP3 as analysed by Principal Co-ordinates (PCO).[1]

Abbreviations: A, antibiotics; GI, gavage inoculum; NoA, no antibiotics; NoI, no inoculum; TP, time point; WI, water inoculum.

[1] TP1 - 2 days post inoculum administration and 5 days after antibiotics administration; TP1a – 5 days post inoculum and 8 days after antibiotics administration; TP2 - 8 days post inoculum, 11 days after antibiotics administration; TP3 - 15 days post inoculum, 18 days after antibiotics administration.

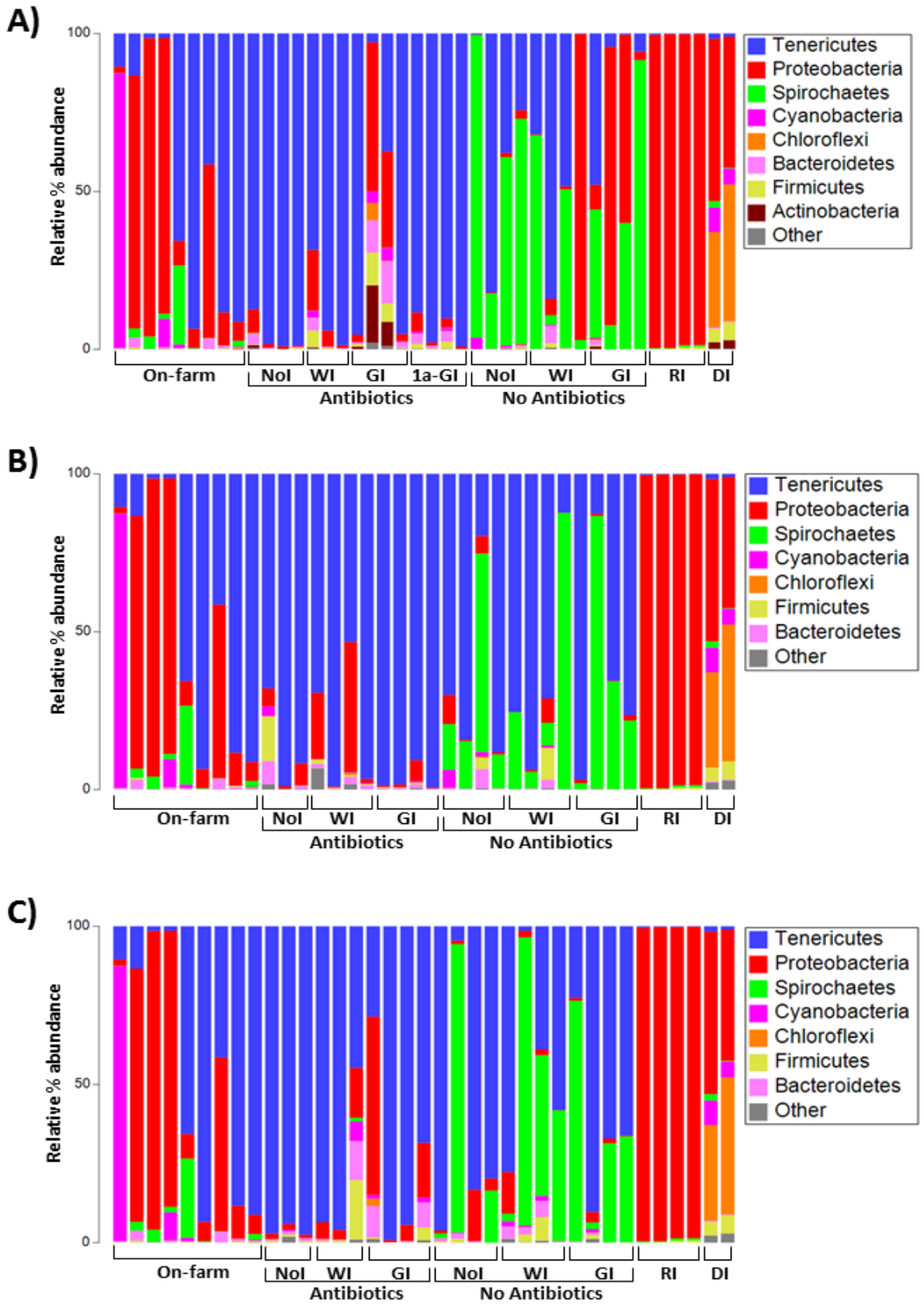


Figure 3.3.1.4.11. Relative percent abundance of bacterial phyla associated with the YTK gut scrapings of no inoculum, water inoculum and gavage inoculum samples with and without antibiotics at A) TP1, B) TP2 and C) TP3.[1]

Abbreviations: 1a, time point 1a; DI, DNA inoculum; GI, gavage inoculum; NoI, no inoculum; RI, RNA inoculum; WI, water inoculum.

[1] TP1 - 2 days post inoculum administration and 5 days after antibiotics administration; TP1a – 5 days post inoculum and 8 days after antibiotics administration; TP2 - 8 days post inoculum, 11 days after antibiotics administration; TP3 - 15 days post inoculum, 18 days after antibiotics administration.

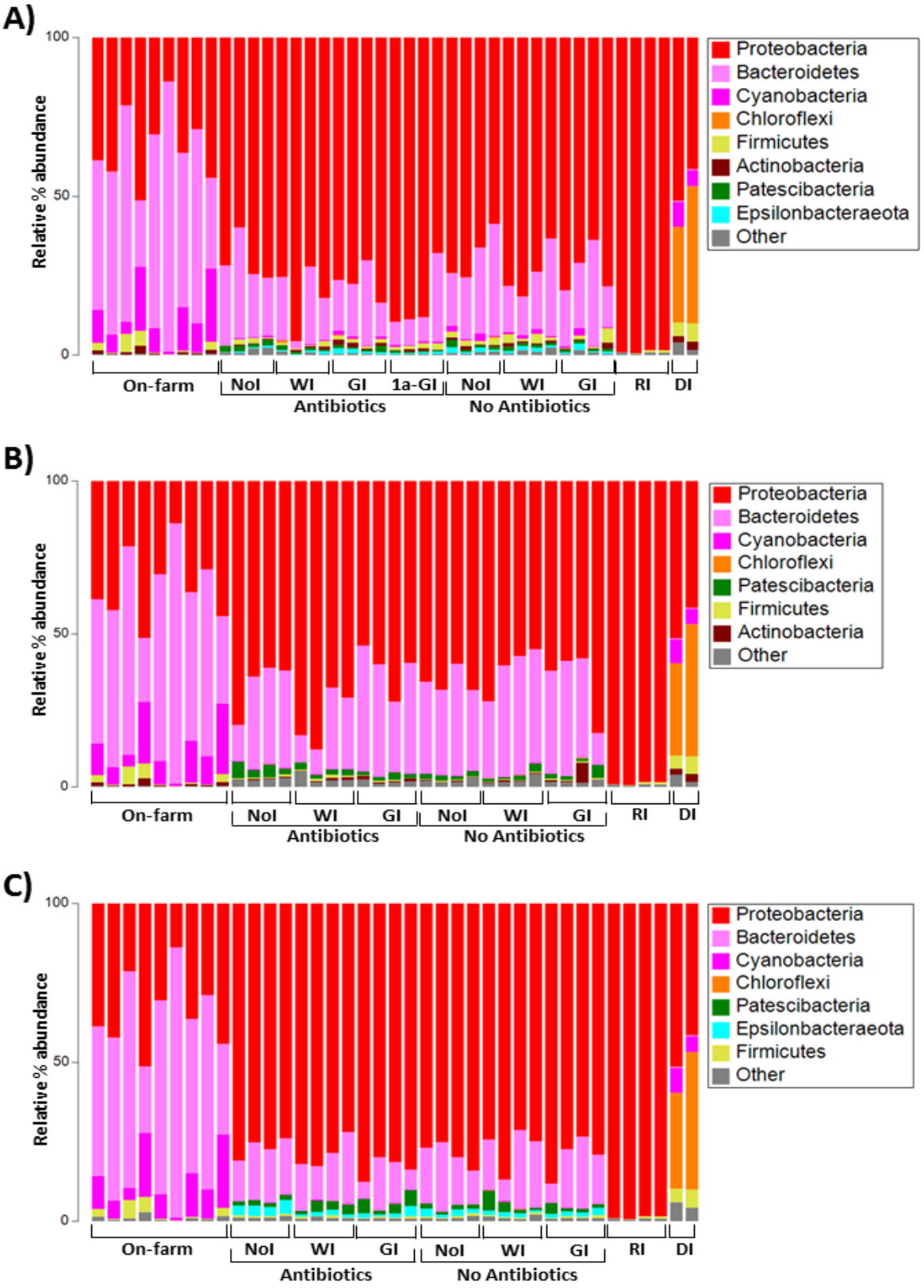


Figure 3.3.1.4.12. Relative percent abundance of bacterial phyla associated with the YTK skin swabs of no inoculum, water inoculum and gavage inoculum samples with and without antibiotics at A) TP1, B) TP2 and C) TP3.[1]

Abbreviations: 1a, time point 1a; DI, DNA inoculum; GI, gavage inoculum; NoI, no inoculum; RI, RNA inoculum; WI, water inoculum.

[1] TP1 - 2 days post inoculum administration and 5 days after antibiotics administration; TP1a – 5 days post inoculum and 8 days after antibiotics administration; TP2 - 8 days post inoculum, 11 days after antibiotics administration; TP3 - 15 days post inoculum, 18 days after antibiotics administration.

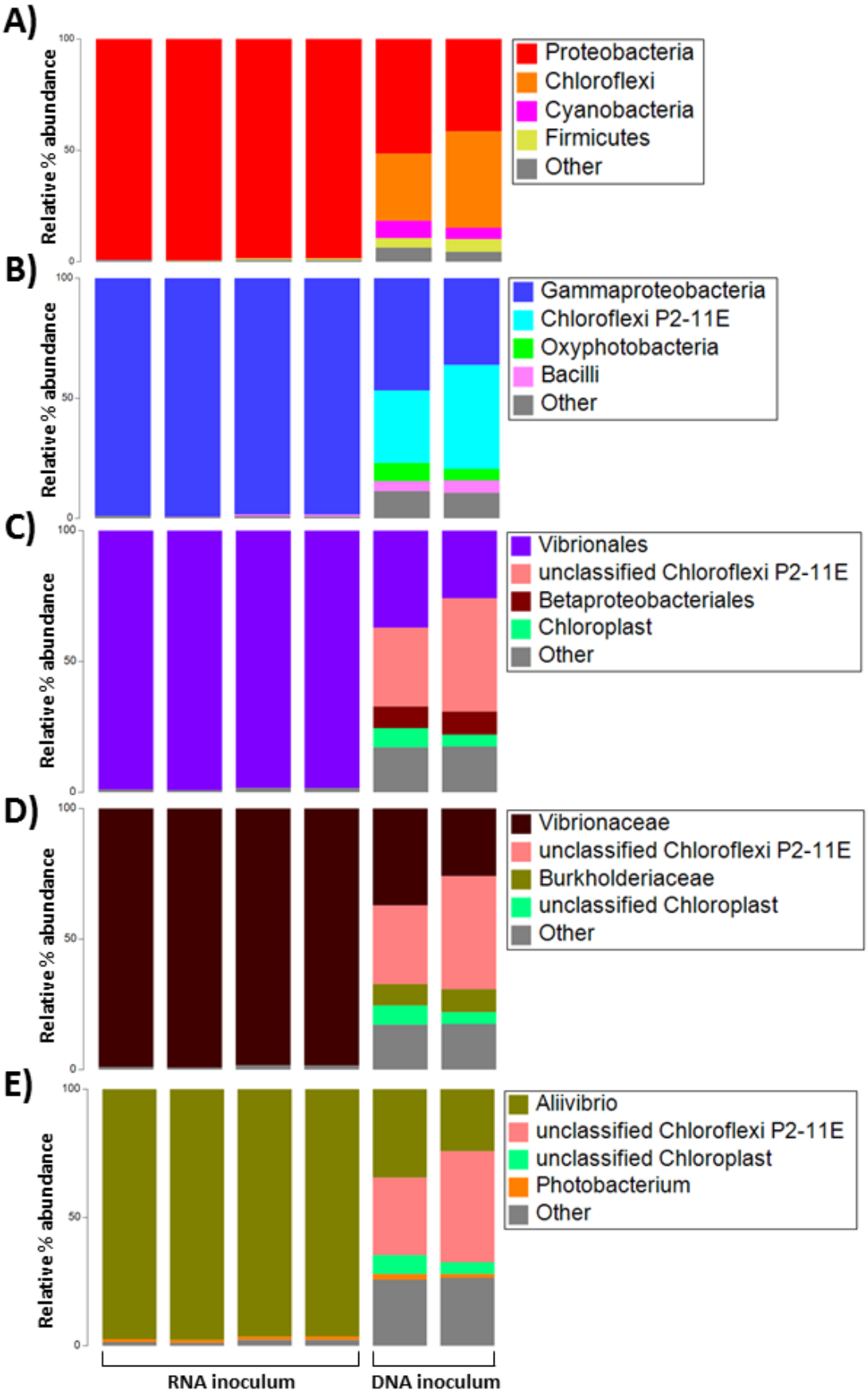


Figure 3.3.1.4.13. Relative percent abundance of bacterial taxa associated with inoculum (RNA and DNA) samples at the A) phylum, B) class, C) order, D) family and E) genus levels.

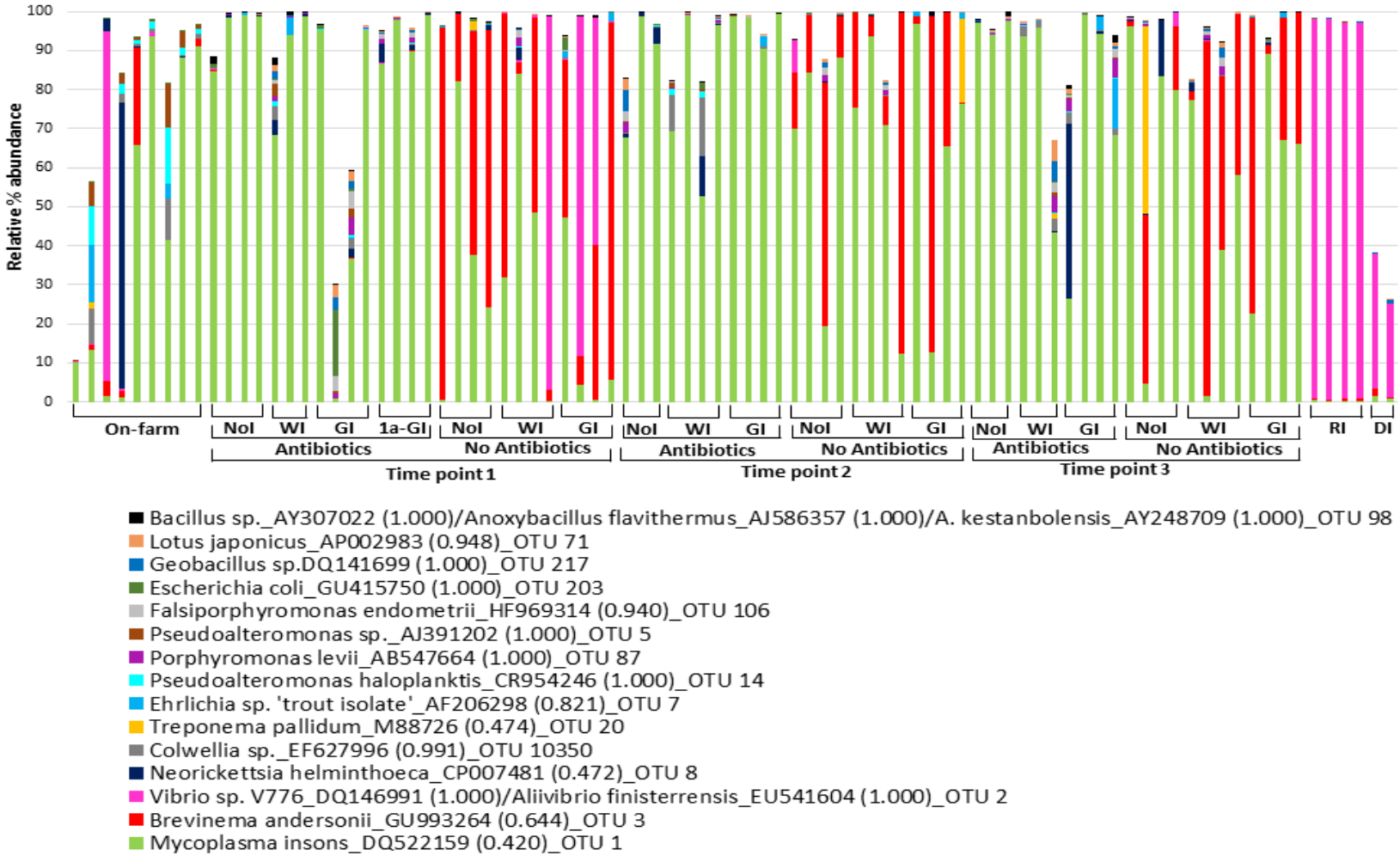


Figure 3.3.1.4.14. Relative percent abundance of the 15 most abundant bacterial OTUs in the on-farm, no inoculum, water inoculum and gavage inoculum gut scraping samples with and without antibiotics at the three sampling time points.[1,2]

Abbreviations: 1a, time point 1a; DI, DNA inoculum; GI, gavage inoculum; NoI, no inoculum; RI, RNA inoculum; WI, water inoculum.

[1] TP1 - 2 days post inoculum administration and 5 days after antibiotics administration; TP1a – 5 days post inoculum and 8 days after antibiotics administration; TP2 - 8 days post inoculum, 11 days after antibiotics administration; TP3 - 15 days post inoculum, 18 days after antibiotics administration.

[2] Relative percent abundance of these top 15 gut OTUs are also shown for the RNA and DNA inoculum samples.

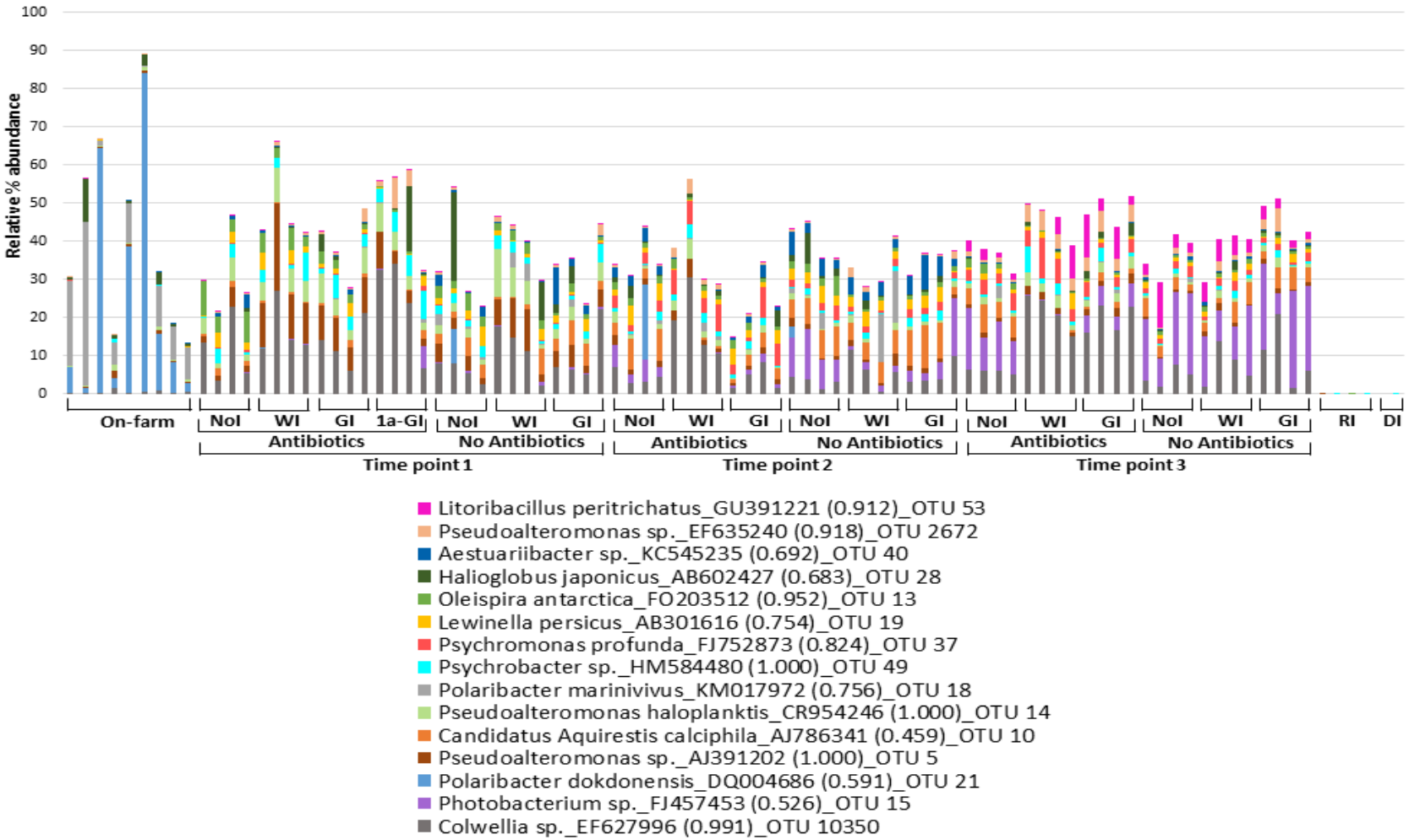


Figure 3.3.1.4.15. Relative percent abundance of the 15 most abundant bacterial OTUs in the on-farm, no inoculum, water inoculum and gavage inoculum skin swab samples with and without antibiotics at the three sampling time points.[1,2]

Abbreviations: 1a, time point 1a; DI, DNA inoculum; GI, gavage inoculum; NoI, no inoculum; RI, RNA inoculum; WI, water inoculum.

[1] TP1 - 2 days post inoculum administration and 5 days after antibiotics administration; TP1a – 5 days post inoculum and 8 days after antibiotics administration; TP2 - 8 days post inoculum, 11 days after antibiotics administration; TP3 - 15 days post inoculum, 18 days after antibiotics administration.

[2] Relative percent abundance of these top 15 skin OTUs are also shown for the RNA and DNA inoculum samples.

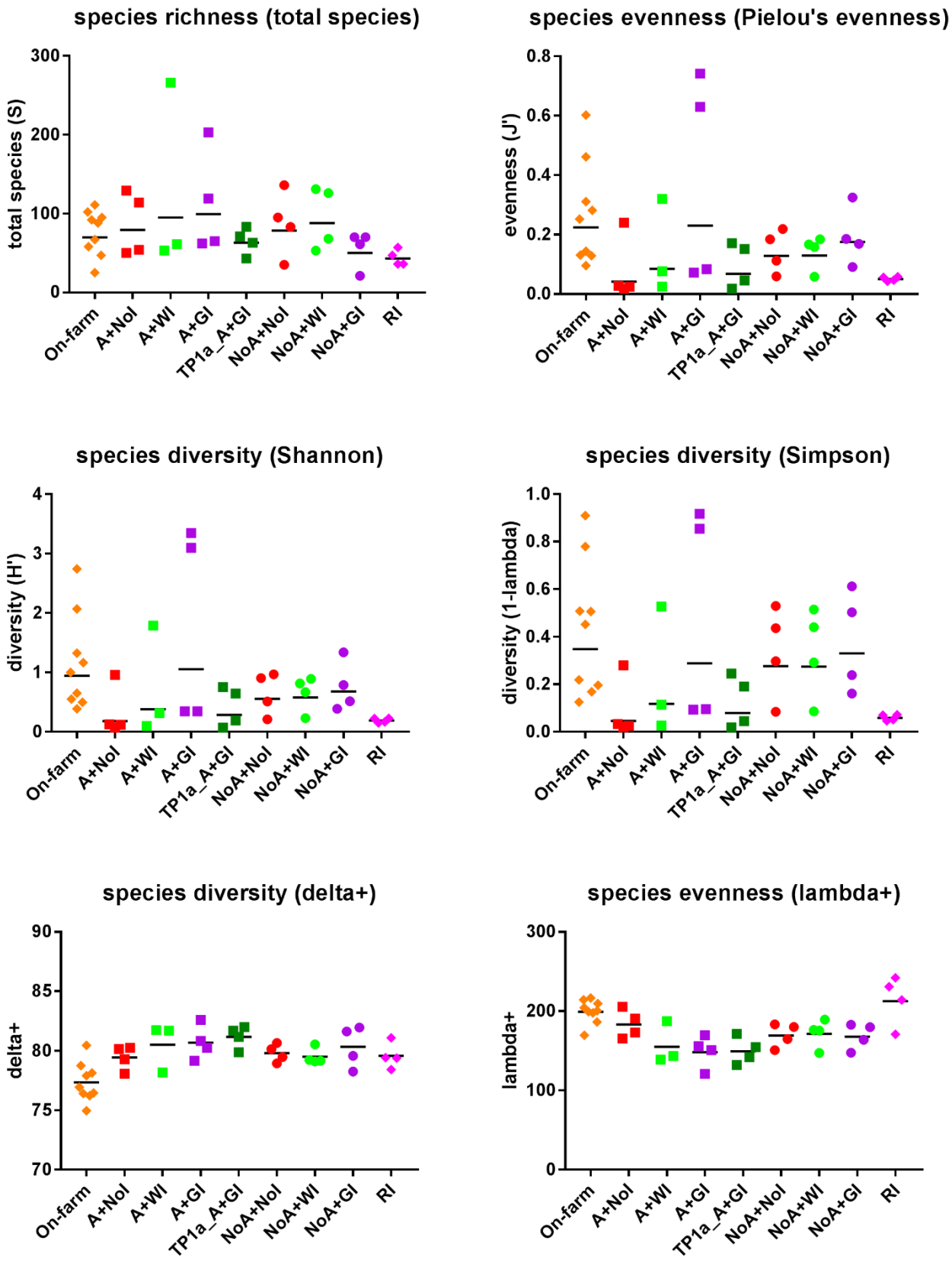


Figure 3.3.1.4.16. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for on-farm, no inoculum, water inoculum and gavage inoculum gut scraping samples with and without antibiotics at time point 1.[1]

Abbreviations: 1a, time point 1a; GI, gavage inoculum; NoI, no inoculum; RI, RNA inoculum; WI, water inoculum.

[1]TP1 - 2 days post inoculum administration and 5 days after antibiotics administration; TP1a – 5 days post inoculum and 8 days after antibiotics administration.

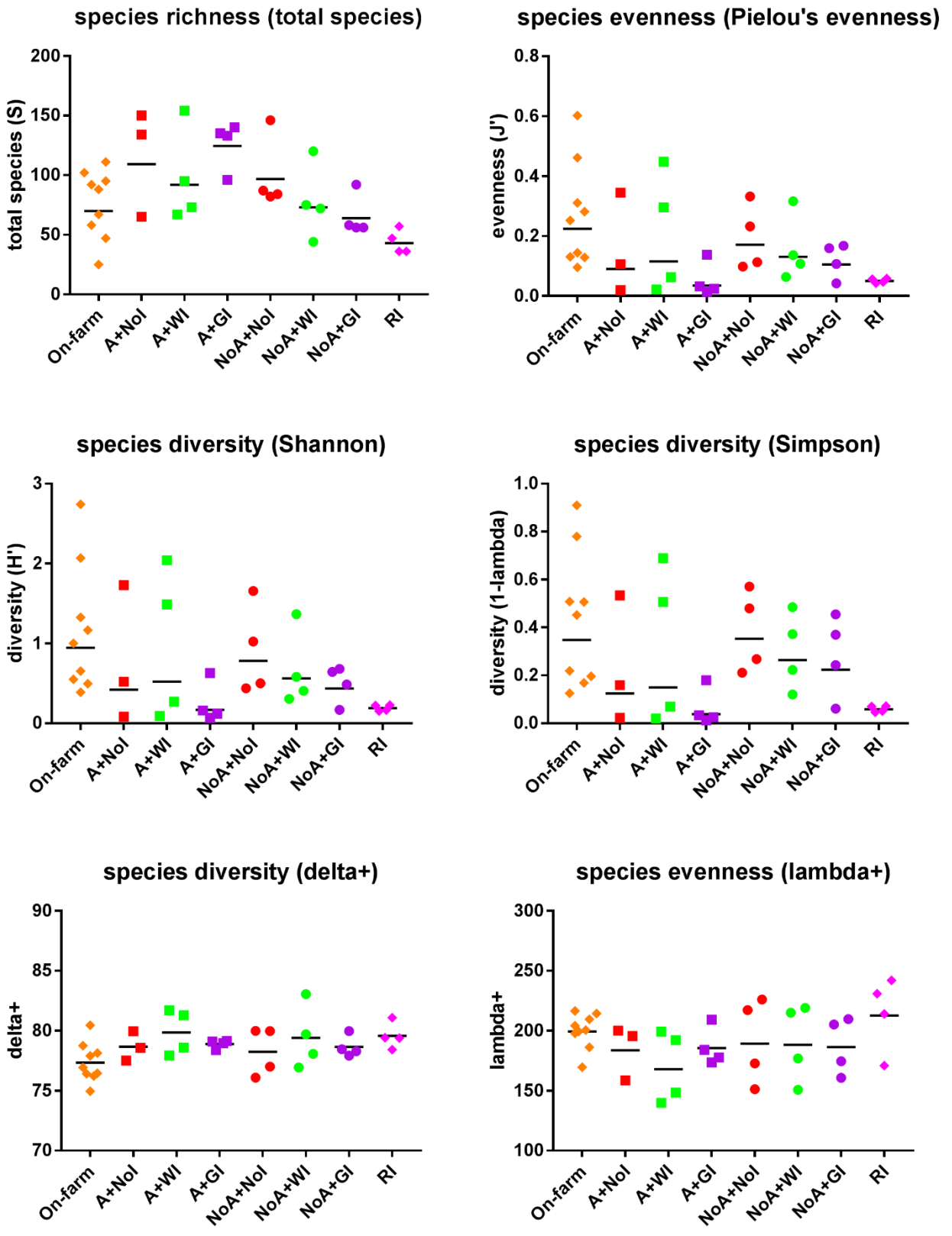


Figure 3.3.1.4.17. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for on-farm, no inoculum, water inoculum and gavage inoculum gut scraping samples with and without antibiotics at time point 2.[1]
Abbreviations: GI, gavage inoculum; NoI, no inoculum; RI, RNA inoculum; WI, water inoculum.
[1] TP2 - 8 days post inoculum, 11 days after antibiotics administration.

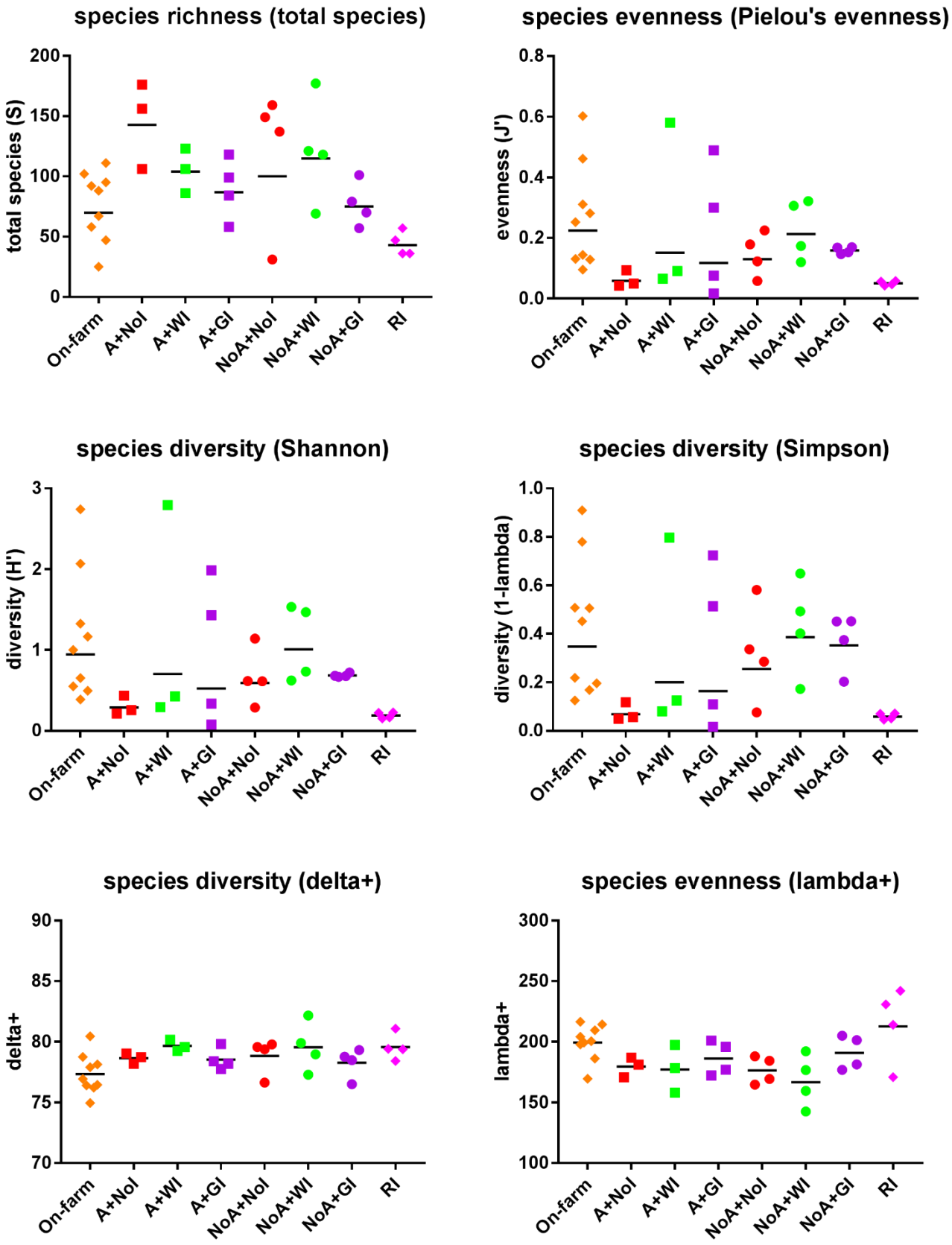


Figure 3.3.1.4.18. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for on-farm, no inoculum, water inoculum and gavage inoculum gut scraping samples with and without antibiotics at time point 3.[1]
Abbreviations: GI, gavage inoculum; NoI, no inoculum; RI, RNA inoculum; WI, water inoculum.
[1] TP3 - 15 days post inoculum, 18 days after antibiotics administration.

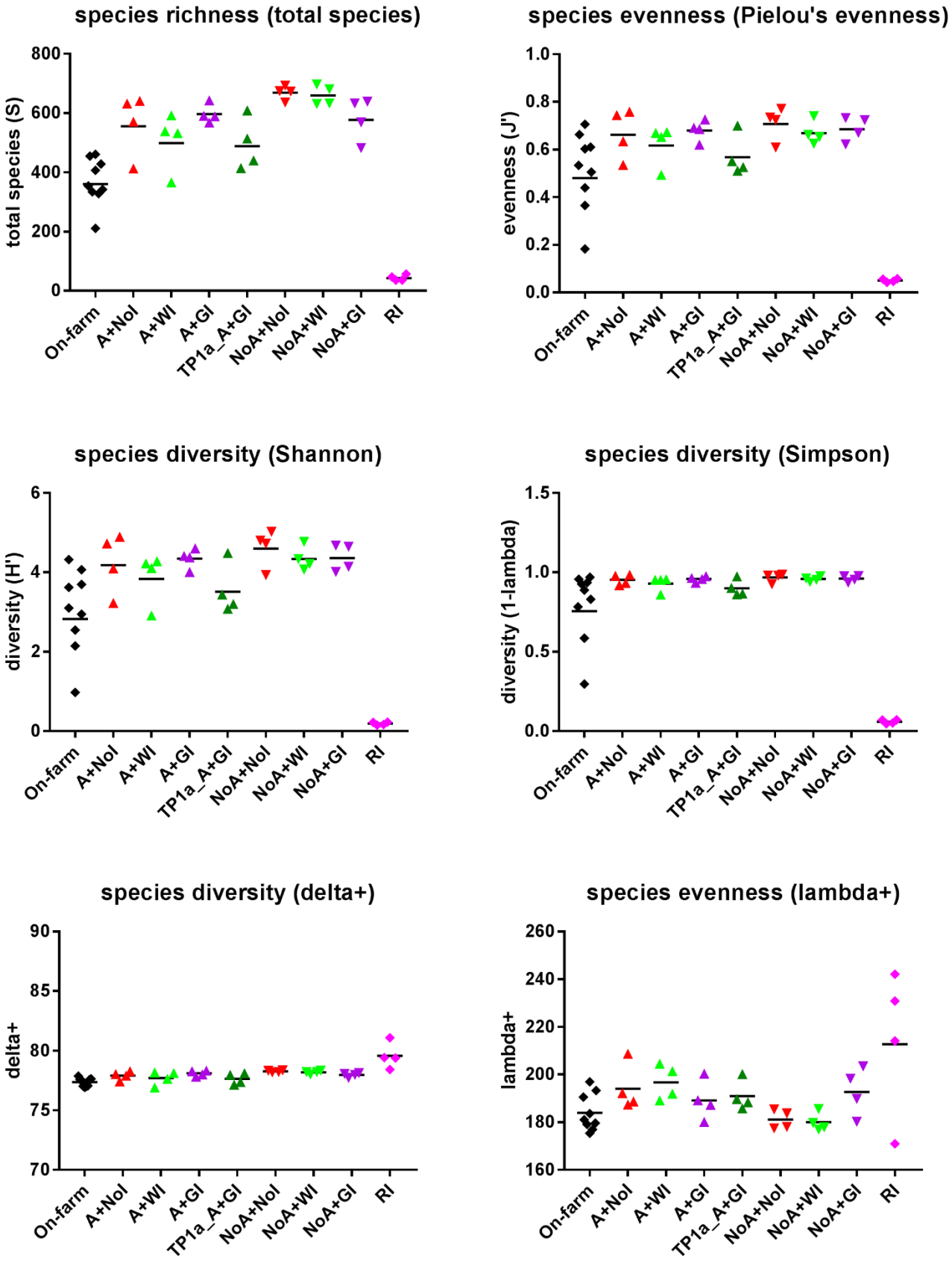


Figure 3.3.1.4.19. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for on-farm, no inoculum, water inoculum and gavage inoculum skin swab samples with and without antibiotics at time point 1.[1]

Abbreviations: 1a, time point 1a; GI, gavage inoculum; NoI, no inoculum; RI, RNA inoculum; WI, water inoculum.

[1] TP1 - 2 days post inoculum administration and 5 days after antibiotics administration; TP1a – 5 days post inoculum and 8 days after antibiotics administration.

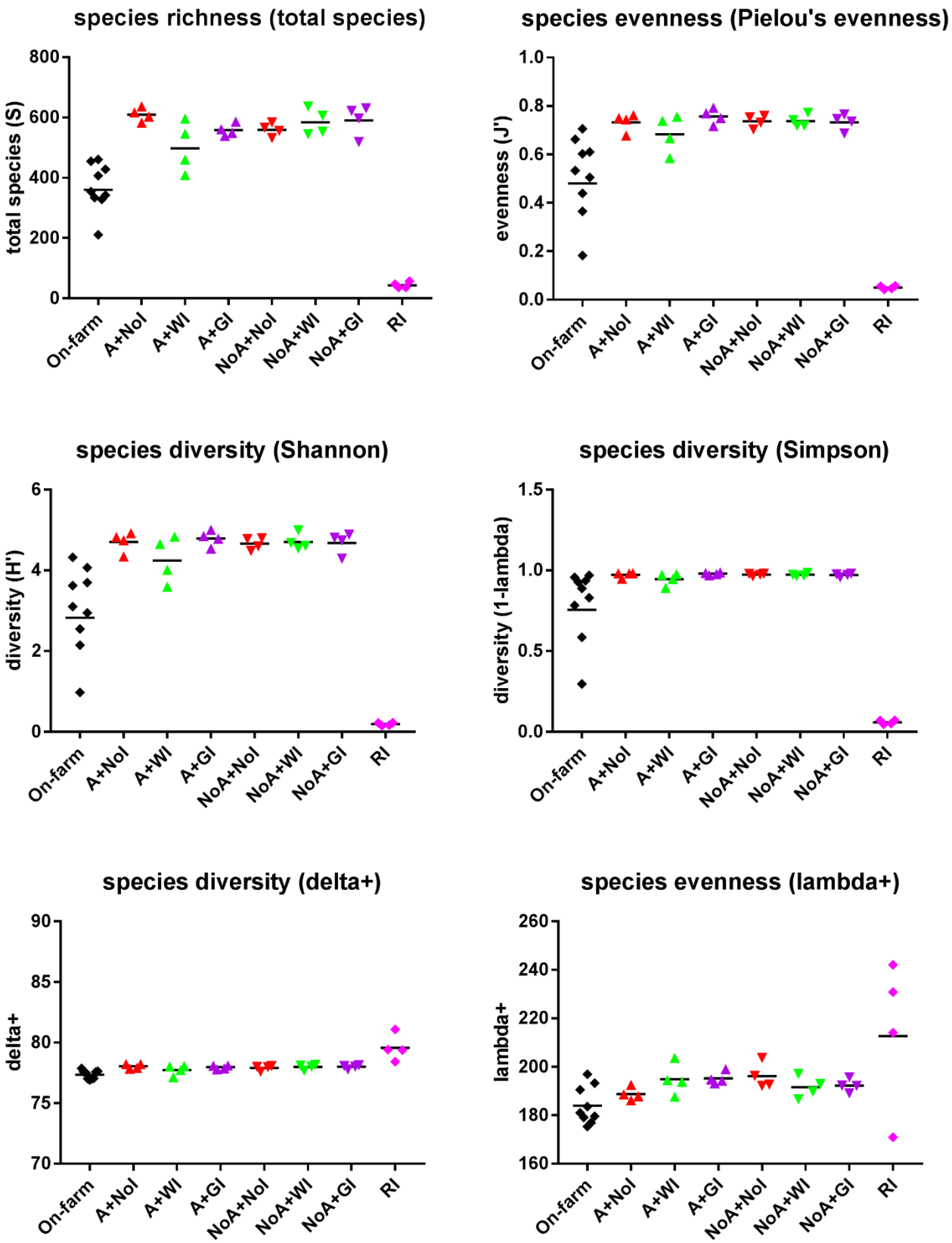


Figure 3.3.1.4.20. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for on-farm, no inoculum, water inoculum and gavage inoculum skin swab samples with and without antibiotics at time point 2.[1]
Abbreviations: GI, gavage inoculum; NoI, no inoculum; RI, RNA inoculum; WI, water inoculum.
[1] TP2 - 8 days post inoculum, 11 days after antibiotics administration.

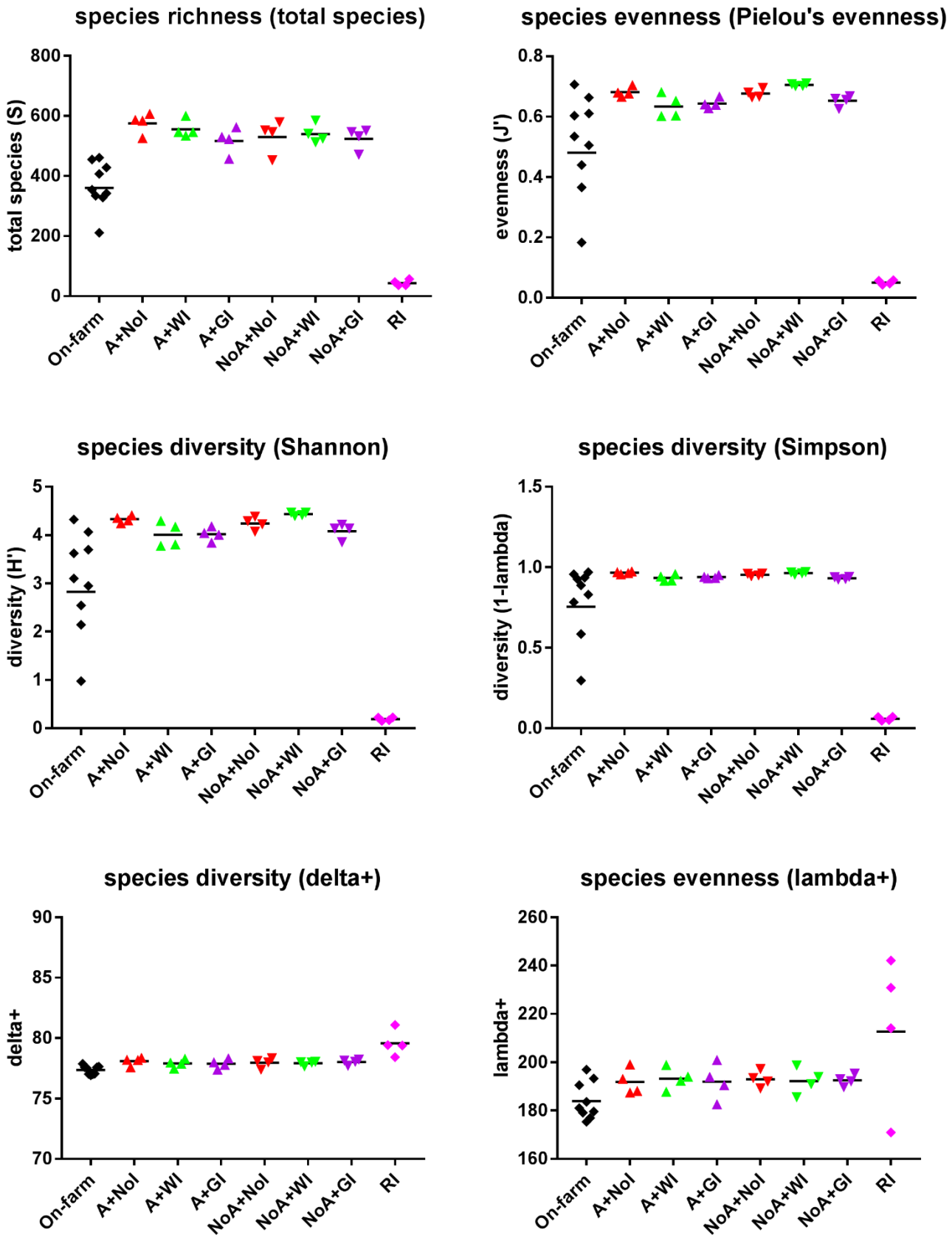
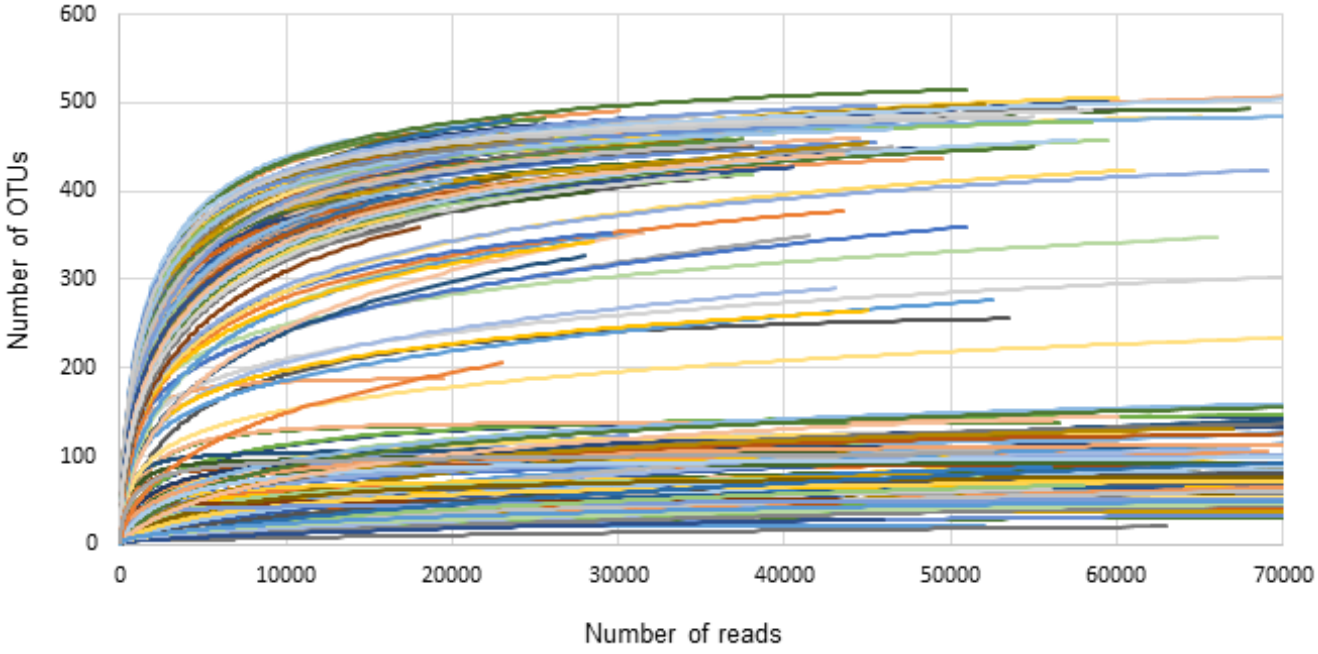


Figure 3.3.1.4.21. Measures of species richness (total species), evenness (Pielou's and lambda+) and taxonomic diversity (Shannon, Simpson and delta+) for on-farm, no inoculum, water inoculum and gavage inoculum skin swab samples with and without antibiotics at time point 3.[1]
Abbreviations: GI, gavage inoculum; NoI, no inoculum; RI, RNA inoculum; WI, water inoculum.
[1] TP3 - 15 days post inoculum, 18 days after antibiotics administration.

Appendix 1. Rarefaction curves portraying the number of resolved OTUs against sequencing depth of each sample (hindgut scraping, skin swab and inoculum) collected from the microbiome manipulation trial.



3.3.2. Chapter - Investigating the microbiome and methods to assess the health of Yellowtail Kingfish (*Seriola lalandi*) from wild and farmed stocks.

3.3.2.1. Manuscript - A challenge model to understand the immune influences of Yellowtail Kingfish diets.

Emmanuelle Zoccola[a], Andrew Barnes[a], Matthew S. Bansemer [b], Marty R Deveney[b]

[a] The University of Queensland, School of Biological Sciences and Centre for Marine Studies, Brisbane, QLD 4072, Australia

[b] South Australian Research and Development Institute, South Australian Aquatic Sciences Centre, West Beach, SA 5024, Australia

This manuscript may be referenced as: Zoccola, E., Barnes, A., Bansemer, M.S. and Deveney, M.R. (2019). 3.3.2.1. Manuscript – A challenge model to understand the immune influences of Yellowtail Kingfish diets (Output 4a). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.760-774.

Abstract

Immunity to infectious disease is a critical energy-dependent system in animals. Immunity has short-term energy and long-term raw material requirements. Adequate supply of essential and conditionally-essential nutrients is a prerequisite to a functional immune response, and dietary limitation of key nutrients may result in a defective or depleted response to challenge. We investigated if a challenge using a killed bacterial vaccine (100 µL killed *P. damselae piscicida* 1×10^{16} cells L^{-1} culture), and analysis of the subsequent specific antibody and molecular measures of immune signalling molecules, could show differences in immune response between diets. Compared to the control diet, the selected experimental diets had a detrimental effect on the way in which Yellowtail Kingfish (*Seriola lalandi*; YTK) responded to vaccination. For further development of the challenge test we recommend further use and supplementation of the suite of genes, with refinement of the trial design and sampling to reduce population variance and increase statistical power.

Introduction

The immune system of higher animals comprises a complex network of cells, proteins and peptides that protect the animal from disease and exogenous toxins, and promotes wound repair. As this system is highly energy dependent, it is modulated by stress hormones and other processes that allocate intake energy on an urgent need basis through the neuro-endocrine-immune axis of transcriptional regulators. Consequently, the immune system and its ability to respond to threat can be employed as an indicator of energy supply, and therefore, of the nutritional health of the animal. Indeed, the study of the influence of diet on immunity in humans and animals has become very popular in recent years, giving rise to the field of immuno-nutrition. This field has become relevant in aquaculture with the drive to reduce both the environmental footprint and costs associated with fish feeds. While the total proportions of key components of diets for a particular species can be largely maintained during substitution of fishmeal with more sustainably sourced ingredients, the availability of essential and semi-essential nutrients is

harder to balance, particularly where the requirement for a particular nutrient is not yet understood for that species.

Immuno-nutrition has identified a range of influences on fish immune systems. For example, in red snappers, improved performance in terms of ability of immune effector cells to respond to stimulation was detected within three days of transferring onto new winter feeds. In contrast, the performance in terms of weight gain was only detected after 60 days on the same diets (Cook et al., 2003). Thus, the operational efficacy of the innate immune system responds rapidly to the nutritional status of the animal. The adaptive immune system, on the other hand, takes time to develop and requires maintenance of a population of memory lymphocytes that can initiate a rapid and massive response upon secondary exposure to a previous infection, and forms the basis of vaccination (dos Santos et al., 2000; dos Santos et al., 2001). Whilst formation of the initial adaptive response is intimately linked to the initial innate inflammatory response, and is consequently reflective of the health of the animal at the time of vaccination, the maintenance of the population of memory cells and subsequent proliferation is dependent upon the health of the animal over the duration of its life. Therefore, any assessment over time of performance of diet in terms of general immunological health should assay both the innate and adaptive responses to challenge. The former will provide indication of current ability to respond, whilst the latter will indicate how well animals have maintained a population of memory B and T lymphocytes over time.

Aim

We aimed to develop a challenge model that could assess if Yellowtail Kingfish (*Seriola lalandi*; YTK) fed different diets showed differeing immune responses, to provide an independent indicator of the effects of dietary substitution on YTK robustness and health.

Materials and Method

Diets, samples and fish

Samples were obtained from the N5/2 trial (3.1.3.1). The control diet was a commercial diet (Ridley Clean Seas Pelagica; 30% wild derived (WD) fish meal (FM) manufactured by Ridley (Narangba, QLD, Australia)). Diet 5 was a 10% WD FM diet with the other 20% replaced with digestible poultry meal protein (= 22.64% dietary inclusion level) manufactured by Skretting Australia (Cambridge, TAS, Australia). Diet 8 was a 10% WD FM diet with the other 20% replaced with digestible FM by-product protein (= 10.70% dietary inclusion level) and digestible soy protein concentrate protein (SPC) (= 10.88% dietary inclusion level) manufactured by Skretting Australia (Cambridge, TAS, Australia). The 10% WD FM diets were chosen as in most cases this level of WD FM substitution with alternative protein sources may be limiting to sub-adult YTK growth (Manuscript 3.1.3.1, this report). The 48 YTK (~2.5 kg fish^{-1}) used in this trial were removed from the 9 month N2/N5 trial after 3 months due to poor performance. The 24 control fish were selected at the same time. Once removed from the N5/N2 trial the fish were held for an additional 4 weeks in a separate system, weighed, measured and tagged and then used for this immune challenge trial.

To infer immune performance, the immune systems of half of the fish fed each diet were challenged by vaccination with a formalin inactivated *Photobacterium damselae piscicida* vaccine (100 µL killed *P. damselae piscicida* 1×10^{16} cells L^{-1} culture) by intraperitoneal injection (12 fish for the control diet; 12 fish for Diet 5; and 12 fish for Diet 8). An identical group of fish from each diet received sterile PBS as a handling control. Injections were performed on YTK anaesthetised with Aqui-S on 18th June 2017. One day post-injection (19th June 2017), six fish per treatment and six fish from handling controls per diet were euthanased with an overdose of anaesthetic (Aqui-S) and blood was sampled by caudal venipuncture. Blood was allowed to clot at 4 °C overnight and serum was collected the following day by centrifugation. From the same fish, pronephros (HK) and spleen were collected by aseptic dissection

and placed in RNAlater on dry ice. The remaining 36 fish were sampled on 7th July (approximately 350 degree days later). Serum samples were retained for enzyme linked immunosorbent assay (ELISA). Tissues samples were retained for RNA extraction and qPCR analysis.

Bacterial culture for vaccination and ELISA

Photobacterium damselae (strain QMA0365) was grown overnight at room temperature (RT) on Tryptone Soy Agar supplemented with 2% w/v NaCl (TSA2) before being inoculated in 10 mL of Tryptone Soy Broth supplemented with 2% w/v NaCl (TSB2) and grown overnight with agitation at 22 °C. The next day, 1% (0.42 mL) of overnight culture was inoculated into 41.58 mL fresh TSB2 and grown with agitation at 22 °C for 18 h. The culture was then cooled rapidly on ice and inactivated with 250 µL of formaldehyde solution (40%) for at least 48 h at 4 °C. Sterility of the culture for vaccination was determined by plating 200 µL aliquots on TSA2 and incubating for 1 week at 22 °C. Absence of colonies was deemed confirmatory of sterility. The inactivated culture was then stored in the dark at 4 °C until required. Cultures for ELISA plate coating were prepared similarly to the culture conditions used for the formulation of the vaccine.

ELISA and plate coating

An aliquot of 10 mL of formalin-killed cells were vortexed and centrifuged at 4 °C, 3,500 rpm for 25 min before the supernatant was removed and the pellet was re-suspended in 10 mL of carbonate-bicarbonate buffer. A 96 well high-binding ELISA plate was coated with the cell suspension in carbonate-bicarbonate buffer at 100 µL per well before being spun for 5 min, 1,500 rpm, 5 acc. 0 br. to put cells in contact with bottom of plate and being incubated overnight at 4 °C. The plates were then dried and frozen upside down at -20 °C until needed.

ELISA protocol

Plates were blocked overnight at 4 °C with 1% BSA (100 µL per well) before being washed three times in Tris-buffered saline 0.05% Tween (TBST) for 5 min. Subsequently, sera (primary antibodies) were added to each well in triplicate at 100 µL/well (diluted 1:32 in TBST) and incubated overnight at 4 °C. Primary antibodies were removed and plates washed three times as described above before the secondary sheep anti-barramundi IgM (1:2,000 in TBST) antibody was added at 100 µL/well and plates incubated for 1 h at RT on the rocker. Following a further three washes in TBST, plates were incubated with 100 µL of donkey anti-sheep IgG alkaline phosphatase conjugate (1:15,000 in TBST) for 1h at RT on the rocker. After a final three washes in TBST and two washes in TBS (to remove the Tween), 50 µL of p-nitrophenyl phosphatase (pNPP) liquid substrate was added to each well and subsequently read at 405 nm every 10 min for 40 min with a BMG Fluostar Optima spectrophotometer.

RNA extraction and cDNA synthesis

RNA was extracted from 144 tissue samples in RNAlater using the Maxwell automated RNA extraction kit and robot (Promega). The RNA was quantified in each sample by Qubit fluorimetry (Thermofisher) and then 200 ng were reverse transcribed using the QuantiTect reverse transcription kit (Qiagen). The resulting cDNAs were stored in aliquots at -20 °C.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Immune response was assessed by quantitative reverse transcription real-time PCR (qRT-PCR) to quantify expression of a cohort of genes involved in the inflammatory response to infection. The mRNA transcripts of three potential internal reference genes were also determined across the template samples to identify the most stable candidate to employ as an internal reference. In this way, we ensured accurate relative quantification of the transcripts of interest (immune-related genes), which cannot be done if the internal references are not first ascertained to be stable across all treatment and control samples.

Primer design

The YTK genome has not yet been published and there is no publically available YTK transcriptome. Consequently, we were unable to design optimal primer sequences against cDNA and gDNA sequences, failing to ensure that the resulting PCR products span across an intron-exon boundary. Therefore, a cohort of ten potential primer pairs were collated from the literature on YTK and other yellowtail species (Table 3.3.2.1.1). These comprised three potential internal reference genes and seven genes from the pro-and anti-inflammatory pathways predominantly found in cells of the monocyte lineage (macrophages, monocytes, DCs and subclasses) and in lymphocytes (B and T cells and subclasses) (Table 3.3.2.1.1).

Primer optimisation

Primers were initially optimised on a blended template for each organ sampled (HK and spleen), comprising equal aliquots of every treatment and control cDNA. First a standard PCR reaction was completed with each primer set at annealing temperatures of 57 °C and 60 °C, and amplicons analysed in 1.2% agarose genes (E-Gel, Thermofisher). Next, serial dilutions of template DNA from head kidney and from spleen were prepared with five concentrations ranging from 0.03125 to 5 ng of cDNA and the reaction efficiency and uniformity of melt-curves determined with each primer set by qRT-PCR (Figures 3.3.2.1.1 and 3.3.2.1.2). Based on these analyses, subsequent qRT-PCR runs were set with an annealing temperature of 57 °C and three potential reference genes (GAPDH, EF1α and β-actin) were included in the experimental design along with four immune genes of interest (IL-1β, IL-8, IL-10 and TNF-α) (Figure 3.3.2.1.2). IFNγ, RAG-1 and RAG-2 were rejected based on low reaction efficiency and/or non-specificity of the reaction (Figure 3.3.2.1.2).

qRT-PCR reactions

Gene expression was quantified in 10 µL reactions using SYBR chemistry on the ViAA7 qPCR systems. Reaction mixtures were prepared with the epMotion 5075 Robot to avoid pipetting errors in 384-well PCR plates, and thermal cycling was performed by the ABI ViiA7. Primer and input cDNA per reaction were optimised at 5 µM and 2 ng, respectively. In each reaction, there was 0.5 µL of forward primer, 0.5 µL of reverse primer, 5 µL of SYBR Green, 2 µL of cDNA (1 ng μL^{-1}), and 2 µL of nuclease free water. Cycling parameters were: 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s, 57 °C for 20 sec and 60 °C for 20 sec, then a final melt curve at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. All temperature cycling was performed with acceleration at 1.6 °C s^{-1}.

Technical C_T values were averaged for each sample after removal of outliers. For each sample, the $2^{\Delta CT}$ was calculated with $\Delta C_T = C_T$ average gene of interest – C_T average reference gene for the same sample. Those values for control and vaccinated fish were then averaged separately from the six biological replicates. Finally, a ratio of the average ($2^{-\Delta CT}$) vaccinated / average ($2^{-\Delta CT}$) control was determined to give the final $2^{-\Delta CT}$ value.

Statistical analysis

For ELISA data, the effect of vaccination over PBS control was determined by two-way analysis of variance (ANOVA) with diet and time as the additional independent variables with intergroup comparisons using the Holm-Sidak t-test method, with alpha = 0.05. Each row was analyzed individually, without assuming a consistent SD.

For qPCR, threshold values for all sample templates derived from the three internal reference primer sets were normalised by geometric averaging and comparison of variance across all samples. Effect of treatment (vaccination) was compared relative to controls (non-vaccinated) for each diet using all biological replicates by randomization and bootstrapping performed in REST (Pfaffl, 2001; Pfaffl et al., 2002). Column graphs were plotted indicating the log (2) relative fold change in gene expression in vaccinated fish relative to non-vaccinated fish from the same diet group (i.e. response to vaccination). Effect of diet in unvaccinated fish was also determined for each organ and time point using REST by comparing experimental diets to the control diet. Column graphs were plotted indicating the log (2) relative fold change in gene expression in experimental diet fish relative to control diet fish from the same time point and organ.

Results

Antibody response to vaccination

In vaccinated fish specific antibody titres were elevated one day post vaccination but decreased by day 19 (Figure 3.3.2.1.3). In YTK fed control diets natural antibody levels in unvaccinated fish were high one day following vaccination, and remained high 19 days post vaccination, with a small increase in mean antibody titre. There were no significant differences in specific or natural antibody levels in control fish.

In fish fed experimental diets, vaccinated fish showed no marked specific antibody response one day post vaccination, but displayed an increase in specific antibody titre 19 days post vaccination, which was more marked for Diet 5 than Diet 8. Antibody levels in unvaccinated fish fed experimental diets were similar to those in fish fed control diets on day 1, but fell over the duration of the trial in fish fed Diet 5 and to a lesser extent in Diet 8 (Figure 3.3.2.1.3).

PCR Optimisation

The melt curves (Figure 3.3.2.1.1 and Figure 3.3.2.1.2) showed that the primers for GAPDH, IL-8, B actin, IL-10, EF1a, TNF-α, IL-1β functioned reliably in head kidney and spleen. Primers for IFNg, RAG1 and RAG2 did not provide homogeneous melt curves (Figure 3.3.2.1.1 and Figure 3.3.2.1.2), indicating that the primers do not match the YTK sequences for these cytokines. The poorly performing cytokine primers were not used in the final analyses of the experimental samples.

Effect of vaccination and diet on immune gene expression

When cytokine expression was analysed pairwise as the difference between unvaccinated and vaccinated fish, changes in expression of IL-1β were not statistically significant. IL-8 was significantly upregulated in spleen in vaccinated control fish on day 1; IL-10 was significantly upregulated in control and experimental fish in head kidney on day 19 and in the spleen of control fish on day 19; and TNF-α was significantly upregulated in control fish in spleen on day 19 (Figure 3.3.2.1.4).

When cytokine expression was analysed as the difference between the control diet and the experimental diets, no significant differences were found. This was largely due to the variability in the effect between samples (Figure 3.3.2.1.5).

Discussion

The experimental diets, where two thirds of the WD FM protein was substituted with either poultry meal or FM by-product and SPC, had a detrimental effect on the immunity of YTK in terms of the way in which the animals responded to vaccination. The levels of natural immunoglobulin fell as specific antibody response rose, suggesting the building blocks for Ig secretion were limiting in fish fed WD FM experimental diets relative to the control diet. Further investigation into the purine and pyrimidine nucleotides required during cell proliferation in the fish may be informative. The qPCR experiment worked well as the inflammatory response proceeded as expected, evidenced by the pro-inflammatory signal seen on day 1 followed by onset of inflammatory regulation. In cytokine production there was also a small but significant influence of diet. Overall, the selected experimental diets appeared to be detrimental to immune function, but were not significantly different to each other.

Natural antibodies are antibodies that are circulating in the animal without any pre-stimulation; these are the primary line of defence in vertebrate innate humoral system, including fish (Coutinho et al., 1995; Casali and Schettino, 1996). Natural antibodies are polyreactive and have low affinity (strength of target binding) (Lalor et al., 1989; Boes, 2000; Katzenback et al., 2013). The polyreactive property of natural IgM allows it to bind to different unrecognised foreign particles and more than one antigen copy at a time (Baumgarth et al., 2005). It triggers preliminary elimination of the potential pathogen, prior to the production of the specific monoreactive antibodies by adaptive immunity (Boes, 2000). Fish are thought to have a high level of circulating natural antibody (Stavnezer and Amemiya, 2004), in part reflecting the nature of the aquatic environment in which they are continuously exposed to potentially pathogenic microorganisms (Gonzalez et al., 1988; Magnadottir et al., 2009). Consequently, the reduction in natural IgM in the YTK fed the experimental diets as specific Ig increases is strongly suggestive of resource limitation for production of the IgM molecule, or maintenance of the B-cells that produce it. It may be that a critical micronutrient is limiting in the experimental diets. The lack of change in specific antibody over time in vaccinated fed the control diet was influenced by high sample variability, including two anomolous fish samples that had high titres 1 day post vaccination. As it is almost impossible to exclude prior exposure to *Photobacterium*, it is possible that these two animals had been recently exposed, but it is difficult to envisage how this could have occurred in the experimental system without some of the other animals being exposed.

Twenty-four hours post-vaccination, the significantly increased IL-8 expression in spleen in fish fed the control diet, but not in fish fed Diets 5 and 8 indicates decreased ability of fish fed experimental diets to stimulate immune cells. By day 19, TNF-α expression was significantly up-regulated in the spleen of control fish, consistent with maturing cells in the secondary lymphoid system, but the lack of this response in fish fed the experimental diets is further evidence of the lack of capacity to stimulate and maintain the immune response in fish fed the experimental diets.

When considering the effects of diet alone on the expression of immune genes there was no statistically significant variations either in spleen or head-kidney (Figure 3.3.2.1.4). The overall patterns are consistent with inflammatory responses to vaccination (DeForge et al., 1993; Lukens et al., 2014). The reversal of IL-8 regulation is likely to have occurred in response to oxidative stress (DeForge et al., 1993), which can be influenced by diet composition (Vetrani et al., 2013). In mice, diets that do not provide enough saturated fats and cholesterol can severely affect microbial communities and significantly increase IL-1β expression, potentially leading to inflammatory diseases (Lukens et al., 2014) but the observations we made do not differentiate between downregulation and the inability of the fish to further produce a particular cytokine. Elevated IL-10 is associated with immunodeficiency and increased allergic reactions (reviewed in Pestka et al., 2004) and the lower levels of IL-10 induced by Diet 8 may have a positive influence on growth relative to Diet 5.

The qPCR method appears to be robust; the internal references were stable over treatments and replicates, and the responses amongst the cytokine genes confirm each other in accordance with

theoretical immune function. This indicates that the data reflect biological reality and that the tool is sensitive to the immune status of the fish. Small changes in signalling molecules can have substantial biological functions and the lack of significance indicates high fish-to-fish variation rather than that the experiment was unsuccessful. A larger sample size for future experiments would be likely to provide better confidence about the results.

Conclusions and Recommendations

The immune challenge model we developed produces a measureable, although variable, immune response in YTK. The variability we observed among control fish shows that attempting to assess nutritional immune status in fish whose immune system has not been stimulated is unlikely to provide useful outputs. While the system we developed requires fish to be captured, challenged, released, maintained and recaptured, providing some limits for its applicability on farms, it provides reliable outputs which are not obtainable from a single measurement approach. We recommend further use and refinement of the challenge model, including understanding the time-temperature relationships between challenge and response for YTK and supplementation of the suite of genes used here, with a larger sample size to reduce population variance and increase statistical power. Measurement of B cells by flow cytometry in experimental fish would provide information about the source of the impaired ability of the fish fed the experimental diets to produce antibodies, as would finer dietary analysis. The combined assays are a very useful tool for assessing the effects of nutrition on fish health status.

Findings

- Vaccination with 100 µL killed *P. damselae piscicida* 1×10^{16} cells L^{-1} culture produced a reliable immune response in YTK
- This immune challenge test provides a viable mechanism for assessing YTK health and robustness, despite high between fish variability
- Measurement of the antibody response showed that fish fed the experimental diets did not maintain their baseline immune response when responding to vaccination
- The experimental diets had a detrimental effect on the immunity of YTK

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project was supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The authors would also like to acknowledge the support of the South Australian Research and Development Institute (SARDI) and Marine Innovation Southern Australia for their financial support of Assoc. Prof. Marty Deveney and the provision of the SARDI South Australian Aquatic Sciences experimental facilities at West Beach, South Australia. We would also like to thank Dr Richard Smullen and Dr Michael Salini of Ridley and Dr Nicole Ruff and Dr Leo Nankervis of Skretting Australia for

their input into experimental diet formulation and manufacture. We also thank Paul Skordas, Assoc. Prof. James Harris, Wayne Hutchinson, Jessica Buss, Nicole Thompson, Samantha Chown, John Faltin, Noah Boldt and Mark Purvis for technical assistance during the experiment.

References

Baumgarth, N., Tung, J.W., Herzenberg, L.A., 2005. Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. Springer Seminars in Immunopathology 26, 347–362.

Boes, M., 2000. Role of natural and immune IgM antibodies in immune responses. Molecular Immunology 37, 1141–1149.

Broomfield, G. R., 2015. Molecular Characterisation of Immune Genes in Yellowtail Kingfish (*Seriola lalandi*) (Thesis, Master of Science (Research) (MSc Research)). University of Waikato, Hamilton, New Zealand.

Casali, P., Schettino, E., 1996. Structure and function of natural antibodies. Current Topics in Microbiology and Immunology Journal 210, 167–179.

Cook, M.T., Hayball, P.J., Hutchinson, W., Nowak, B.F., Hayball, J.D., 2003. Administration of a commercial immunostimulant preparation, EcoActiva(TM) as a feed supplement enhances macrophage respiratory burst and the growth rate of snapper (*Pagrus auratus*, Sparidae (Bloch and Schneider)) in winter. Fish and Shellfish Immunology 14, 333-345.

Coutinho, A., Kazatchkine, M.D., Avrameas. S., 1995. Natural autoantibodies. Current Opinion in Immunology 7, 812–818.

DeForge, L.E., Preston, A.M., Takeuchi, E., Kenney, J., Boxer, L.A., Remick, D.G., 1993. Regulation of interleukin 8 gene expression by oxidant stress. Journal of Biological Chemistry 268, 25568-25576.

Dos Santos, N.M.S., Romano, N., de Sousa, M., Ellis, A.E., Rombout, J.H.W.M., 2000. Ontogeny of B and T cells in sea bass (*Dicentrarchus labrax*, L.). Fish and Shellfish Immunology 10, 583-596.

Dos Santos, N.M.S., Taverne-Thiele, J.J., Barnes, A.C., Ellis, A.E., Rombout, J., 2001. Kinetics of juvenile sea bass (*Dicentrarchus labrax*, L.) systemic and mucosal antibody secreting cell response to different antigens (*Photobacterium damselae* spp. *piscicida*, *Vibrio anguillarum* and DNP). Fish and Shellfish Immunology 11, 317-331.

Gonzalez, R., Charlemagne, J., Mahana, W., Avrameas, S., 1988. Specificity of natural serum antibodies present in phylogenetically distinct fish species. Immunology 63, 31–36.

Jirapongpairoj, W., Kobayashi, K., Fukuda, Y., Takano, T., 2015. Development of consensus qPCR primers to detect cytokine genes in three amberjack species: *Seriola quinquradiata*, *S. lalandi* and *S. dumerili*. Fisheries Science 81, 907-914.

Katzenback, B.A., Plouffe, D.A., Belosevic, M., 2013. Goldfish (*Carassius auratus* L.) possess natural antibodies with trypanocidal activity towards *Trypanosoma carassii* in vitro. Fish and Shellfish Immunology 34, 1025–1032.

Lalor, P.A., Herzenberg, L.A., Adams, S., Stall, A.M., 1989. Feedback regulation of murine Ly-1 B cell development. European Journal of Immunology 19, 507–513.

Lukens, J.R., Gurung, P., Vogel, P., Johnson, G.R., Carter, R.A., McGoldrick, D.J., 2014. Dietary modulation of the microbiome affects autoinflammatory disease. Nature 516, 246-249.

Magnadottir, B., Gudmundsdottir, S., Gudmundsdottir, B.K., Helgason, S., 2009. Natural antibodies of cod (*Gadus morhua* L.): Specificity, activity and affinity. Comparative Biochemistry and Physiology, Part B 154, 306–316.

Muncaster, S., Kraakman, K., Gibbons, O., Mensik, K., Forlenza, M., Jacobsen, G., Bird, S., 2018. Antimicrobial peptides within the Yellowtail Kingfish (*Seriola lalandi*). Developmental and Comparative Immunology 80, 67-80.

Pestka, S., Krause, C.D., Sarkar, D., Walter, M.R., Shi, Y., Fisher, P.B., 2004. Interleukin-10 and related cytokines and receptors. Annual Reviews in Immunology 22, 929-979.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29, e45.

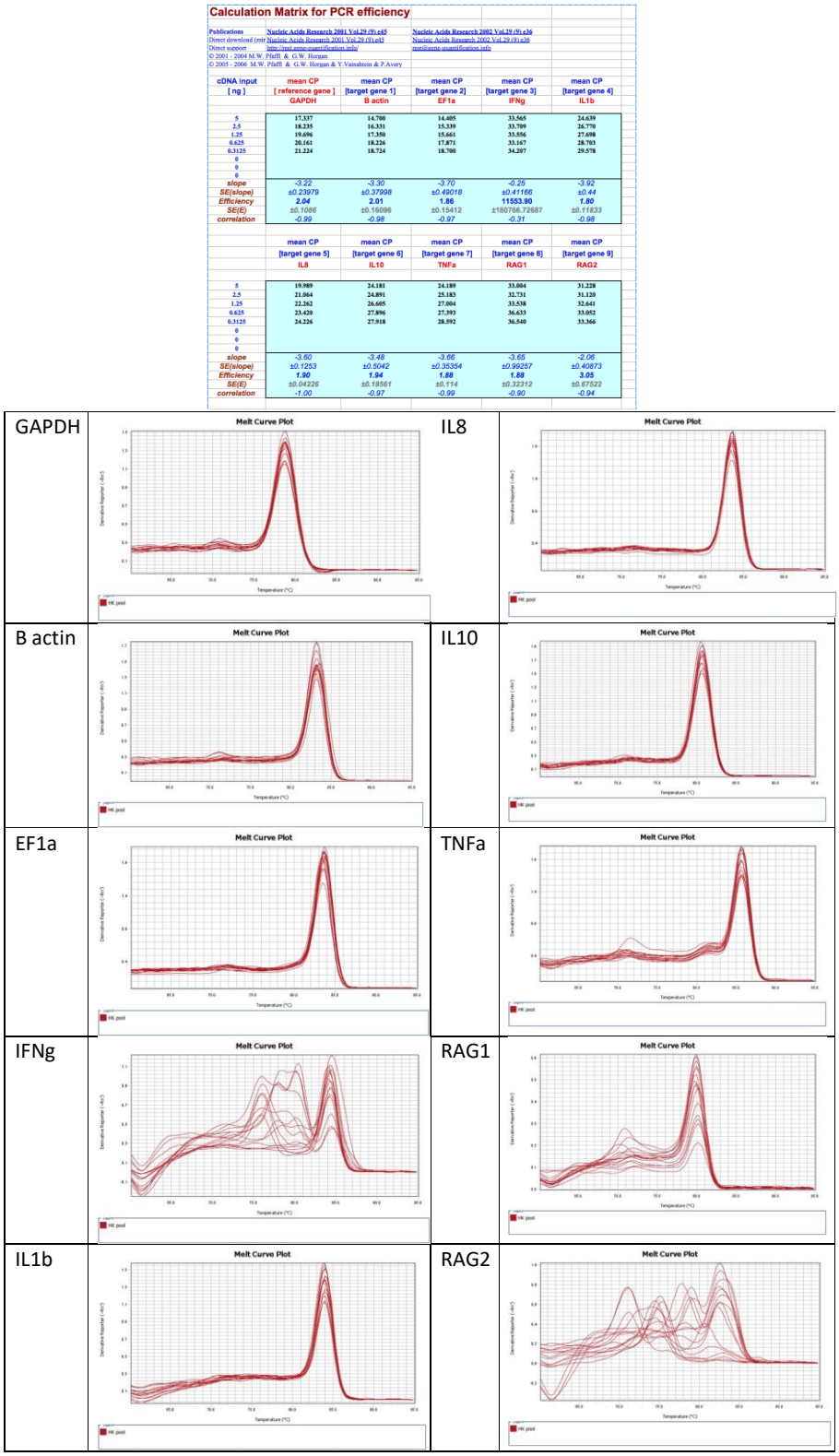
Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research 30, e36.

Stavnezer, J., Amemiya, C.T., 2004. Evolution of isotype switching. Seminars in Immunology 16, 257–275.

Vetrani, C., Costabile, G., Di Marino, L., Rivellese, A.A., 2013. Nutrition and oxidative stress: a systematic review of human studies. International Journal of Food Science and Nutrition 64, 312-326.

Table 3.3.2.1.1. Genes for which primer pairs were collated based on literature.

Gene	Function/cells or tissues producing	Reference
EF1α	Potential internal reference / all tissues	Jirapongpairoj et al., 2015
GAPDH	Potential internal reference / all tissues	Muncaster et al., 2017
β-actin	Potential internal reference / all tissues	Muncaster et al., 2017
IL-1β	Pro-inflammatory mediator of pyroptosis / activated macrophages	Jirapongpairoj et al., 2015
IL-8	Neutrophil chemotaxin / macrophages/endothelium/epithelium	Jirapongpairoj et al., 2015
IL-10	Down-regulator of inflammation / many tissues	Jirapongpairoj et al., 2015
TNF-α	Pro-inflammatory /macrophages and monocytes	Jirapongpairoj et al., 2015
IFNγ	Activator of macrophages, NK and B cells / Th1, Tc, macrophages	Jirapongpairoj et al., 2015
RAG-1	Ig variable recombination / B-lymphocytes	Broomfield, 2015
RAG-2	Ig variable recombination / B-lymphocytes	Broomfield, 2015



Calculation Matrix for PCR efficiency

Publications	Nucleic Acids Research 2001 Vol.29 (9) e45	Nucleic Acids Research 2002 Vol.29 (9) e36
Direct download (mir	Nucleic Acids Research 2001 Vol.29 (9) e45	Nucleic Acids Research 2002 Vol.29 (9) e36
Direct support	http://rest.gene-quantification.info/	rest@gene-quantification.info
© 2001 - 2004 M.W. Pfaffl & G.W. Horgan		
© 2005 - 2006 M.W. Pfaffl & G.W. Horgan & Y.Vainshtein & P.Avery		

cDNA input [ng]	mean CP [reference gene] GAPDH	mean CP [target gene 1] B actin	mean CP [target gene 2] EF1a	mean CP [target gene 3] IFNg	mean CP [target gene 4] IL1b
5	17.337	14.700	14.405	33.565	24.639
2.5	18.235	16.331	15.339	33.709	26.770
1.25	19.696	17.350	15.661	33.556	27.698
0.625	20.161	18.226	17.871	33.167	28.703
0.3125	21.224	18.724	18.700	34.207	29.578
0					
0					
0					
slope	-3.22	-3.30	-3.70	-0.25	-3.92
SE(slope)	±0.23979	±0.37998	±0.49018	±0.41166	±0.44
Efficiency	2.04	2.01	1.86	11553.90	1.80
SE(E)	±0.1686	±0.16696	±0.15412	±180766.72687	±0.11833
correlation	-0.99	-0.98	-0.97	-0.31	-0.98

	mean CP [target gene 5] IL8	mean CP [target gene 6] IL10	mean CP [target gene 7] TNFa	mean CP [target gene 8] RAG1	mean CP [target gene 9] RAG2
5	19.989	24.181	24.189	33.004	31.228
2.5	21.064	24.891	25.183	32.731	31.120
1.25	22.262	26.605	27.004	33.538	32.641
0.625	23.420	27.896	27.393	36.433	33.052
0.3125	24.226	27.918	28.592	36.540	33.366
0					
0					
0					
slope	-3.60	-3.48	-3.66	-3.65	-2.06
SE(slope)	±0.1253	±0.5042	±0.35354	±0.99257	±0.40873
Efficiency	1.90	1.94	1.88	1.88	3.05
SE(E)	±0.04226	±0.18561	±0.114	±0.32312	±0.67522
correlation	-1.00	-0.97	-0.99	-0.90	-0.94

Figure 3.3.2.1.1. Quantitative polymerase chain reaction (qPCR) optimisation diagram showing melt curves for PCR product from head kidney of sub-adult YTK.

Calculation Matrix for PCR efficiency

cDNA input [ng]	mean CP [reference gene] GAPDH	mean CP [target gene 1] B actin	mean CP [target gene 2] EF1a	mean CP [target gene 3] IL1b	mean CP [target gene 4] IL8
5	18.703	15.427	15.920	25.737	21.427
2.5	19.744	16.553	16.867	26.632	22.450
1.25	20.720	17.343	17.866	27.650	23.577
0.625	21.718	18.377	18.899	29.042	24.511
0.3125	22.595	19.479	19.783	29.669	25.467
0					
0					
0					
slope	-3.24	-3.30	-3.24	-3.41	-3.37
SE(slope)	±0	±0.08117	±0	±0.16853	±0
Efficiency	2.03	2.01	2.03	1.96	1.98
SE(E)	±0	±0.03451	±0	±0.07312	±0
correlation	-1.00	-1.00	-1.00	-1.00	-1.00

	mean CP [target gene 5] IL10	mean CP [target gene 6] TNFa	mean CP [target gene 7] RAG1	mean CP [target gene 8] IFNg	mean CP [target gene 9] RAG2
5	22.886	23.310	37.002	32.698	33.325
2.5	23.612	23.917	36.965	33.224	32.477
1.25	24.844	24.710	36.226	32.638	33.520
0.625	25.744	25.017	37.589	34.685	33.025
0.3125	26.777	26.918		34.707	34.560
0					
0					
0					
slope	-3.29	-2.76	-0.34	-1.82	-1.00
SE(slope)	±0.11473	±0.45391	±0.66432	±0.65328	±0.69398
Efficiency	2.01	2.30	874.02	3.54	9.92
SE(E)	±0.049	±0.31513	±11568.04334	±1.60899	±15.75409
correlation	-1.00	-0.96	-0.24	-0.84	-0.62

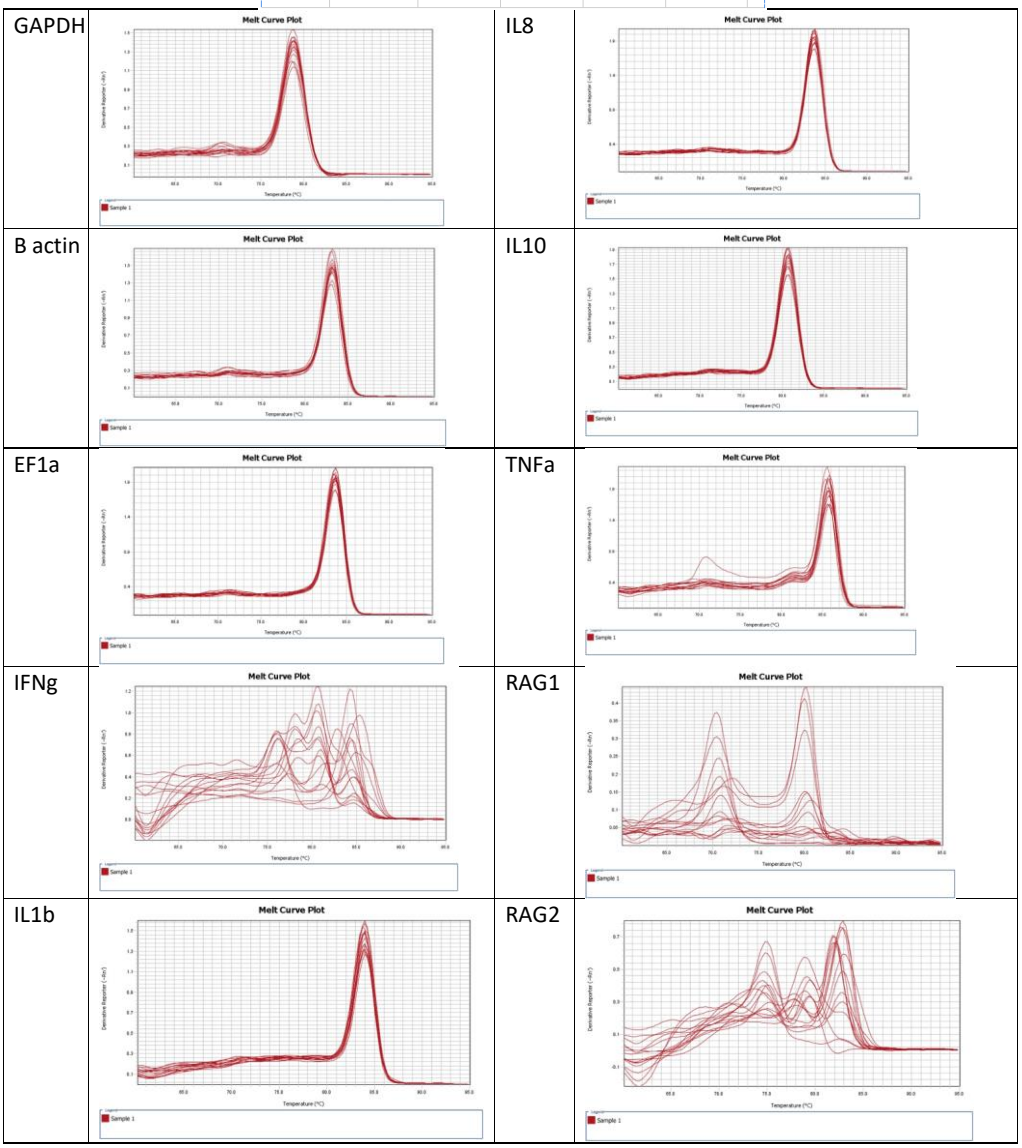


Figure 3.3.2.1.2. Quantitative polymerase chain reaction (qPCR) optimisation diagram showing melt curves for PCR product from head kidney and spleen.

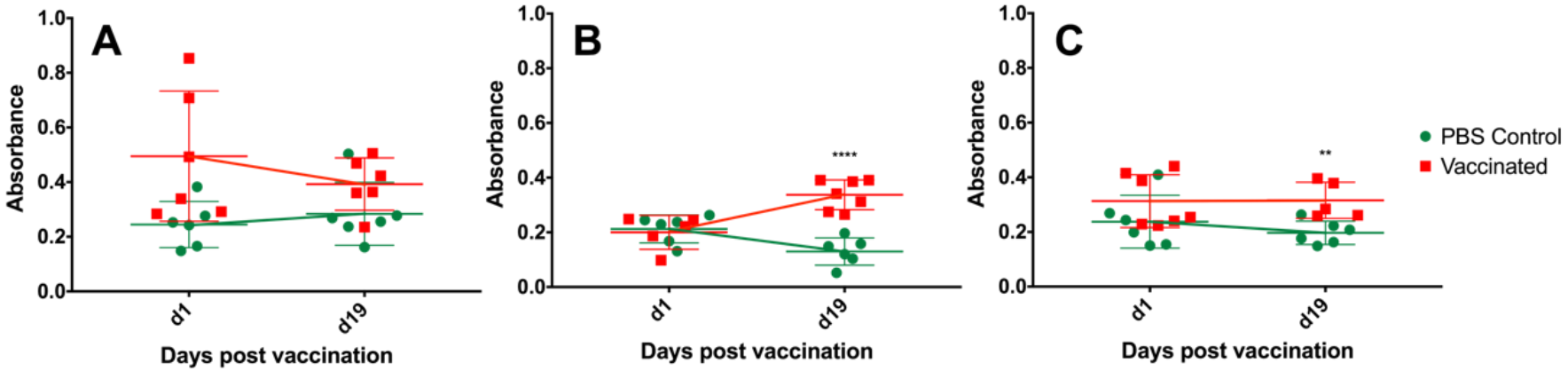


Figure 3.3.2.1.3. Antibody titres (mean ± SD) from sub-adult YTK determined by whole-cell ELISA for control diet (A), Diet 5 (B) and Diet 8 (C). Non-vaccinated fish (PBS controls) are represented in green and fish vaccinated with *P. damselae* are represented in red. Significant differences using Holm-Sidak t-test between PBS control and vaccinated are represented by ** for $P < 0.01$ and **** for $P < 0.001$.

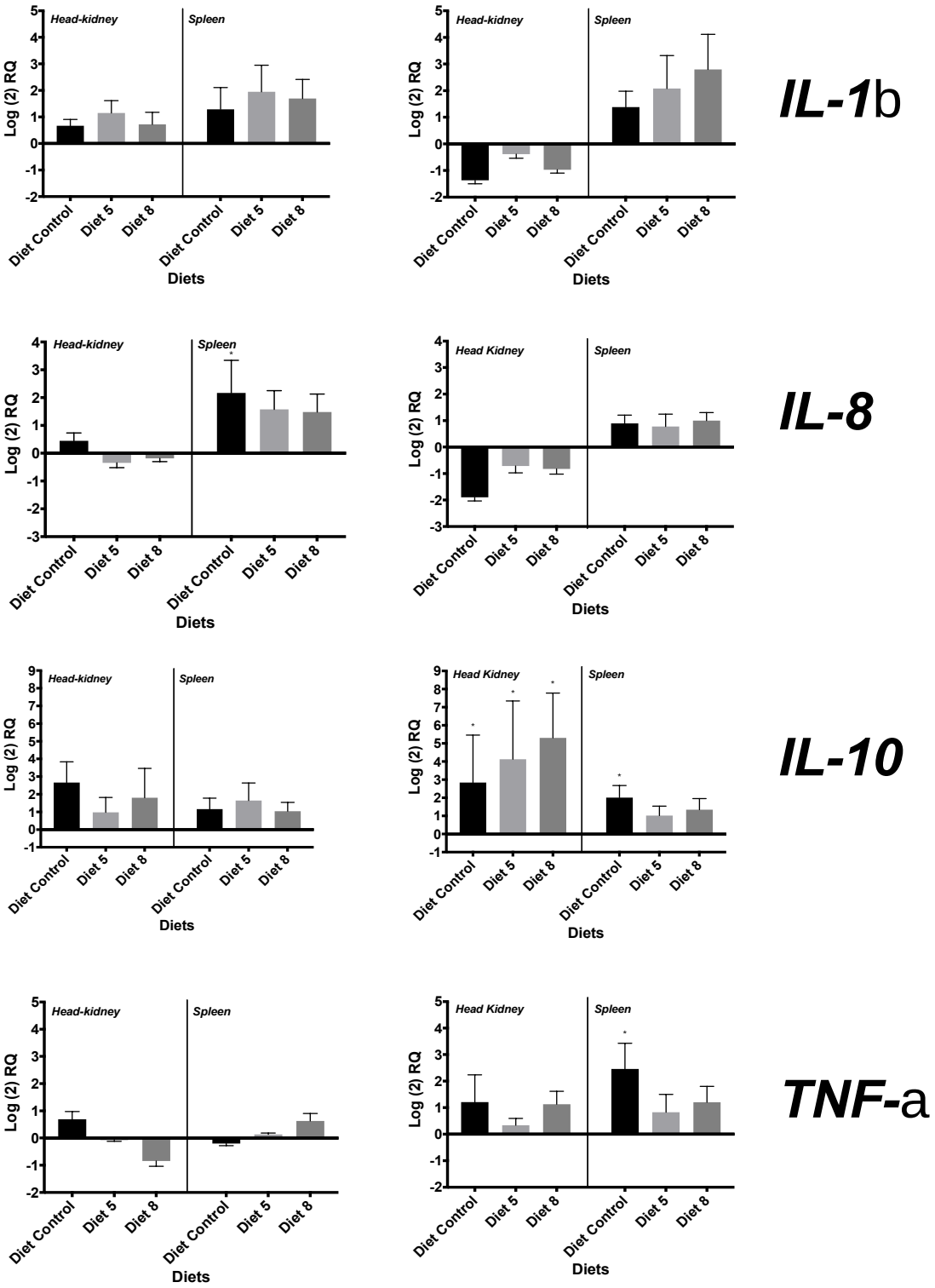


Figure 3.3.2.1.4. Log (2) fold change in expression of immune gene transcripts in head kidney and spleen of vaccinated sub-adult YTK relative to unvaccinated (control) fish fed control and experimental diets. Samples collected 24 h (left) and 19 days (right) post-vaccination. (Mean ± SD, n = 6). Data analysed with REST with *: $P < 0.05$ for the difference between vaccinated and unvaccinated fish.

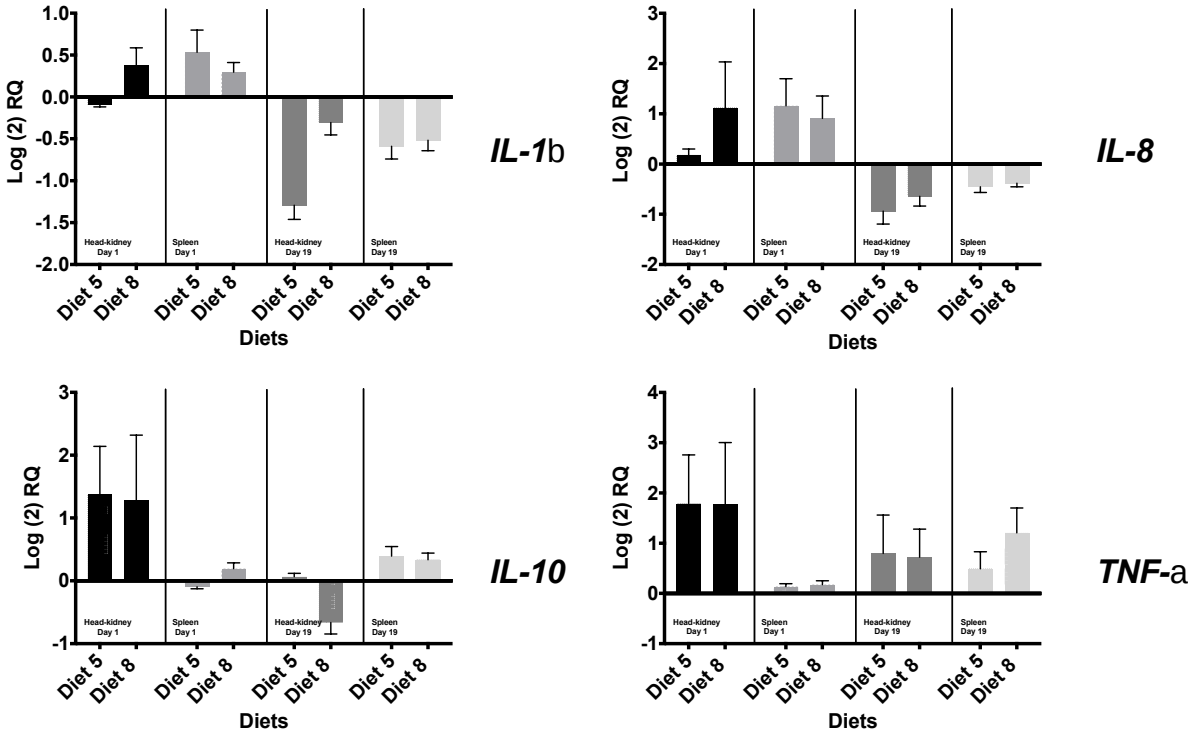


Figure 3.3.2.1.5. Log (2) fold change in expression of immune gene transcripts in head kidney and spleen of sub-adult YTK fed Diet 5 and 8 compared to the control diet (Mean ± SD, *n* = 6) for vaccinated fish. Data analysed with REST showed no statistically significant differences.

3.3.2.2. Manuscript - Improved treatment efficacy and surveillance for skin and gill flukes of Yellowtail Kingfish.

Marty R Deveney[a], Matthew Landos[b], Matthew S. Bansemer[a]

[a] South Australian Research and Development Institute, South Australian Aquatic Sciences Centre, West Beach, SA 5024, Australia

[b] Future Fisheries Veterinary Services Pty Ltd, East Ballina, NSW, 2478.

This manuscript may be referenced as: Deveney. M.R., Landos, M. and Bansemer, M.S. (2019). 3.3.2.2. Manuscript – Improved treatment efficacy and surveillance for skin and gill flukes of Yellowtail Kingfish (Output 4a). In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2019, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.775-781.

Abstract

Skin and gill flukes remain problematic in Yellowtail Kingfish (*Seriola lalandi*; YTK) aquaculture, and their management incurs substantial costs. Novel treatments are required, but incremental improvement of current approaches can also decrease management costs and increase flexibility. We found that bathing in 2.5 mg L^{-1} praziquantel for 30 min was > 99% effective against gill flukes and 100% effective against skin flukes, and provides a viable alternative to hydrogen peroxide. We also found that a standard statistical approach accurately describes the sample size required to provide a given level of confidence about estimates of fluke abundance given some population data. These data provide improvement and better flexibility in fluke management approaches for YTK.

Introduction

Management of skin (*Benedenia seriolae*) and gill (*Zeuxapta seriolae*) flukes causes substantial increases in production cost for the Yellowtail Kingfish (*Seriola lalandi*; YTK) aquaculture industry. In South Australia, management of gill flukes is more problematic than management of skin flukes. Existing management relies on bathing fish in hydrogen peroxide (H_2O_2) (HP), but poorly managed HP exposure can cause mass fish mortalities, particularly if the dose of HP is miscalculated or the oxygen supply during the bath is insufficient for the biomass. At low temperatures, furthermore, HP becomes less effective and the margin between the effective dose and the dose where HP toxicity to the fish narrows. In vitro exposure to 1 mg L^{-1} praziquantel ((RS)-2-(Cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a]isoquinolin-4-one) (CAS 55268-74-1) was 100% effective against skin flukes *in vitro* (Hirazawa et al.., 2013), and Sharp et al. (2004) found that 2.5 mg L^{-1} praziquantel was > 99% effective against skin and gill flukes, but exposures > 30 to 45 min are impractical for sea-cage use.

Accurate monitoring of parasite intensity on fish in sea-cage production systems is essential for informed treatment decisions and appropriate parasite control (Whittington, 2005). Treatment is triggered by assessing fluke burdens, but some aspects of fluke monitoring need validation or refinement to provide confidence in assessment of infestation, efficacy assessment and development of treatment triggers. Caraguel et al. (2015) evaluated the precision, accuracy and bias of industry assessments of gill and skin fluke intensity in a YTK sea-cage population and found that a major management gap was understanding sample size for certainty about fluke abundances. Choices in industry about sampling are, however, largely made based on convenience, and a practical method for understanding the accuracy of sampling for fluke burdens, the first step in making decisions about treatment, is lacking.

Aim

We aimed to assess the efficacy of immersion treatments of praziquantel for treating skin and gill flukes following the World Association for the Advancement of Veterinary Parasitology (WAAVP) guideline for testing the efficacy of ectoparasiticides of fish (Sommerville et al., 2016). We also aimed to better understand methods to calculate a required sample size to achieve a certain level of confidence for fluke abundance.

Materials and Method

Fish and treatment

Sub-adult YTK (1-4 kg) infected with skin and gill flukes were brought to SARDI as part of the K4P project. Praziquantel (99.9% powder) was obtained from Ningbo Samreal Chemical Co. (Ningbo, People's Republic of China) and assessed for purity by Redox Pty Ltd (Minto, NSW, Australia). Based on an unpublished pilot study by Williams et al. (2007), we dissolved 35 g praziquantel L^{-1} in propylene glycol (Propane-1,2-diol) (CAS 57-55-6) (Redox, Minto NSW). Doses were based on measurement of tank volume and calculated as an amount of 35 g L^{-1} solution, but are described here in terms of doses in mg L^{-1}.

On arrival at SARDI, ten fish were euthanased (overdose of Aqui-S) and seven days later a further five fish were euthanased for a general health check. All fish were checked under a stereomicroscope for the enumeration of skin and gill flukes. These fluke data were used as a basis for power analysis and other analyses to determine sample sizes.

Treatment

The designated amount of praziquantel in propylene glycol was added to a bucket of water and mixed into the treatment tank. Further mixing was achieved by aeration and movement of the fish. Sample sizes were based on power analysis of skin and gill fluke abundance, assuming an effect size of 80%. Six fish with a mean weight of 1.2 ± SEM 0.2 kg (Range 0.975 to 1.4 kg) were randomly assigned to three treatments and exposed to 2.5 mg L^{-1} praziquantel for 30 min. A further six fish were added to three identicle control tanks that underwent the same mixing process and received the corresponding amount of propylene glycol without praziquantel. After treatment, fish were returned to a new tank, run on flow through. The next day, they were sampled for flukes.

Fluke sampling

Fish for fluke assessment were acclimated at SARDI for 8 weeks, then captured from their tank by net and transferred to an inflatable bath containing 1,000 L of anaesthetic (7.5 mg $.L^{-1}$ Aqui-S®, Aqui-S New Zealand Ltd) until anaesthesia reached the stage characterised by lateral recumbency and slowing of opercular movement (Sharp, 2004). Anaesthetised fish were transferred to a 50 L tank containing 5 mg L^{-1} praziquantel in seawater for 10 min, then to a 50 L bath containing deionised water for 5 min. The praziquantel bath dislodges gill flukes and the freshwater bath dislodges the skin flukes. The bath solutions were filtered through a 40 µm mesh sieve to collect dislodged flukes. Flukes were counted individually per fish. This protocol was shown to be effective by Chambers and Ernst (2005), Mooney et al. (2006) and Williams et al. (2007). The filtrate was transferred into 70 mL screw top sample containers for subsequent pooled microscopic counting.

When YTK were needed to survive the experiments, they were checked for flukes, transferred from the freshwater bath to a recovery tank until they were assessed as having recovered from anaesthesia, and released back into another tank.

Treatment

The designated amount of praziquantel in propylene glycol was added to a bucket of water and mixed into the treatment tank. Further mixing was achieved by aeration and movement of the fish. Sample sizes were based on power analysis of skin and gill fluke abundance, assuming an effect size of 80%. 120 fish with a mean weight of 1.2 ± SEM 0.2 kg (Range 0.975 to 1.4 kg) randomly assigned to three treatment and three control 5,000 L tanks were exposed to 2.5 mg.L^{-1} praziquantel for 30 min. Control groups underwent the same mixing process and received the corresponding amount of propylene glycol without praziquantel. After treatment, fish were returned to a new tank, run on flow through. The next day, they were sampled for flukes.

Efficacy statistics

Normality of the data was tested using the Shapiro-Wilk test and variances were tested using Levene's test. Data that did not conform to homoscedasticity were log (y + 1)-transformed, where y is parasite abundance, prior to analysis. Differences in mean abundance between treatment groups were analysed using one-way ANOVA. Where significant differences were detected, post-hoc comparisons were made via Tukey's tests. SPSS 20 statistical analysis software was used to analyse the data and significance for all tests was judged at $P < 0.05$.

During in vivo trials, the efficacy of each treatment was assessed as a percentage reduction in mean skin and gill fluke abundance, relative to the control groups, and was calculated by adapting the formula of Stone et al. (1999) given below:

$$\%\, efficacy = 100 - \left(100 \times \frac{mean\ parasite\ intensity\ of\ treatment}{mean\ parasite\ intensity\ of\ control}\right)$$

Understanding the accuracy of abundance estimates

A variety of methods were trialled for understanding the relationship between variability in fluke abundance and sample size, including modified hypergeometric exact, simple binomial, and formula-based sample size calculations. Assessments were made using FreeCalc (Ausvet Services, 2018) based on a series of fluke burden counts from this study and field assessments of individual fish made since 2000 on commercial YTK farms in Australia.

Results

Efficacy

Mean abundance of *B. seriolae* was was 22.2 ± 6.7 (15 - 68) in the control groups and 0 (0 - 0) in fish treated with 2.5 mg praziquantel L^{-1} for 30 min, which was significantly different between groups (one-way ANOVA; $F_{3,12} = 16.513$; $P < 0.001$). Efficacy against *B. seriolae* was 100%.

Mean abundance of *Z. seriolae* was 105.6 ± 29.4 (26 - 413) in the control groups and 2 ± 2.1 (0 - 9) in fish treated with 2.5 mg Praziquantel L^{-1} for 30 min, which was significantly different between groups (one-way ANOVA; $F_{3,52} = 51.524$; $P < 0.001$). Efficacy against *Z. seriolae* was 98.1%. All flukes remaining after treatment were immature.

Fluke counts

All subsampling methods tested provided estimates of sample sizes within ± 1 SE of actual counts of 100 fish. On this basis we chose the simplest approach, based on the formula from Cochran (1977):

$$n = \left(\frac{(t_{\alpha/2})\, s}{B}\right)^2$$

Where $t_{\alpha/2}$ is the t-value at n-1 degrees of freedom, s is the population standard deviation, and B is the acceptable margin of error. The formula assumes the sample is random or each sample is as independent from each other as they are from the total population, and that the population is either normally distributed or $n \geq 30$. We built a tool in Microsoft Excel using this formula (Figure 3.3.2.2.1) which can accept up to 1,000 sample values and includes a histogram in the sheet for quick visual assessment of normality.

Discussion

Immersion treatment using Praziquantel is effective against skin and gill flukes. Bathing fish however, remains operationally challenging, labour intensive and can be ineffective if managed inappropriately (Whittington and Chisholm, 2008). Australian YTK farms, however, have equipment suitable for bathing and experience in applying immersion treatments. Praziquantel immersion treatment, furthermore, can be used as an alternative to HP. The principal problem with HP treatment is oxidative toxicity to the fish, which, along with efficacy, varies with dose, time and temperature (Rach et al., 2007). Hydrogen peroxide appears to be most toxic to YTK in winter during periods of low water temperatures (unpublished data), and although the limits of dose and duration are not defined for YTK, praziquantel is a useful addition to the treatment capacity of Australian YTK farms.

The gill flukes that were not detached from the fish by treatment were all small juveniles. This has been observed in other monogeneans (Chisholm, 2002; Forwood et al., 2013a), and occurs because the small parasites remain between the secondary gill lamellae and are not exposed to the treatment. Treating monogeneans requires timed repeat treatments (Tubbs et al., 2005), and that some parasites are likely to be juvenile survivors of treatment should be taken into account when planning strategic treatments, rather than assuming that all parasites will be post-treatment recruits.

The best sampling approaches for YTK flukes to inform treatment remain unknown. The sample size formula we assessed provides a number of fish to provide a pre-determined confidence about the accuracy of an estimate from a sample with respect to the whole population of fish, but only after the variability in the fluke population is characterised, or relative to a model or standard population. Understanding how to sample to estimate fluke burdens from a sample of fish is always problematic. Further refinement of approaches using a model similar to that developed by Shaw et al. (2005), or the statistical approach developed by Forwood et al. (2013b), could provide a better method for understanding fluke abundance from a subsample.

Conclusions and Recommendations

Praziquantel is an effective immersion treatment for skin and gill flukes. 2.5 mg L^{-1} praziquantel for 30 min is an effective dose and duration for practical use in sea cages and hatcheries. This dose and duration should be assessed for field use and incorporation into an Australian Pesticides and Veterinary Medicines Authority (APVMA) Minor Use Permit for this product and pattern of use.

The statistical approach outlined provides a method for understanding the robustness of sampling and understanding the accuracy of estimates of fluke populations from a subsample of fish. Further refinement using modelling or statistical approaches, and populated by a range of data sets from field assessment of flukes, would provide an improved method for understanding fluke abundance and lead to better decision making about treatment.

Findings

- Immersion treatment in 2.5 mg L^{-1} praziquantel for 30 min is > 90% effective against skin and gill flukes of YTK.

- The statistical approach outlined provides a method for understanding of the robustness of sampling and understanding the accuracy of estimates of fluke populations from a subsample of fish.

Publications

No publications have resulted from this R&D to date.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The authors would also like to acknowledge the support of the South Australian Research and Development Institute (SARDI) and Marine Innovation Southern Australia for their financial support of Assoc. Prof. Marty Deveney and the provision of the SARDI South Australian Aquatic Sciences experimental facilities at West Beach, South Australia. We would also like to thank Paul Skordas, Assoc. Prof. James Harris, James Forwood, Jessica Buss, John Faltin, Noah Boldt and Mark Purvis for assistance.

References

Ausvet Services, 2018. http://epitools.ausvet.com.au/content.php?page=FreeCalc2

Caraguel, C., Fensham, J., Bubner, E., Landos, M., D'Antignana, T. 2015. Evaluation of sampling strategies to assess skin and gill fluke intensity in farmed Yellowtail Kingfish. In FRDC Seafood CRC 2013/731: Improving fluke control in Yellowtail Kingfish culture.

Chambers, C.B., Ernst, I. 2005. Dispersal of the skin fluke Benedenia seriolae (Monogenea: Capsalidae) by tidal currents and implications for sea-cage farming of *Seriola* spp. Aquaculture 250, 60-69.

Chisholm, L.A. 2002. Efficacy of Praziquantel Bath Treatments for Monogenean Infections of *Rhinobatos typus*. Journal of Aquatic Animal Health, 14, 230-234.

Cochran, W.G. 1977. Sampling Techniques (3rd edition). John Wiley &Sons, New York.

Forwood, J.M., Harris, J.O., Deveney, M.R. 2013a Efficacy of bath and orally administered praziquantel and fenbendazole against *Lepidotrema bidyana* Murray, a monogenean parasite of silver perch, *Bidyanus bidyanus* (Mitchell). Journal of Fish Diseases, 36, 939-947.

Forwood, J.M., Harris, J.O., Deveney, M.R. 2013b. Validation of a rapid counting method for assessing treatment efficacy against *Lepidotrema bidyana* infecting silver perch *Bidyanus bidyanus*. Diseases of Aquatic Organisms, 105, 253-257.

Hirazawa, N., Akiyama, K., Umeda, N. 2013. Differences in sensitivity to the anthelmintic praziquantel by the skin-parasitic monogeneans *Benedenia seriolae* and *Neobenedenia girellae*. Aquaculture, 404, 59-64.

Mooney, A.J., Ernst, I., Whittington, I.D. 2006. An egg-laying rhythm in *Zeuxapta seriolae* (Monogenea: Heteraxinidae), a gill parasite of yellowtail kingfish (*Seriola lalandi*). Aquaculture 253, 10-16.

Sharp, N.J., Diggles, B.K., Poortenaar, C.W., Willis, T.J. 2004. Efficacy of Aqui-S, formalin and praziquantel against the monogeneans, *Benedenia seriolae* and *Zeuxapta seriolae*, infecting yellowtail kingfish *Seriola lalandi lalandi* in New Zealand. Aquaculture 236, 67-83.

Shaw, J.C., Aguirre-Macedo, L., Lafferty, K.D. 2005. An efficient strategy to estimate intensity and prevalence: sampling metacercariae in fishes. Journal of Parasitology, 91, 515-21.

Sommerville, C., Endris, R., Bell, T.A., Ogawa, K., Buchmann, K., Sweeney, D. 2016. World association for the advancement of veterinary parasitology (WAAVP) guideline for testing the efficacy of ectoparasiticides for fish. Veterinary Parasitology, 219, 84-99.

Stone, J., Sutherland, I.H., Sommerville, C.S., Richards, R.H., Varma, K.J. 1999. The efficacy of emamectin benzoate as an oral treatment of sea lice, *Lepeophtheirus salmonis* (Kroyer), infestations in Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 22, 261-270.

Tubbs, L.A., Poortenaar, C.W., Sewell, M.A., Diggles, B.K. 2005. Effects of temperature on fecundity in vitro, egg hatching and reproductive development of *Benedenia seriolae* and *Zeuxapta seriolae* (Monogenea) parasitic on yellowtail kingfish *Seriola lalandi*. International Journal for Parasitology, 35, 315–327.

Williams, R.E., Ernst, I., Chambers, C.B., Whittington, I.D. 2007. Efficacy of orally administered praziquantel against *Zeuxapta seriolae* and *Benedenia seriolae* (Monogenea) in yellowtail kingfish *Seriola lalandi*. Diseases of Aquatic Organisms, 77, 199–205.

Whittington, I.D. 2005. Monogenea Monopisthocotylea (ectoparasitic flukes). In: Marine Parasitology. Rohde K. (Ed.), pp. 63–72. CSIRO Publishing, Melbourne, Australia.

Whittington, I.D., Chisholm, L.A. 2008. Diseases caused by Monogenea. In: Fish Diseases Volume 2. Eiras, J.C., Segner, H., Wahlii, T., Kapoor B.G. (Eds.), pp. 683–816 (Chapter 13). Science Publishers, Inc., New Hampshire, USA.

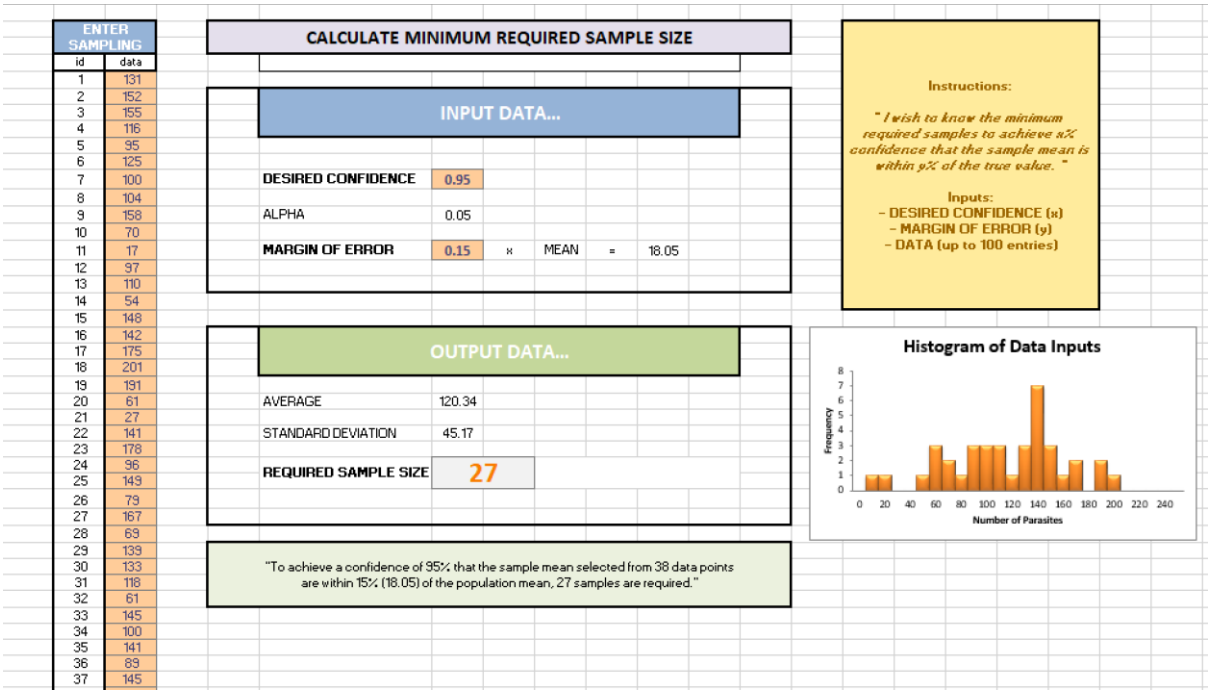


Figure 3.3.2.2.1. Screen shot of sample size calculator.

4. Student Activities

4.1. Introduction

This project included a commitment to engage three Postdoctoral Fellows (or equivalent), and up to six PhD students and 12 Honours students. The three Postdoctoral Fellows were employed early in the project and were associated with a much of the research undertaken and its dissemination in a wide range of forums. The six PhD students were all engaged within the first two years of the project and have contributed to the core research (those associated with NSW DPI) and/ or broadened the scope and depth of research possible (those associated with SARDI). Four Honours student were engaged, with three achieving 1st Class Honours, and two MSc students. Most Postdoctoral Fellows and Honours, Masters and PhD students participated in the three personal development workshops arranged by this project (K4P) and the four annual K4P Research Workshops.

4.2. Students

4.2.1. Honours

Mr Jackson Doherty (Flinders University, SA) - (achieved 1st)

Mr Leigh Kuerschner (Flinders University, SA) - (achieved 2A)

Mr Thibault Legrand (University of Adelaide, SA) - (achieved 1st)

Mr Aaron Teoh (Flinders University, SA) - (achieved 1st)

4.2.2. MSc

Ms Caroline Candebat (Macquarie University, NSW) - (completed)

Ms Marina Rubio (Wageningen University and Research Centre, Holland) – (MSc research intern activity completed)

4.2.3. PhD

Ms Caroline Candebat (James Cook University, QLD)

Ms Dam Thi My Chinh (University of the Sunshine Coast, QLD)

Ms Samantha Chown (University of Adelaide, SA)

Mr Ben Crowe (Flinders University, SA)

Mr Thibault Legrand (University of Adelaide, SA)

Ms Angela Liu (University of New South Wales, NSW)

4.2.4. Postdoctoral Fellowships (or equivalent)

Dr Sarah Catalano (SARDI Aquatic Sciences)

Dr Matthew Bansemer (SARDI Aquatic Sciences)

Dr Igor Pirozzi (New South Wales DPI)

4.3. Student Project Summaries

4.3.1. Jackson Doherty, Honours

Affiliation: Flinders University, College of Science and Engineering

University supervisors: Assoc. Prof. James Harris and Assoc. Prof. Catherine Abbott (Flinders University, College of Science and Engineering)

Industry supervisors: Assoc. Prof. David Stone and Dr Matthew Bansemer (SARDI Aquatic Sciences)

Honours thesis title: Digestive enzyme activity of Yellowtail Kingfish (*Seriola lalandi*) in response to different feeding strategies

K4P project link: This Honours project was linked to the K4P project Feeding Strategy theme (Output 3b) and Manuscript 3.2.3.2: Effect of feeding frequency on the growth and feed utilisation for large Yellowtail Kingfish (*Seriola lalandi*) at warm water temperatures.

Research aims and objectives

Research was designed to quantify the relationship between the feeding frequency and relative digestive efficiency and subsequent enzymatic profiles within Yellowtail Kingfish (YTK) cultured in controlled conditions to represent commercial feeding scenarios.

Specific research aims included:

1. Investigating the digestive efficiency of adult YTK via specific digestive enzyme activity (trypsin, lipase and dipeptidyl peptidase-4 [DPP4]) in response to increasing levels of feed intake and split-rationing representing commercial feeding frequencies.
2. Evaluating for the first time in YTK, the distribution of specific DPP4 activity within the membrane and cytoplasm of gastrointestinal tissues, as well as in blood serum.

Trypsin, lipase and DPP4 were measured within four regions of the gastrointestinal tract (pyloric caecae, foregut, midgut and hindgut), and DPP4 was also measured in blood plasma to determine the physiological response to these feeding strategies.

Research outcomes

The primary findings from this study included:

- Neither lipase or trypsin activity within the gastrointestinal tract was significantly affected by feeding treatment ($P > 0.05$).
- Lipase and trypsin activities were significantly higher in the pyloric caecae than posterior regions of the gastrointestinal tract ($P < 0.01$).
- Trypsin activity had a positive correlation with important growth parameters, including feed intake ($P = 0.018$, $r = 0.757$) and feed conversion ratio ($P = 0.043$, $r = 0.682$), corroborating findings from other studies involving YTK.
- DPP4 was measured in adult YTK for the first time and was shown to significantly increase as feed intake increased with feeding treatment ($P < 0.049$).

- The physiological distribution of DPP4 activity within the gastrointestinal tract was highest in the pyloric caecae (6.12 nm U mg protein^{-1}) and lowest in the middle of the gastrointestinal tract (0.44 nm U mg protein^{-1}).
- Cytoplasmic DPP4 activity was not significantly different between any gastrointestinal sections within any feeding treatment ($P > 0.076$) and DPP4 activity within blood serum was not significantly affected by feeding treatment ($P > 0.666$).
- Recorded activities of DPP4 were highest in the membrane-bound component of gastrointestinal tissues and lowest in the blood serum of YTK.
- Cytoplasmic DPP4 activity was negatively correlated with specific growth rate ($P = 0.006$, $r = -0.824$) (warrants further investigation into role of DPP4 in regulating bioactive peptides).

This research advanced our understanding of the physiological responses of cultured YTK within the context of commercially relevant feeding strategies. All enzyme activities, with the exception of cytoplasmic DPP4, were found to be higher in the pyloric caecae than other regions of the gastrointestinal tract and blood serum. This suggests further enzymology studies should target this area in the future. This study also provided new insight into YTK digestion by investigating the digestive distribution of DPP4 within cytoplasmic and membrane-bound components of the gastrointestinal tissues of YTK for the first time.

Benefits gained by student

This project provided many new and exciting experiences for myself in numerous areas important to the commercial production process for YTK. Some of these new experiences included taking part in health checks, sample taking and regular weigh and measures, as well as helping with the operation and maintenance of the SARDI pool-farm facility. During these experiences I was also able to further my understanding of the systems and technologies utilised by large scale experimental trials.

The friends and contacts within the industry I made by undertaking this project have also been one of the greater benefits to myself, in retrospect. Seeing the organisation, commitment and passion of other scientists and students towards this project was vital to me shaping and fine-tuning my own project and research style. The many discussions with other students about their projects have further intensified my interest in numerous other areas within aquaculture, as it seems there is still much to learn.

Whilst undertaking my Honours study as part of a larger experimental and industry supported project seemed daunting at first, I know that the experience I have gained and contacts I have made during this process will hold me in good stead for a future career in higher research or aquaculture.

Project progress and thesis citation

The project was completed in June 2018. The Honours student graduated with 1st Class Honours.

Honours thesis citation: Doherty, J.G., 2018. Digestive enzyme activity of Yellowtail Kingfish (*Seriola lalandi*) in response to different feeding strategies, Honours Thesis, Flinders University, South Australia, Australia. 74 pp.

Acknowledgements

This project was supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

Thanks also to Dr Leo Nankervis and Dr Nicole Ruff of Skretting Australia for input into experimental design, diet formulation and diet manufacture. We would also thank Dr Richard Smullen and Dr Michael

Salini of Ridley and Dr Trent D'Antignana for input into experimental design and diet formulation. I wish to offer my sincerest gratitude to my supervisors Assoc. Prof. James Harris, Assoc. Prof. David Stone, Assoc. Prof. Catherine Abbott and Dr Matthew Bansemer for their guidance and support throughout the year. I would also like to thank the staff at the SARDI Aquatic Science Centre for the use of their facilities and the hardworking team at the SARDI pool-farm (Paul Skordas, Leigh Kuerschner, Mark Purvis, Ben Crowe, Krishna-Lee Currie, Jessica Buss, Filipa Isabel Neto Duarte, Nicole Thomson, and Thibault Legrand) who always made working on this project entertaining and whose advice was highly valuable and most appreciated. I would also like to thank the AJ and AM Naylon Honours Scholarship (Flinders University) for providing me with financial support. Finally, I wish to thank my family and friends, whose support was invaluable.

4.3.2. Leigh Kuerschner, Honours

Affiliation: Flinders University, College of Science and Engineering

University supervisor: Assoc. Prof. Kathy Schuller (Flinders University, College of Science and Engineering)

Industry supervisor: Assoc. Prof. David Stone (SARDI Aquatic Sciences)

Honours thesis title: Effect of feed restriction and re-feeding on mitochondrial activity in Yellowtail Kingfish, *Seriola lalandi*

K4P project link: This Honours project was linked to the K4P project Feeding Strategy theme (Output 3B) and Manuscript 3.2.3.1: Optimising feeding strategies for Yellowtail Kingfish (*Seriola lalandi*) at winter water temperatures.

Research aims and objectives

The overall objective of this project was to examine the effects of feed restriction and apparent satiation feeding regimes on muscle growth and mitochondrial abundance in Yellowtail Kingfish (YTK) at winter water temperatures. Mitochondrial abundance was measured by assaying selected mitochondrial enzymes of importance.

The specific aims of this project were:

1. To investigate the effects of feed restriction on mitochondrial metabolic activity and mitochondrial abundance in YTK red muscle, white muscle and liver tissues. Two treatments from Manscript 3.2.3.1 were examined in the study:

    - Treatment 1 (satiation): Formulated diet fed to apparent satiation six days week^{-1}.
    - Treatment 7 (restricted): Formulated diet fed at 0.12% body weight (BW) six days week^{-1}.

    Treatments were investigated by analysing the enzyme activity for the enzymes citrate synthase (CS) and cytochrome *c* oxidase (COX). CS is a key regulatory enzyme in the citric acid cycle in the mitochondria and COX is a complex of the mitochondrial electron transport chain within the mitochondrial inner membrane. Both enzymes were investigated as indicators of mitochondrial metabolic activity and mitochondrial abundance. It was predicted that feed restriction would increase mitochondrial enzyme activity, indicating an increase in mitochondrial abundance in YTK red muscle, white muscle and liver tissue. If there was an increase in mitochondrial abundance under restricted feeding conditions, this would indicate that the fuel reserves in the body (fatty acids) were being utilised as a source of energy. If this was the case then it is likely that these fish would also have a reduction in growth or even a weight loss.

Research outcomes

The primary findings and outcomes from this study included:

- Citrate synthase activity in red and white muscle was not significantly affected by feeding regime. However, CS activity increased in the liver for YTK when fed a restricted ration. In contrast, COX enzyme activity decreased in the red muscle, remained constant in the white muscle and increased in the liver when fed a restricted ration.
- Results indicate that feed restriction resulted in an increase in mitochondrial metabolic activity in the liver. Providing an indication that fish were utilising fuel reserves in addition to feed as an energy source, contributing to weight loss.

Benefits gained by student

While undergoing my study I gained vital knowledge in the areas of research such as how to design and run a project, complete fieldwork and collect samples for analysis. Prior to completing this study, I had only been involved in the technical side of running a study including feeding the YTK and maintaining water quality. Gaining an insight into the steps necessary for designing of a study gave me more appreciation for my supervisors and how difficult it can be to get a project up and running. After collecting the samples for the study, I had to learn the correct techniques for molecular biology sampling and enzyme assay methodology which I had very limited experience with. I learnt techniques from other students and adapted them to complete the laboratory work for the study. Similarly, I increased my knowledge of statistical analyses throughout the study by conversing with other students and gained a higher understanding of what the data indicates.

Project progress and thesis citation

This Honours project was completed in June 2016. The Honours student graduated with 2A Class Honours.

Honours thesis citation: Kuerschner, L., 2016. Effect of feed restriction and re-feeding on mitochondrial activity in Yellowtail Kingfish, *Seriola lalandi.* Honours Thesis. Flinders University, South Australia, Australia. 77 pp.

Acknowledgements

This project was supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

To my university supervisor Assoc. Prof. Kathy Schuller, thank you for the opportunity and privilege to work with you during this study. Your guidance and patience encouraged me throughout this study, giving me confidence in my limited ability of an area of science unfamiliar to me at the beginning of my honours. It has been an honour to work under you during this study. To my industry supervisor Assoc. Prof. David Stone, thank you for giving me the push I needed to get into honours. I thank you for also funding my project via SARDI SAASC, Clean Seas Seafood and the Australian Seafood CRC, and it has been a pleasure to work under you during this study. Thanks also to Dr Matthew Bansemer, Paul Skordas and Krishna Lee Currie for their assistance with running the experiments and sample collection at the SARDI SAASC. To Arif Malik at Flinders University, thank you for all your guidance with lab protocols and assistance. Being a novice in molecular biology at the beginning of my honours, your patience and simple teaching techniques were a large part of the completion of my honours. Your knowledge was invaluable and was appreciated greatly. Also your sense of humour kept things interesting throughout the study and broke up the times when problems occurred. To Sarmad Al-Asadi at Flinders University, thank you for your knowledge and patience when helping me with the statistical

analyses. To fellow Honours students, Ben Crowe and India Attwood-Henderson, thank you for being relaxed and comforting when times went south. Studying together since undergraduate level, you build up solid friendships that you don't take for granted. Your motivation in your own projects rubs off on others and gave me the confidence to complete this study.

4.3.3. Thibault Legrand, Honours

Affiliation: Flinders University, College of Science and Engineering

University supervisor: Prof. Jian Qin (Flinders University, College of Science and Engineering)

Industry supervisors: Dr Andrew Oxley and Assoc. Prof. David Stone (SARDI Aquatic Sciences)

Honours thesis title: The inner workings of the outer surface: mucosal barrier bacterial assemblages as indicators of changing health status in Yellowtail Kingfish, *Seriola lalandi*

K4P project link: This Honours project represents an extension of activities as part of Nutritional Health Theme Task H3: Elucidate the role of the gut microbiome in Yellowtail Kingfish gastrointestinal health; and aligns with Output 4d: Collect baseline data to differentiate the effects of the environment, Yellowtail Kingfish growth and farm production cycle, disease and different genetic cohorts on the microbiome; and Output 4b: Collect histopathology and blood chemistry data of diseased and healthy Yellowtail Kingfish to characterise the general health of Yellowtail Kingfish used in tank based nutrition and feeding strategy R&D.

Research aims and objectives

The mucosal surfaces and associated microbiota of fish are an important primary barrier and provide the first line of defence against potential pathogens. An understanding of the skin and gill microbial assemblages and the factors which drive their composition may provide useful insights into the broad dynamics of fish host-microbial relationships, and may reveal underlying changes in health status. This is particularly pertinent to cultivated systems whereby various stressors may led to conditions (like enteritis) which impinge on productivity. As an economically important species, this honours project assessed whether the outer-surface bacterial communities reflect a change in gut health status of cultivated Yellowtail Kingfish (YTK, *Seriola lalandi*). The specific aims of the project were to: 1) Elucidate the normal skin and gill mucosal microbiota of YTK; 2) Assess whether the outer mucosal surface bacterial communities reflect changes in gut health, and 3) Identify possible bacterial biomarkers that may be used as indicators for underlying chronic conditions in YTK.

Research outcomes

This project identified core and variable bacterial assemblages across the outer (skin and gill) mucosal surfaces of YTK in health and during early and late stage enteritis. Specifically, *Proteobacteria* and *Bacteroidetes* were predominant in both the skin and gills, with enrichment of key *β-proteobacteria* in the gills (*Nitrosomonadales* and *Ferrovales*). Fish exhibiting early stage chronic lymphocytic enteritis comprised markedly different global bacterial assemblages compared to those deemed healthy and exhibiting late stages of the disease. This corresponded to an overall loss of diversity and enrichment of *Proteobacteria* and *Actinobacteria*, particularly in the gills. In contrast, bacterial assemblages of fish with late stage enteritis were generally similar to those of healthy individuals, though with some distinct taxa. In conclusion, gut health status is an important factor which defines the skin and gill bacterial assemblages of fish and likely reflects changes in immune states and barrier systems during the early onset of conditions like enteritis. Potential biomarkers for early detection of gut enteritis were also revealed and included the *Proteobacteria:Bacteroidetes* ratio, and a few members of the *α-proteobacteria*, *γ-proteobacteria* and *Actinobacteria* phyla.

Benefits gained by student

The benefits gained by the student in this project included significant contribution to his professional development and learning. In this project, the student was able to learn state-of-the-art (next generation sequencing, NGS) methodologies as an approach for assessing host-microbiota relationships and aquatic animal health. Specific skills obtained from the project included: 1) Sample collection and handling procedures for molecular and histological analyses; 2) Methods for the extraction of nucleic acids and the preparation 16S rRNA gene amplicon libraries; 3) Post-processing of sequencing data, including the ability to access and interrogate public data repositories for assigning taxonomy and depositing sequences; 4) Conducting and interpreting multivariate sequence datasets; 5) Participating in industry forums, workshops and national conferences (including a poster presentation at the inaugural *Australian Microbial Ecology Conference*, AusME 2017); and 6) Completion of a Honours dissertation (Class I) and a peer-reviewed publication in the prestigious journal *Frontiers in Microbiology* (https://doi.org/10.3389/fmicb.2017.02664).

Project progress and thesis citation

This Honours project was awarded 1st Class Honours and was completed in November 2016.

Honours thesis citation: Legrand, T., 2016. Effects of health status on the microbiome of the skin and gill mucosa of Australian Yellowtail Kingfish. Honours Thesis (1st Class). Flinders University, South Australia, Australia. 53 pp.

Acknowledgments

This project was supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The student also wishes to acknowledge Flinders University and SARDI Aquatic Sciences (Molecular Sciences Subprogram) for supporting his candidature; and Dr Andrew Oxley, Prof Jian Qin and Assoc. Prof David Stone for their mentorship. The student also kindly thanks Dr Fran Stephens for assisting with the histological analyses, and Dr Sarah Catalano and Dr. Melissa Wos-Oxley for guiding the laboratory and statistical components of the project. Finally, a warm thanks is extended to Clean Seas Seafood for supporting the project and kindly providing samples.

4.3.4. Aaron Teoh, Honours

Affiliation: Flinders University, College of Science and Engineering

University supervisor: Prof. Graham Mair and Assoc. Prof. James Harris (Flinders University, College of Science and Engineering)

Industry supervisor: Assoc. Prof. David Stone (SARDI Aquatic Sciences)

Honours thesis title: Physiological response to stress in Yellowtail Kingfish (*Seriola lalandi*) at different oxygen levels under summer temperatures

K4P project link: This Honours project was linked to the K4P project Feeding Strategy theme (Output 3a) and Manuscript 3.2.1.1: Intermittent feed-induced hypoxia effects the growth and feed utilisation of large Yellowtail Kingfish at warm water temperatures.

Research aims and objectives

Currently, there is a lack of published literature on the effects of intermittent hypoxia on the physiological stress responses and dietary nutrient digestibility in Yellowtail Kingfish (YTK). The overall objective of this project was to examine the effects of long term feed induced intermittent hypoxia on the physiological stress responses and dietary nutrient digestibility in YTK. In addition, the effects of short term acute repeated stressing events on the physiological stress responses in YTK that had been exposed, long term, to feed induced intermittent hypoxia were also investigated. This simulated anthropogenic stressing events that the fish may experience on-farm culture conditions such as grading or weight checks.

Research outcomes

The primary findings and outcomes from this study included:

- An insight into digestibility and physiological stress response of YTK subjected to long term feed induced intermittent hypoxia and repeated anthropogenic stressors. Nutrient digestibility tended to be compromised by long-term intermittent hypoxia, however, this was not significantly different between treatments. Results from the physiological response analysis indicated that YTK were unable to acclimatise to the physiological stress of irregular hypoxia/re-oxygenation challenges as indicated by significantly higher oxidative stress and significantly lower antioxidant activity compared to other treatments. Mitochondrial abundance may have decreased as a physiological response to reduce oxidative capacity when challenged with long-term intermittent hypoxia, however this was not significantly different between treatments.
- An insight into the effects of repeated handling stress which significantly increased the oxidative stress in the liver of YTK exposed to hypoxia/re-oxygenation but not in the treatment representative of culture conditions without oxygen fluctuations. However, there were no synergistic effect of repeated handling stress and intermittent hypoxia. GPx activity, a key antioxidant enzyme, was unchanged in hypoxia/re-oxygenation challenged fish but was significantly decreased in fish with constant oxygen saturation. This emphasises the important of understanding the overall antioxidant response, more research in this area is warranted.

The oxygen fluctuations in this study were not as harsh as situations experienced in South Australian YTK sea-cage culture conditions, which may have a more prominent effect on digestibility and physiological stress response. It would be beneficial in future studies to perform on-farm studies at multiple potential site locations to investigate the effects of real life oxygen fluctuations on digestibility and physiological stress response in YTK.

Benefits gained by student

Aaron graduated from Flinders University in 2016 with 1st Class Honours for his project. Aaron plans to make a significant contribution towards a manuscript arising from Manuscript 3.2.1.1 of this report. Aaron will be a co-author on said manuscript. Aaron has completed his project and is currently self-employed and was not available to make comment in this section.

Project progress and thesis citation

This Honours project was awarded 1st Class Honours and was completed in December 2016.

Honours thesis citation: Teoh, A.Y.L., 2016. Effects of feed induced intermittent hypoxia on digestive physiology and stress response in Yellowtail Kingfish, *Seriola lalandi*. Honours Thesis. Flinders University, South Australia, Australia. 86 pp.

Acknowledgements

This project was supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The author also wishes to acknowledge Professor Graham Mair, Associate Professor David Stone, Associate Professor Kathy Schuller, Associate Professor James Harris, Dr Matthew Bansemer, Krishna-Lee Currie, Paul Skordas, Arif Malik, Samad Al-Asadi, Leigh Kuerschner, Jess Buss and Nicole Thompson for their academic, technical, and statistical assistance.

4.3.5. Caroline Candebat, MSc

Affiliation: University of Hamburg, Germany in collaboration with Macquarie University, Australia

University supervisors: Prof. Dr Angelika Brandt (University of Hamburg) and Dr Jane Williamson (Macquarie University)

Industry supervisors: Dr Igor Pirozzi and Dr Mark Booth

Master thesis working title: Effect of lipid sources and temperature on the critical oxygen level and routine metabolic rate of juvenile Yellowtail Kingfish (*Seriola lalandi*)

K4P project link: This Masters project is linked to the K4P Project Feeding strategies Theme (Output 3c) and Manuscript 3.2.2.3, which details research to elucidate the critical oxygen threshold in juvenile Yellowtail Kingfish.

Research aims and objectives

Low concentrations of dissolved oxygen are one of the first limiting abiotic factors in aquaculture and mariculture systems, impacting the health and wellbeing of target-species. Yellowtail Kingfish (YTK, *Seriola lalandi*; YTK) are a high energy demand species and commercial aquaculture is rapidly expanding globally, yet, no information on the hypoxia tolerance for this species is available. YTK aquaculture is commonly carried out in sea pens, in which abiotic factors such as ambient temperature and oxygen can fluctuate substantially, with oxygen in particular becoming critically limiting. YTK diets contain relatively high levels of crude fat. The move away from marine fish oils to terrestrial oil sources in aquafeeds implies a change in intake of dietary fatty acid (FA) profiles. This shift in dietary FA concentration can impart physiological effects impacting on the stress tolerance of the animal.

My Master's project assessed the hypoxia tolerance in YTK with respect to temperature and dietary lipid source. Additionally, observations on visual and behavioural patterns were made with respect to progressive hypoxia exposure. Results on the hypoxia tolerance and behavioural changes provide essential species-specific information for the YTK industry, emphasising the importance of adequate DO levels in farm settings and contributing to risk management for farm managers. My Masters project focused on the following:

1) Determine the critical oxygen threshold ([02]crit) of YTK, acclimated to two different temperature regimes (15 °C and 20 °C) and dietary lipid sources (fish and poultry oil).
2) Assess the visual and behavioural changes in YTK when exposed to progressive hypoxia.

Research outcomes

The study confirms that YTK is hypoxia sensitive but regulates the oxygen consumption to 1.84 – 2.24 mg oxygen L^{-1} (22 - 38% saturation), which strongly depends on the acclimation temperature. Warmer acclimation temperatures led to less hypoxia tolerance while colder temperatures resulted in a higher degree of hypoxia tolerance. Dietary oil source had no significant effect on the critical oxygen threshold, even though YTK fed a poultry-oil based diet showed a strong deviation in routine metabolic rate and $[O2]_{crit}$. The first behavioural response exhibited by YTK when passing the $[O2]_{crit}$ threshold is exaggerated gulp ventilation, then mouth breathing on the surface, and operculum movements, followed by a visual change in coloration. Rapid re-oxygenation of the system is critical at this behavioural change as further responses such as burst swimming quickly lead to the final stages of hypoxia.

Benefits gained by student

The K4P project gave me the opportunity to grow professionally by developing existing skills, learning new skills, meet other professionals, take on challenges, enhance my presentation skills and gain work experience. Most importantly was the financial support which led to an experiment with a meaningful research outcome. The direct supervision of Dr Igor Pirozzi and Dr Mark Booth taught me more about fish bioenergetics, measuring and analytical methods and how to apply these. Additionally, I have learned how to produce aquafeed pellets and how to maintain a RAS. The annual K4P Research Workshop participation taught me a valuable lesson on how to present clearly to an aquaculture industry audience.

Project progress and thesis citation

- Project completed.
- Master of Science thesis 2017: "Effect of lipid sources and temperature on the critical oxygen level ($_{Pcrit}$), hypoxia tolerance and routine metabolic rate of juvenile Yellowtail Kingfish (*Seriola lalandi*) completed.
- Manusript 3.2.2.3 of this report: "The critical oxygen threshold and hypoxia tolerance of Yellowtail Kingfish (*Seriola lalandi*)" completed.
- Manuscript in preparation: Candebat, C., Booth, M., Williamson, J., Pirozzi, I. (In prep.) The critical oxygen threshold and hypoxia tolerance of Yellowtail Kingfish (*Seriola lalandi*).

Acknowledgements

I am extremely grateful to the following groups for funding my research; the Australian Government Department of Agriculture and Water Resources, the Fisheries Research and Development Corporation (FRDC), the South Australian Research and Development Institute (SARDI), Clean Seas Seafood, the New South Wales Department of Primary Industries (NSW DPI) and Huon Aquaculture.

Ridley (Dr Michael Salini) and Skretting Australia have also contributed actively to my project through the input of technical information and the provision of experimental feed ingredients and feeds. I would also like to thank the University of Hamburg and Macquarie University for their collaborative work and for sending me to Australia to develop my professional skills. I would like to thank my current primary James Cook University supervisor Dr Igor Pirozzi and NSW DPI industry supervisor Dr Mark Booth for their patience and guidance throughout the K4P project. I would also like to thank my ex-supervisors, Prof. Angelika Brandt and Dr Jane Williamson, for their collaborative work. I am grateful that I was a team member of the nutrition team at the Port Stephens Fisheries Institute (PSFI) and I would like to thank Dr Basseer Codabaccus, Ian Russell, and Brendan Findlay for their technical assistance. My sincere thanks also go to Dr Wayne O'Connor, who was my first correspondence with the K4P project and who forwarded me to my current supervisors.

Special thanks go also to Jodie Rummer from Centre of Excellence for Coral Reef Studies, James Cook University, who contributed adequate equipment for the oxygen measurements (FireSting O2 Units from Pyroscience).

My biggest thanks go to my family in Germany: My mother and sister who supported me spiritually but also helped me rehearse my presentations from time to time. In closing I would like to personally thank the wider Project Group for providing me with the opportunity to pursue a Master thesis. I am hopeful my research will make a valuable contribution to the development of the Australian YTK industry.

4.3.6. Marina Rubio Benito, MSc intern placement (May-August 2016)

Affiliation: Wageningen University, Aquaculture and Fisheries. The Netherlands

University supervisors: Prof. Dr Johan Verreth

Industry supervisors: Dr Igor Pirozzi and Dr Mark Booth

Major thesis working title: The effect of dissolved oxygen and feed intake on Yellowtail Kingfish (*Seriola lalandi*) growth and performance

K4P project link: Feeding strategies Theme (Output 3c) and Manuscript 3.2.2.1.

Research aims and objectives

Fluctuations in environmental conditions may affect abiotic factors which can potentially show a negative impact on Yellowtail Kingfish (YTK, *Seriola lalandi*) sea-cage aquaculture. Dissolved oxygen (DO) is a critical factor as important processes like nutrient metabolism require oxygen uptake. Knowledge on feeding requirements based on FCR and utilization efficiency of nutrients when abiotic conditions vary is valuable information to optimise YTK feed management. By feeding various ration levels it is possible to assess the individual effect of DO on nutrient utilization efficiencies across the range of feeding levels.

The aim of this study was to determine the effect of DO concentration and feed intake on YTK growth and FCR. Two DO saturation regimes (~100%) and (~60%) were selected to reflect the concentrations that can be encountered during fish farming operations.

Research outcomes

Low DO at 60% saturation negatively affected the nutrient and energy utilisation efficiencies in YTK with this response tending to be more pronounced with increasing nutrient and energy intake. However DO did not significantly affect feed intake. This study provides insight into the effects of abiotic factors on the nutritional physiology of YTK. Data generated from this study will be used to improve feed models for YTK facilitating better feed management and feed formulation through a better understanding of the influence of abiotic conditions on nutrient demand and utilisation.

Benefits gained by student

Skills in aquaculture research including planning and running a manipulative experiment with juvenile YTK. Fish husbandry, handling and anesthetisation and managing a recirculating aquaculture system (RAS). Experience with working as part of a research team and more broadly an important industry related National project. Experience with the tools of aquaculture nutrition research including feed making, fish feeding, statistics and report writing.

Project progress and thesis citation

- Project completed.

- This project contributes towards Manuscript 3.2.2.1. "Protein, amino acid and energy utilisation and maintenance requirements of juvenile Yellowtail Kingfish (*Seriola lalandi*): quantifying abiotic influences."

Acknowledgements

The Australian Government Department of Agriculture and Water Resources, the Fisheries Research and Development Corporation (FRDC) funded this project. Special thanks are to NSW DPI for providing me with the opportunity to do my internship at an internationally recognised aquaculture institute. Special thanks to Dr Mark Booth and Dr Igor Pirozzi for sharing their knowledge and providing me the opportunity to share in the research and to complete this report. Also thanks to Ian Russell and Brendan Findlay for their technical assistance. Dr. Stewart Fielder and the marine fish team at PSFI produced the YTK used in this study. Ridley contributed YTK feeds for this project. Wageningen University, Aquaculture and Fisheries, The Netherlands provided the networking opportunity and funding for this internship placement.

4.3.7. Caroline Candebat, PhD

Affiliation: James Cook University

University supervisors: Dr Igor Pirozzi and Prof. Dean Jerry

Industry supervisors: Dr Mark Booth

PhD thesis working title: Sulfur amino acid requirements in Yellowtail Kingfish (*Seriola lalandi*): optimizing aquafeeds for the Australian Yellowtail Kingfish industry

K4P project link: This PhD project is linked to the K4P Project Nutrition Theme (Output 2d) and Chapter 3.1.5, which details research to elucidate the sulfur amino acid requirements of juvenile Yellowtail Kingfish.

Research aims and objectives

My PhD project will determine requirements, interactions and modulations on regulatory biosynthetic mechanisms of sulfur amino acids; specifically methionine, cysteine and taurine. Results will improve the formulation of aquafeeds resulting in more efficient utilization of nutrients and will increase industry profitability and sustainability. My PhD is focused on the following;

1) Investigate sulfur amino acid requirements and precursor effects of sulfur amino acids in sub-adult Yellowtail Kingfish (YTK).
2) Map the regulative mechanisms of taurine metabolism.
3) Evaluate the effect of sulfur amino acid deficiencies on the health of fish.

Research outcomes

To date, I have completed two major practical experiments. The first experiment determined the taurine requirement of juvenile YTK and the second experiment determined the methionine requirement of juvenile YTK. Both experiments significantly increased our understanding of taurine and methionine utilisation and their interactions with other important amino acids such as cysteine. Preliminary recommendations on suitable dietary amounts of taurine and methionine have been made to our industry partners. I am now completing my research at James Cook University by examining specific enzymes involved in taurine metabolism such as cysteine dioxygenase (CDO) and cysteinesulfinate decarboxylase (CSD).

Benefits gained by student

The K4P project gave me the opportunity to grow professionally by developing existing skills, learning new skills, meet other professionals, take on challenges, enhance my presentation skills and gain work experience. Most importantly was the financial support which led to two successful experiments, which I will be able to include in my PhD thesis and will help me to develop my career in fish nutrition. Additionally, this project finances further work on the sulfur amino acid metabolism in YTK through research conducted at James Cook University College of Public Health, Medical and Veterinary Sciences at the proteomics facilities in collaboration with Dr Andreas Lopata and Elicia Johnston.

This project has also helped me develop my English skills in an Australian research environment and gave me a better understanding of how research is conducted outside of Germany. Dr Mark Booth and Dr Igor Pirozzi played a significant role by teaching me a set of new skills such as modelling data, formulating experimental diets, producing aquafeed pellets, maintaining good water quality in a recirculating aquaculture system (RAS), designing experiments, analysing collected data for standard values, how to collect blood and faecal samples from fish. The Yellowtail Kingfish Health Training Workshop, organised by Steven Clarke, taught me more about the anatomy, histology and microbiology of YTK and how to interpret them. Participation at the annual K4P Research Workshops, and personal development workshops, and attendance at an international conference taught me valuable lessons on how to present clearly and confidently. Participating at an international conference also gave me the opportunity to meet fish nutritionists and other experts and professionals from the aquaculture industry, expanding my professional network and advertising myself.

Project progress and thesis citation

Benchtop studies related to K4P project data are being completed at James Cook University (Townsville Campus). PhD thesis preparation and development of draft manuscripts on the first two requirement studies is underway. The PhD is due for completion in 2020.

Acknowledgements

I am extremely grateful to the following groups for funding my research; the Australian Government Department of Agriculture and Water Resources, the Fisheries Research and Development Corporation (FRDC), the South Australian Research and Development Institute (SARDI), Clean Seas Seafood, the New South Wales Department of Primary Industries (NSW DPI) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to my project through the input of technical information and the manufacture of experimental feeds.

Foremost, I would like to thank the people from whom I learned the most during this project: My industry supervisor Dr Mark Booth and my primary university supervisor Dr Igor Pirozzi. I am very grateful for their guidance, patience, great knowledge and continued support I received by working with them on the K4P project. My sincere thanks also go to Dr Wayne O'Connor, who was my first correspondence with the K4P project and who forwarded me to my current supervisors.

I would also like to thank the nutrition team at Port Stephens Fisheries Institute (PSFI) for their technical assistance. I am very grateful that I was part of a team that was very committed to get all the experiments done. My special thanks from the team go to Dr Basseer Codabaccus, Luke Vandenberg, Ian Russell, Brendan Findlay and Justin Tierney for giving me not only information on YTK, but also guidance, commitment, motivation, team spirit and coffee sessions.

Special thanks to Dr Michael Salini from Ridley for providing many of the raw material used in the experimental diets and for organizing the rather spontaneous K4P gathering at the International Symposium on Fish Nutrition and Feeding in Spain.

I thank Dr Fran Stephens for interpretation and advice of histological samples. I also thank Barney Hines (CSIRO) for conducting biochemical analyses of my experiment samples.

I would also like to thank James Cook University for awarding me with the 'Postgraduate Research Scholarship' providing me with food, a place to live and a waiver for the student fees.

My biggest thanks goes to my family in Germany: My mother and sister who supported me spiritually but also helped me rehearse my presentations from time to time.

In closing I would like to personally thank the wider Project Group for providing me with the opportunity to pursue a PhD, attend an international conference and participate in project annual personal development workshops and Research Workshops. I am hopeful my research will make a valuable contribution to the development of the Australian YTK industry.

4.3.8. Dam Thi My Chinh, PhD

Affiliation: University of the Sunshine Coast

University supervisors: Professor Abigail Elizur and Dr Tomer Ventura

K4P project supervisors: Dr Mark Booth and Dr Igor Pirozzi

PhD working thesis title: Nutritional and molecular approaches to optimise feed in farmed Yellowtail Kingfish *(Seriola lalandi)*: The impacts of raw materials on digestion, gut microbiota and transcriptomic responses.

K4P project link: This PhD project is linked to the K4P project Nutrition theme (Output 2a) and Chapter 3.1.4, which details research on the apparent digestibility of common raw materials fed to Yellowtail Kingfish.

Research aims and objectives

The overall objective of this research is to contribute to the development of sustainable, environmentally friendly feeds for sub-adult Yellowtail Kingfish (YTK). Specifically, this study has three major aims;

1) To determine apparent digestibility coefficients of nutrients and energy from common raw materials by YTK.
2) To determine the effects of raw materials on the diversity of gut microbiota composition and function of YTK.
3) To investigate the effects of raw materials on the transcriptomics response in YTK.

Research outcomes

During my PhD I completed two major digestibility experiments with YTK. These experiments determined the apparent digestibility of gross nutrients (e.g. dry matter, protein, and lipid etc.), amino acids and gross energy in 14 common raw materials fed to YTK. Additional samples from these experiments were taken to investigate the effect of raw material inclusion on the gut microbiome of YTK as well as the impacts on the underlying functionality of genes involved in digestion. The results will assist in the formulation of research and commercial aquaculture feeds for YTK and greatly improve our understanding of the interactions between raw material selection and gut health of YTK.

Benefits gained by student

As an international PhD student it has been a great opportunity to be involved in the K4P Research Project. I have gained many benefits and advantages that will accelerate my career path such as:

Personal Development
Learn valuable working skills (diet formulation, experimental design, faecal collection technique, digestibility data analysis) from nutritional experts (Dr Mark Booth and Dr Igor Pirozzi).

Learn new skills on molecular laboratory (DNA, RNA extraction, PCR, etc) and bioinformatics analysis (QIIME, CLC genomics workbench) at the University of the Sunshine Coast (USC).

Learn health management skills (i.e. histology and microbiology preparation, diagnosis on and off- farm, disease management, etc) from the Yellowtail Kingfish Health Training Workshop organised by Steven Clarke, SARDI. Learn and practice professional influence and communication skills through Student Personal Development Workshop in Adelaide organised by the K4P project.

Opportunity to practice presenting skills throughout the annual K4P Research Workshops and conference (World Aquaculture Conference) funded by the K4P project.

Networking
I have had great opportunities to meet nutritionists, microbiologists and molecular experts through conferences, training and annual K4P Science Workshops to share the results from my PhD research and get their valuable advice and feedback.
At the K4P Research Workshops I have had opportunities to meet representatives of our industry partners (i.e. Ridley, Skretting Australia, Huon Aquaculture and Clean Seas Seafood) and others at the Yellowtail Kingfish Health Training Workshop (Marine Produce Australia, Pacific Reef Fisheries).

I have had the benefit of being a member of the K4P PhD student network, which has allowed me to share knowledge and experiences on a varied set of experiments and studies, greatly helping in the interpretation of my own research.

Funding support
Funding support for two large scale digestibility experiments at PSFI.

Funding support for next generation sequencing of microbiomes and transcriptomes sections.

Funding support to attend an international conference.

Project progress and thesis citation

PhD Thesis preparation is underway and due for completion in mid-2019.

Acknowledgements

My PhD research is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, New South Wales Department of Primary Industries (NSW DPI) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

I am sincerely grateful to my supervisor's Professor Abigail Elizur, Dr Tomer Ventura and Dr Mark Booth for the continuous support of my PhD study, for their patience, motivation, and immense knowledge. Their guidance helped me in all the time of my PhD project. I would like to special thanks to Dr Igor Pirozzi for his support throughout my research. I also would like to gratefully thank the Biotechnology Program of Ministry of Agriculture and Rural Development (MARD), Vietnamese International Education Department (VIED) and University of the Sunshine Coast (USC) for providing me with my PhD scholarship.

My sincere thanks also go to Ridley for supplying raw materials for my research (especially Dr Michael Salini, Simon Tabrett and Dr Richard Smullen). I would also like to acknowledge David Blyth (CSIRO) for manufacturing some of my experimental diets. I would like to acknowledge Dr Basseer Codabaccus, Brendan Findlay, Ian Russell and Steven Gamble (PSFI) for their valuable technical assistance. I would also like to thank Cedric Simon and Barney Hines (CSIRO) for undertaking the biochemical analysis on feedstuffs, feeds and faecal material. Thanks to Dan Power (USC) for training me in laboratory and bioinformatics skills.

Last but not least, I would like to thank my parents, my husband and my children for supporting me spiritually throughout my PhD and my life in general.

4.3.9. Samantha Chown, PhD

Affiliation: University of Adelaide, School of Food, Agriculture and Wine

University supervisors: Prof. Robert Gibson (University of Adelaide, School of Food, Agriculture and Wine), Dr Todd McWhorter (University of Adelaide, School of Animal and Veterinary Sciences) and Dr John Carragher (University of Adelaide, School of Food, Agriculture and Wine)

Industry supervisors: Assoc. Prof. David Stone (SARDI Aquatic Sciences)

PhD thesis title: Understanding omega 3 long chain polyunsaturated fatty acids (n-3 LC PUFA) utilisation in large Yellowtail Kingfish (*Seriola lalandi*)

K4P project link: This PhD project is linked to the K4P project Nutrition theme (Output 2c) and Manuscript 3.1.1.1: Practical dietary long-chain omega-3 polyunsaturated fatty acids levels large Yellowtail Kingfish at warm water temperatures; and Manuscript 3.1.1.2. Evaluation of alterative oils for large Yellowtail Kingfish at winter water temperatures.

Research aims and objectives

The primary aim of my PhD research was to investigate the utilisation of dietary lipids by large Yellowtail Kingfish (YTK, *Seriola lalandi*). During my candidature this was refined to focus on the utilisation of dietary omega 3 long chain polyunsaturated fatty acids (n-3 LC PUFA). The objective was to increase our knowledge of how YTK digest, distribute, accumulate and process n-3 LC PUFA within their body, with the aim of informing the YTK industry on how to best utilise the n-3 LC PUFA that they source from marine resources and put in to their feeds in the most responsible manner. This is an extremely important aspect of YTK nutrition, given that dietary fish oil and thus, dietary n-3 LC PUFA is continually being reduced in commercial fish feeds in order to improve the environmental and economical sustainability of these feeds.

Research outcomes

During my candidature I have completed five main studies, with specific outcomes that include: Quantifying the fatty acid profile of wild South Australian YTK:

- Quantifying the fatty acid profile of aquaculture YTK fed commercially formulated diets with graded levels of n-3 LC PUFA.
- Defining the rate at which n-3 LC PUFA accumulate and dissipate in the white muscle tissue of YTK.
- Assessing the digestibility of dietary fatty acids from commercially formulated diets and measuring and quantifying free fatty acids and oxylipins in YTK blood plasma.

This research in collaboration with the primary K4P trials will positively expand our knowledge of YTK nutrition and improve the commercial aquaculture production of this species.

Benefits gained by student

As a student, my participation in the project has been incredibility beneficial. From an experimental perspective, being able to be involved in and collect samples from the large-scale feed trials that were run during the K4P project was invaluable. In terms of resource availability, facilities and expertise, the K4P project has provided everything that I could have needed. And lastly in relation to the K4P participants, the academics, industry partners and students, the knowledge that has been shared and the support that has been given has been amazingly helpful and has made this last three years enjoyable and fulfilling.

At the K4P Research Workshops I have had opportunities to meet representatives of our industry partners (i.e. Ridley, Skretting Australia, Huon Aquaculture and Clean Seas Seafood) and others at the Yellowtail Kingfish Health Training Workshop (Marine Produce Australia, Pacific Reef Fisheries).

Project progress and thesis citation

PhD Thesis preparation is underway and due for completion in mid-2019.

Acknowledgements

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

I would also like to thank the University of Adelaide for their financial support. My supervisors and my fellow PhD students at the University of Adelaide and SARDI Aquatic Sciences. Kristina Hickson and Ela Zielinski from Waite Lipid Analysis Services for sharing their expertise and supporting me during my candidature.

4.3.10. Benjamin Crowe, PhD

Affiliation: Flinders University, College of Science and Engineering

University supervisors: Assoc. Prof. James Harris (Flinders University, College of Science and Engineering), Dr Todd McWhorter (University of Adelaide, School of Animal and Veterinary Sciences)

Industry supervisors: Assoc. Prof. David Stone and Dr Matthew Bansemer (SARDI Aquatic Sciences)

PhD thesis title: Bile and cholesterol metabolism in Yellowtail Kingfish, *Seriola lalandi*.

K4P project link: This PhD project is linked to the K4P project Nutrition theme (Output 2b, 2c, 2d and 2e) and Chapter 3.1.2: Emulsifiers and protein and energy levels for large Yellowtail Kingfish. More specifically, Ben's project work is related to: Manuscript 3.1.1.1: Lipid and fatty acid requirements for large Yellowtail Kingfish, Practical dietary long-chain omega-3 polyunsaturated fatty acids requirements for large Yellowtail Kingfish at warm water temperatures (Output 2c); Manuscript 3.1.2.1: Evaluation of dietary lipid levels and emulsifiers on growth and feed utilisation in large Yellowtail Kingfish at cold water temperatures (Output 2b and 2e); and Manuscript 3.1.2.2: Effects of graded dietary protein and lipid levels on growth performance, feed utilisation and gut health in large Yellowtail Kingfish at summer water temperatures (Output 2b); Manuscript 3.1.5.3: Requirement studies for juvenile Yellowtail Kingfish. Taurine requirements for juvenile Yellowtail Kingfish (Output 2d).

Research aims and objectives

The overall objective of this research is to investigate changes that may occur in bile acid and cholesterol metabolism in Yellowtail Kingfish (YTK) in relation to dietary fish oil and fish meal replacement. Specifically, this study will investigate the following three questions:

1. How bile acid and cholesterol metabolism are related to fish performance and health?
2. How bile acid and cholesterol metabolism are modulated by inclusion of alternative dietary oils and/or meals?
3. What are the enzymes and other biochemical mechanisms responsible?

Research outcomes

During my candidature I have completed all of my experimental studies, with specific outcomes that include assessing bile acid and cholesterol metabolism and alterations in liver structure and function in relation to fish meal and fish oil replacement in diets with different protein and energy ratios in cultured Yellowtail Kingfish (*Seriola lalandi*). The outcomes of this research, carried out in collaboration with the primary K4P participants, enhance our knowledge of YTK physiology in response to nutrition and will improve the commercial aquaculture production of this species.

Benefits gained by student

I have gained a wide range of benefits while participating as a PhD student in the K4P project. For example:

1. Attending and presenting at the 18th International Symposium on Fish Nutrition and Feeding Conference, Las Palmas de Gran Canaria, Spain, 3rd - 7th June 2018.

The conference provided the opportunity to observe the diversity and depth of research being undertaken globally by different research groups. This allowed for the opportunity to meet many Australian and international scientists and industry persons and discuss methods and analyses appropriate to my research - particularly in the area of nutrition and histological analyses. The trip was very valuable in providing the opportunity to:

- Observe current domestic and international fish nutritional research
- Observe the scale of global research being undertaken
- Present our research to the international community
- Meet and develop relationships with groups from other research facilities and universities
- Discuss techniques for histological analyses of tissues

2. Attending the Yellowtail Kingfish Health Training Workshop, Roseworthy Campus, University of Adelaide, South Australia, 10th-11th September 2018.

This workshop provided the opportunity to learn vital dissection and histological techniques from and network with Australian experts. These meetings have led to longer-term collaboration and training with specific focus on nutrition and histological analyses.

3. Attending two annual professional development workshops in Adelaide run by the K4P project. One professional development workshop focussed on improving participants communication skills, particularly with industry, and the other on learning and practicing how to enhance ones professional influence.
4. Attending and presenting at annual K4P Research Workshops
   Annual K4P Research Workshops provided an excellent forum to meet and get feedback from other students, researchers and industry participants.

Project progress and thesis citation

PhD Thesis preparation is underway and due for completion in late 2019.

Acknowledgements

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The authors would also like to acknowledge the support of the South Australian Research and Development Institute (SARDI) for the provisions of the SARDI SAASC experimental facilities at West Beach, South Australia. The authors would also like to acknowledge the support of Marine Innovation Southern Australia in association with Flinders University, NSW Department of Primary Industries, Clean Seas Seafood, Huon Aquaculture, Ridley and Skretting Australia. Thanks also to Dr Leo Nankervis and Dr Nicole Ruff of Skretting Australia for input into experimental design and diet formulation and manufacture, Dr Richard Smullen and Dr Michael Salini of Ridley, and Dr Trent D'Antignana for input into experimental design and diet formulation. Also acknowledged is the support of Yvette DeGraaf and Pat Vilimas from Flinders Microscopy Biomedical Services for the training and use of histology processing equipment and Paul Skordas for running and maintaining the K4P experiments at SARDI. Finally, we would like to thank SARDI, Flinders University and the University of Adelaide aquaculture students, including Leigh Kuerschner, Jackson Doherty, Filipa Duarte, Krishna-Lee Currie, Mark Purvis, Jessica Buss, Nicole Thompson and Samantha Chown for their technical assistance during the experiments.

4.3.11. Mr Thibault Legrand, PhD

Affiliation: The University of Adelaide

University supervisor: Dr Laura Weyrich (University of Adelaide)

Industry supervisors: Dr Andrew Oxley (SARDI Aquatic Sciences) and Dr James Wynne (CSIRO, Hobart)

PhD thesis title: The functional role of mucosal microbiomes in fish health

K4P project link: This PhD project represents an extension of activities as part of the Nutritional Health theme Task H3: Elucidate the role of the gut microbiome in Yellowtail Kingfish gastrointestinal health; and aligns with Output 4d: Collect baseline data to differentiate the effects of the environment, Yellowtail Kingfish Yellowtail growth and farm production cycle, disease and different genetic cohorts on the microbiome; and Output 4b: Collect histopathology and blood chemistry data of diseased and healthy Yellowtail Kingfish to characterise the general health of Yellowtail Kingfish used in tank based nutrition and feeding strategy R&D.

Research aims and objectives

The microbiome is known to contribute in facilitating the numerous metabolic and immunological processes vital for fitness and survival in teleost, and following the earlier findings of the project's Nutritional Health theme, it has been shown that altered (dysbiotic) microbiomes are a feature of changing health in Yellowtail Kingfish (YTK, *Seriola lalandi*) (see publication of Legrand et al 2018). Therefore, it was decided to evaluate the underlying functional (transcriptionally-mediated) mechanisms responsible for the observed changes between the relevant gut, skin and/or gill mucosal barrier systems;

facilitating a highly detailed and holistic view of host-microbiome interactions during changing health state. Specifically, as an extension of the earlier (Honours) project, the aim of this PhD is to investigate the functional association of the mucosal microbiome and YTK.

Research outcomes

The outcomes of the project are yet to be detailed as the project is still underway and is not expected to be completed until July 2020. Nevertheless, it is anticipated that this project will identify the underlying microbial functions and host responses that contribute to key homeostasis processes and the emergence of gut enteritis. More broadly, the impacts of cultivation will also be explored through comparisons with samples obtained from wild YTK. In doing so, the project will provide a greater understanding of the functional involvement of the microbiome in relation to cultivation, health and specific disease processes, and will aid in the identification of genes associated with normal and dysfunctional host-microbiome responses. Select genes may represent potential biomarkers for health assessment and/or the screening of broodstock. Furthermore, select microbiota exhibiting beneficial metabolic or immunoregulatory functions may represent novel candidates for selective enrichment as e.g. downstream probiotic therapies. Thus far, the student has undertaken a critical review of the literature (for submission to *Reviews in Aquaculture*, Dec 2018) and has completed a critical component of his lab work; generating a tremendous amount of sequence data (> 350 Gb; ~1.5 billion sequence reads) which will be used in the forthcoming stages of his candidature for identifying key differentially expressed genes in health and disease, and for elucidating host-microbiota interactions.

Benefits gained by student

The benefits gained by the student include the further contribution to his professional development and learning. In this project, the student will learn more advanced omics-based procedures (metatranscriptomics) as tools for discerning the functional involvement of the YTK microbiota and associated host-microbiota interactions in health and disease. Specific skills obtained from the project will include: 1) Methods for the preparation and qualitative assessment of metatranscriptomic (RNASeq) libraries from total RNA; 2) Bioinformatic processing of sequencing data, including the ability to quality filter and assign reads to reference genomes, accessing and interrogating public data repositories for assigning gene function/s (COG/KEGG orthology); 3) Undertaking differential gene expression and pathway analyses; 4) Conducting and interpreting multivariate sequence datasets; 5) Participating in industry forums, workshops and national/international conferences; and 5) Completing a dissertation and publishing a min. of 3 peer-reviewed manuscripts.

Thus far, the student has presented an overview of his PhD as part of the *Australian Marine Sciences Association, From Canyons to Coasts* meeting held in Adelaide (July, 2018). Furthermore, he attended and presented at the *Aquatic Animal Health Training Scheme* held at the University of Wageningen, The Netherlands (May 2018). As led by leading authorities on fish immunology, the student participated in theoretical and practical training components covering mucosal immunity and its stimulation, and technologies for its evaluation (transcriptomics). The workshop represented an excellent opportunity to showcase the work of the K4P Nutritional Health theme and for the student to engage with leading authorities and to establish new networks in the field.

The student was also awarded a CSIRO Postgraduate Top-Up Scholarship within the CSIRO Theme - Agriculture and Food 4, Biologicals: Harnessing microbes for the benefit of Agriculture. The award was taken up by the student in late 2017 under the supervision of Dr James Wynne (Aquatic Animal Health Team Leader, Hobart); bringing an additional stipend to the student and $30,000 operating over 3 years. Thereby, enhancing the capacity for the student to extend and address his research aims.

Learning and networking benefits were also gained from attendance at:

1. The Yellowtail Kingfish Health Training Workshop, Roseworthy Campus, University of Adelaide, South Australia, 10th-11th September 2018.
2. One annual professional development workshop on enhancing ones professional influence run by the K4P project in Adelaide.

3. Three annual K4P Research Workshops, which provided opportunities to build presentation skills.

Project progress and thesis citation

This PhD project is in progress and is due for completion in July 2020.

Acknowledgments

This project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, Department of Primary Industries New South Wales (DPI NSW) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

The student wishes to acknowledge The University of Adelaide for providing an International Fellowship, CSIRO for the provision of a Top-Up Scholarship and SARDI Aquatic Sciences (Molecular Sciences Subprogram) for supporting his candidature. The student thanks Dr Andrew Oxley, Dr Laura Weyrich and Dr James Wynne for their mentorship. The student also kindly thanks Drs Sarah Catalano and Melissa Wos-Oxley for guiding the laboratory and statistical components of the project. Finally, a warm thanks is extended to Clean Seas for supporting the project and kindly providing samples.

4.3.12. Angela Liu, PhD

Affiliation: University of New South Wales

University supervisors: Assoc. Prof. Jesmond Sammut (UNSW) and Dr Debashish Mazumder (ANSTO).

Industry supervisors: Dr Mark Booth and Dr Igor Pirozzi

PhD thesis working title: Optimising growth performance and quality of farmed Yellowtail Kingfish (*Seriola lalandi*) through choline supplemented feeds

K4P project link: This PhD project is linked to the K4P project Nutrition theme (Output 2d) and Chapter 3.1.5, which details research on the choline requirements of juvenile Yellowtail Kingfish.

Research aims and objectives

The main aim of my PhD research is to determine the digestible choline requirement of juvenile Yellowtail Kingfish (YTK) and understand how this requirement is affected by water temperature. The specific objectives of my PhD are to:

1) Identify the digestible choline requirement of juvenile YTK and choline-deficiency induced health effect(s).

2) Evaluate the effect of choline supplementation in a practical fishmeal-based aquafeed at different water temperatures.

3) Evaluate the bioavailability of selected choline supplements in a fishmeal-based formulation.

Research outputs and outcomes

During my candidature I have conducted two major experiments with juvenile YTK investigating the use of choline chloride in diets for this species. The first major study employed a dose-response design

to elucidate the choline requirement of rapidly growing juvenile fish at 16°C. The second study investigated whether it was necessary to supplement diets made from commonly available raw materials with choline (as choline chloride). A multitude of additional samples have been generated from these experiments (e.g. histology, liver composition; lipid class analysis, ITRAX; stable isotopes) which will be used to provide a greater understanding of choline utilisation by YTK. These data are being reviewed in order to make recommendations to the Australian YTK industry, particularly the aquafeed sector.

Benefits gained by student

The K4P project allowed me to develop my project management skills including contributing to report writing and delivery of outputs against project milestones. It also allowed me to interact and work with CSIRO technical staff to develop analytical skills in choline determination, an analytical technique that is not routinely conducted in Australia and critically important to current and future research on choline utilisation in fish such as YTK.

Participating in an international conference allowed me and fellow students to showcase the K4P research project and promote Australia's science to an international audience. I had the opportunity to visit impressive aquaculture facilities, exchange ideas and information with fellow K4P members and network with several international nutrition experts and industry participants. These groups provided valuable advice and suggestions that will benefit my PhD research and subsequently the K4P project. It also allowed me to develop professionally in terms of experience, knowledge, science communication and confidence.

Through attendance at the Yellowtail Kingfish Health Training Workshop I gained valuable experience and knowledge on YTK health issues and management, and the skills I developed will help me to interpret histopathology data for my PhD research. This was also a beneficial networking opportunity with a broader range of industry and research stakeholders than just the K4P project.

Attendance at a K4P Professional Development workshop focused on improving ones communication skills and capacity to influence others, as well as at the annual K4P Research Workshops improved my communication skills and presented an opportunity to network with various stakeholders as well as place my research within an industry context. I gained experience on how research outcomes are communicated, delivered and implemented to meet key industry needs.

Project progress and thesis citation

- Poster presentation in 2018 International Symposium on Fish Nutrition and Feeding (ISFNF, Las Palmas, Canary Island) and UNSW 1-minute thesis competition,
- Oral presentation in AINSE Winter School and ANSTO mini-conference (on PhD thesis),
- A total of four manuscripts in preparation for my thesis and peer review publication.

Acknowledgements

My PhD project is supported by funding from the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit programme, the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Institute (SARDI), Clean Seas Seafood, NSW Department of Primary Industries (NSW DPI) and Huon Aquaculture. Ridley and Skretting Australia have also contributed actively to the project through the input of technical information and the manufacture of experimental feeds.

I would also like to acknowledge the valuable feedback received from the K4P Research Committee members. Special thanks to Ridley (especially Dr Michael Salini) for provision of many of the raw materials used in my experiments. I also acknowledge the staff at CSIRO Agriculture and Food (especially Barney Hines and Susan Cheers) for chemical analysis and the Australian Proteome Analysis Facility (APAF) for specialised amino acid analysis (especially Fei Chi and Bernie McInerney). I would like to thank Dr Fran Stephens for providing valuable feedback and guidance on the histology of my

samples and my colleagues at ANSTO (especially Jennifer Van Holst, Robert Chisari, Patricia Gadd) for assistance in running stable isotope and elemental analyses. I would like to extend my special thanks to the great technical team at PSFI without whom my research would not have been possible; Dr Basseer Codabaccus, Luke Vandenberg, Brendan Findlay, Ian Russell, Justin Tierney, Brooke McCartin, Dylan Nash, Steve Gamble and my fellow K4P students Caroline Candebat and Dam Thi My Chinh. I would also like to personally thank the wider Project Group for providing me with the opportunity to pursue a PhD, attend an international conference and participate in training workshops. I am hopeful my research will make a valuable contribution to the development of the Australian YTK industry. Last but not the least, I would like to thank my supervisors Assoc. Prof. Jesmond Sammut, Dr Mark Booth, Dr Igor Pirozzi and Dr Debashish Mazumder for providing a high level of support (technical and financial), guidance, professional development and intellectual contribution to the research and my candidature. I could not ask for a better supervision team and project.

4.4. Student Personal Development Activities

4.4.1. Professional Development Workshops

Communication Workshop, 16th May 2016 (half day)

Prof. Lisa Given (Information Studies, Research Institute for Professional Practice, Learning and Education, Charles Sturt University), who was recommended by the FRDC, presented the workshop "Communicating effectively with industry" at West Beach Parks Resort (adjacent SARDI), West Beach, SA. The workshop was attended by 17 people, which included postdoctoral fellows or equivalents, Honours and PhD students and industry staff from SA, NSW and WA and some K4P project technicians.

Feedback was obtained from about half the attendees, this indicating that while they had definitely gained benefits from the workshop they felt that the absence of participation by aquaculture and fishing executives led to the training being more theoretical than applied, and more research than industry orientated.

Professional Influence Workshop, 29 August 2017 (half day)

Mr Gary Edwards (Leadership, Negotiations, Sales Speaker and Executive Committee Coach), recommended by one of the K4P Postdoctoral fellows as the best presenter he had experienced at the University of Adelaide, presented the workshop "Professional influence - how to be more effective in any situation". At SARDI Aquatic Sciences, West Beach, Adelaide. The workshop was attended by nine K4P participants.

Feedback obtained from attendees indicated that they had found the training worthwhile as it was stimulating and likely to be of future practical use.

Yellowtail Kingfish Health Training Workshop (Aquatic Animal Heath Training Scheme), 10-11th September 2018 (2 days)

The Yellowtail Kingfish Health Training Workshop was initiated by the K4P project in response to a request from researchers and students who were interested in increasing their knowledge and experience in this area. The workshop started at SARDI Aquatic Sciences (West Beach, SA) and was then at the Roseworthy Campus, University of Adelaide, SA, where the specialised teaching facilities of the Livestock and Veterinary Sciences School were used. The key instructors were Dr Fran Stephens (Veterinarian Consultant, WA), and Dr Stephen Pyecroft (Senior Lecturer Veterinary Pathology, School of Animal and Veterinary Sciences, University of Adelaide, SA), Mr Ken Lee (Diagnostic Microbiologist Technician, University of Adelaide, SA), Dr Marty Deveney (Subprogram Leader, Marine Biosecurity at SARDI Aquatic Sciences , SA), Dr Matthew Bansemer (SARDI Aquatic Sciences, SA), Dr Kate Hutson (Marine Parasitology Laboratory, James Cook University, QLD) and Mr Evan Rees (Aquatic Animal Health Officer, PIRSA, SA). The workshop was attended by 26 people from the

aquafeed manufacturing and Barramundi, Cobia and Yellowtail Kingfish (YTK) aquaculture industries from SA, NSW, QLD, TAS and WA and addressed: fish health skills, biosecurity awareness, data collection procedures for diagnostic services, fish anatomy and histopathology. Attendees included academics, and government and industry research and technical personnel, as well as all six PhD students from the K4P project.

Responses to a questionnaire distributed to attendees after the workshop indicated that in general the course rated highly and that all would be interested in further workshops that would add to their knowledge and experience of this area. More hands-on histopathology (preparing, fixing and staining samples) and addressing the specific health issues of other aquatic species were future topics of particular interest.

5. Impact Assessment and Industry Adoption

5.1. Manuscript - An Impact Assessment of Investment in Australian Yellowtail Kingfish aquaculture R&D as part of the Rural R&D for Profit Program.

Talia Hardaker [a] and P. Chudleigh [a]

[a] Agtrans Research, Suite 36, Benson House, Toowoong, QLD, 4066, Australia.

This manuscript may be referenced as: Hardaker, T. and Chudleigh, P. (2019). 5.1. Manuscript – An Impact Assessment of Investment in Australian Yellowtail Kingfish aquaculture R&D as part of the Rural R&D for Profit Program. In: Stone, D.A.J., Booth, M.A. and Clarke, S.M. (eds). South Australian Research and Development Institute (Aquatic Sciences) 2018, *Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market (DAWR Grant Agreement RnD4Profit-14-01-027)*, Adelaide, June. pp.806-861.

Executive Summary

Presented here are the results of an impact assessment of investment in the project "*Growing a profitable, innovative and collaborative Australian Yellowtail Kingfish aquaculture industry: bringing 'white' fish to the market*" (K4P). The project was funded through the Australian Government's Department of Agriculture and Water Resources (DAWR) Rural Research and Development for Profit Program. The project was led by the Fisheries Research and Development Corporation and was funded over the period of July 2015 to April 2019. Total funding from all sources for the project was $7.37 million (present value terms) with DAWR investment in the project totalling $3.65 million. The investment produced estimated total expected benefits of $126.63 million (present value terms). This gave a net present value of $119.26 million, an estimated benefit-cost ratio (BCR) of 17.2 to 1, an internal rate of return of 46.5 % and a modified internal rate of return of 16.1%. The impact assessment followed general evaluation guidelines that are now well entrenched within the Australian primary industry research sector including Research and Development Corporations, Cooperative Research Centres, State Departments of Agriculture, and some universities. The approach includes both qualitative and quantitative descriptions that are in accord with the impact assessment guidelines of the Council of Rural Research and Development Corporations (CRRDC) (CRRDC, 2018). The investment was analysed qualitatively using a logical framework approach that included descriptions of project activities and outputs, outcomes and impacts. Impacts were categorised into a triple bottom line framework. Principal impacts identified were then considered for valuation. Past and future cash flows were expressed in 2017/18 dollar terms and were discounted to the year 2018/19 using a discount rate of 5% to estimate the investment criteria. A number of economic, environmental and social benefits were identified. Three key impacts were valued in monetary terms. Several environmental and social impacts identified were not valued. Non-valuation was generally due to a lack of evidence/data, difficulty in quantifying the causal relationships and pathways between the K4P investment and the impacts, and/or the complexity of assigning monetary values to such impacts. Therefore, when taken in conjunction with the conservative assumptions made for the three impacts valued, the investment criteria as provided by the valuation may be an underestimate of the overall impact of the K4P project investment.

The K4P project produced a number of useful, industry relevant results with respect to nutrition, feeding strategies and nutritional health for farmed Yellowtail Kingfish (YTK) in Australia. The project had a strong focus on industry participation and collaboration and, as a result, findings were translated to industry throughout the duration of the K4P project and some of the key findings already have been adopted by industry and others are likely to be adopted over the next few years. Also, the cross-industry (multiple YTK producers and aquafeed manufacturers) and cross-region (New South Wales [NSW] and South Australia [SA]) collaborations underpinning the K4P project have likely contributed to increased scientific and industry research capacity and supported the training and development of a number of post-graduate students and other aquaculture stakeholders that may contribute to enhanced capability, productivity and profitability of the Australian YTK aquaculture industry. It is worth noting that the results of the current K4P evaluation are highly dependent on the underlying YTK aquaculture production data that includes expected future production for Western Australia (WA). If the outputs of the K4P project are utilised to improve productivity and profitability for YTK production in WA in the future, and the full 48,000 tonnes of projected production is achieved, the estimated investment criteria may be an underestimate of the likely impact of the K4P investment. However, the investment criteria were positive without the inclusion of the WA production data (BCR of 3.0 to 1) and this more conservative result was consistent with the results of previous fisheries research and development evaluations carried out by Agtrans.

Introduction

The Rural R&D for Profit Program

The Rural Research and Development (R&D) for Profit Program, delivered by the Australian Government's Department of Agriculture and Water Resources (DAWR), was created to boost funding to the Rural Research and Development Corporations (RDCs) for nationally coordinated, strategic research that delivers real outcomes for Australian producers. Total funding available for the program is $180.5 million over eight years, ending 30 June 2022 (DAWR, 2019). The Rural R&D for Profit program aims to realise productivity and profitability improvements for primary producers through generating knowledge, technologies, products or processes that benefit primary producers, strengthening pathways to extend the results of rural R&D, including understanding the barriers to adoption, and establishing and fostering industry and research collaborations that form the basis for ongoing innovation and growth of Australian agriculture. Research projects must address one or more of the new rural research development and extension (RD&E) funding priorities announced in the Agricultural Competitiveness White Paper, which fall into four areas; 1) Advanced technology, to enhance innovation of products, processes and practices across the food and fibre supply chains through technologies such as robotics, digitisation, big data, genetics and precision agriculture; 2) Biosecurity, to improve understanding and evidence of pest and disease pathways to help direct biosecurity resources to their best uses, minimising biosecurity threats and improving market access for primary producers; 3) Soil, water and managing natural resources, to manage soil health, improve water use efficiency and certainty of supply, sustainably develop new production areas and improve resilience to climate events and impacts; and 4) Adoption of R&D, focusing on flexible delivery of extension services that meet primary producers' needs and recognising the growing role of private service delivery (DAWR, 2015).

The Yellowtail Kingfish Rural R&D for Profit Project

Yellowtail Kingfish (YTK) farming was identified nationally as a key opportunity for aquaculture growth and development in Australia. A key challenge to achieve this growth is for industry to diversify from supplying only the relatively small volume, high price sashimi market to the larger volume, lower price Australian 'white fish' market. Australia's leading YTK producer identified that a move from the sashimi to the white fish market requires meeting the following YTK production objectives: a fingerling equivalent of 3.0 kg weight per fingerling within 2 years; a feed conversion ratio (FCR) of less than 1.5 and less than 2.2 for fish between 0.01-1.5 kg and 1.5-3.5 kg, respectively; and survival of greater than 90% from the stocking of fingerlings until harvest. In order to meet these objectives, new YTK-specific information was needed. On 6 May 2015, the Minister for Agriculture announced funding of $26.7

million from 2014/15 to 2017/18 for twelve projects for 'round one' of the Rural R&D for Profit Program. One of these projects, led by the Fisheries Research and Development Corporation (FRDC), was titled "*Growing a profitable innovative and collaborative Australian Yellowtail Kingfish aquaculture industry: bringing 'white' fish to the market*". The project, hereafter referred to as the Kingfish for Profit (K4P) project, aimed to develop more cost-effective YTK feeds and feeding strategies to drive productivity gains for YTK aquaculture. An additional focus was to build a YTK aquaculture R&D network to strengthen adoption of research outputs. The project had three interlinked subprojects/themes; 1) Economically sustainable feeds and improved diet formulation (Theme 1: Nutrition); 2) Improved feeding strategies to increase profit (Theme 2: Feeding Strategies), and 3) Improving nutritional health to boost productivity (Theme 3: Nutritional Health). Total project funding was approximately $6.05 million over three years (2015/16 to 2017/18). Project funders and research partners included DAWR, FRDC, the South Australian Research and Development Institute (SARDI), New South Wales Department of Primary Industries (NSW DPI), Clean Seas Seafood, Huon Aquaculture Group Ltd (Huon Aquaculture), Skretting Australia and Ridley Corporation Ltd (Ridley).

Rationale for the Impact Assessment

The Rural R&D for Profit contract between FRDC and DAWR stipulated that the project team (the Grantee), in collaboration with partner organisations, would undertake particular activities, including an end-of-project evaluation. Specifically, 'Activity 1: Project initiation and management - Output 1(e)' of the contract states: "undertake end-of-project evaluation in accordance with Output 1(d) and provide a report to the Department. The evaluation must report on the Project's outcomes against the Programme objective, including quantitative information on the outcomes achieved and independent expert analysis of expected and/or demonstrated quantifiable returns on investment". The current impact assessment report addresses the requirement for 'an independent expert analysis of expected and/or demonstrated quantifiable returns on investment', with the following terms of reference:

1. Undertake an impact assessment of investment in the Rural R&D for Profit Project titled "*Growing a profitable innovative and collaborative Australian Yellowtail Kingfish aquaculture industry: bringing 'white' fish to the market*" led by FRDC.
2. Report investment criteria for the investment including present value of benefits (PVB), present value of investment costs (PVC), net present value (NPV), benefit-cost ratio (BCR), internal rate of return (IRR), and modified internal rate of return (MIRR).
3. Report investment criteria for the total investment by all funding partners, as well as the investment specifically contributed by DAWR alone, and FRDC alone.
4. Provide a written report to FRDC on the impact assessment process (both qualitative and quantitative), assumptions, and findings.

General Method

The impact assessments followed general evaluation guidelines that are now well entrenched within the Australian primary industry research sector including RDCs, Cooperative Research Centres, State Departments of Agriculture, and some universities. The approach includes both qualitative and quantitative descriptions that are in accord with the impact assessment guidelines of the CRRDC (CRRDC, 2018).

The evaluation process involved identifying and briefly describing project objectives, activities and outputs, outcomes, and impacts. The principal economic, environmental and social impacts were then summarised in a triple bottom line framework.

Some, but not all, of the impacts identified were then valued in monetary terms. Where impact valuation was exercised, the impact assessment uses cost-benefit analysis as its principal tool. The decision not to value certain impacts was due either to a shortage of necessary evidence/data, a high degree of uncertainty surrounding the potential impact, or the likely low relative significance of the impact compared to those impacts that were valued. The impacts valued are therefore deemed to represent the

principal benefits delivered by the project. However, as not all impacts were valued, the investment criteria reported for the K4P project potentially represent an underestimate of the performance of the investment.

Project Details

Summary

Project Code: RnD4Profit-14-01-027
Title: *Growing a profitable, innovative and collaborative Australian Yellowtail Kingfish aquaculture industry: bringing 'white' fish to the market*
Lead Organisation: FRDC
Project Executive Officer: Steven Clarke, SARDI
Theme Leaders: David Stone (Theme 1), Mark Booth (Theme 2), Marty Deveney (Theme 3 - Challenge Test) and Andrew Oxley (Theme 3 - Microbiomics)
Period of Funding: July 2015 to December 2018

Objectives

The key objectives of the YTK Rural R&D for Profit Project were divided into five 'activity' categories. The K4P project's key activities/objectives were as follows:

Activity 1: Project initiation and management

Output 1(a) establish steering and research advisory committees and provide their 'terms of reference'.

Output 1(b) execute agreements and contracts with partner organisations and service delivery agents as needed (to be determined by the Grantee).

Output 1(c) finalise an extension and communication strategy. The strategy must include communications and extension activities including, but not limited to, publications, workshops and newsletters.

Output 1(d) create a monitoring and evaluation plan for the Project.

Output 1(e) undertake end-of-project evaluation in accordance with Output 1(d) and provide a report to the Department. The evaluation must report on the Project's outcomes against the Programme objectives, including quantitative information on the outcomes achieved and independent expert analysis of expected and/or demonstrated quantifiable returns on investment.

Activity 2: Identify economically sustainable feeds and improve diet formulation

Output 2(a) evaluate alternative Australian farm protein and oil sources and identify their ideal inclusion levels in juvenile and sub-adult production diets to reduce dependence on fishmeal (FM) and fish oil (FO)

Output 2(b) investigate the protein sparing effect of using higher energy and lower protein diets

Output 2(c) develop diet formulations that use ideal lipid types and levels for less than two-kilogram YTK during periods of suboptimal water temperatures

Output 2(d) determine the dietary requirements of selected essential nutrients for juvenile and sub-adult YTK

Output 2(e) investigate the cost-benefit of using dietary supplements to improve the production of juvenile and sub-adult YTK

Activity 3: Improve feeding strategies to increase profit

Output 3(a) evaluate optimal feeding strategies for juvenile, sub-adult and broodstock YTK, including but not limited to, comparing experimental nutrient-dense and commercially available feeds, floating versus sinking feeds, feed sizes and feeding strategies

Output 3(b) evaluate the cost-benefit of using high versus low energy feeds for juvenile and sub-adult YTK at varying water temperatures

Output 3(c) develop an improved feed ration model for on-farm YTK feed management

Activity 4: Improve nutritional health to boost productivity [amended]

Output 4(a) develop a challenge test method for fish health evaluations associated with tank-based nutrition and feeding strategy R&D

Output 4(b) collect histopathology and blood chemistry data of diseased and healthy YTK to characterise the general health of YTK used in nutrition and feeding strategy R&D

Output 4(c) characterise and understand the microbiome of the digestive system of YTK in particular in relation to different diets and feeding strategies, and how this might be managed to enhance on-farm YTK health, diets or food conversion ratios

Output 4(d) collect baseline data to differentiate the effects of the environment, YTK growth and farm production cycle, disease and different genetic cohorts on the microbiome

Activity 5: Extending YTK capability

Output 5(a) conduct annual workshops and provide peer reviewed publications, Project reports and produce regular articles for the FRDC FISH magazine to extend the outputs from the Project to industry participants, and the broader aquaculture industry, scientific community and public in line with Output 1(c)

Output 5(b) student training to develop the next generation of industry R&D providers, including up to three postdoctoral research fellows, up to six PhD students and up to 12 Honours students

Output 5(c) incorporate the outcomes of the Project into the new sub-program established by the FRDC for the development of new and emerging aquaculture growth opportunities to allow the direct extension and translation of outputs to potential wider 'white' fish and other new and emerging aquaculture opportunities.

Logical Frameworks

Table 5.1.1, Table 5.1.2, and Table 5.1.4 provide a more detailed description of the key RD&E activities of the K4P project (Activity 2 to Activity 5) using a logical framework approach. A detailed description of the activities and outputs associated with Activity 1: Project initiation and management was not included in the following section as the activity was predominantly related to the administration, management and evaluation of the overall K4P project investment. The impact of such activities was taken into account indirectly through the inclusion of management and administration costs in the cost-benefit analysis as per the CRRDC Impact Assessment Guidelines (2018).

Project Investment

Nominal Investment

Table 5.1.5 shows the annual investment (cash and in-kind) in the K4P project by DAWR, FRDC and others. 'Others' included contributions by SARDI, NSW DPI, Clean Seas Seafood, Huon Aquaculture, Skretting Australia and Ridley.

Program Management Costs

For the FRDC investment the cost of managing the FRDC funding was added to the FRDC contribution for the project via a management cost multiplier (1.122). This multiplier was estimated based on the share of 'employee benefits' and 'supplier' expenses' in total FRDC expenditure (5-year average) reported in the FRDC's Cash Flow Statement (FRDC, 2014 to 2018). This multiplier then was applied to the nominal investment by FRDC shown in Table 5.1.5 for the DAWR and Other investment, additional management and administration costs were included by applying a standard multiplier of 1.10 to the values in Table 5.1.5.

Real Investment and Extension Costs

For the purposes of the investment analysis, the investment costs of all parties were expressed in 2017/18 dollar terms using the Implicit Price Deflator for Gross Domestic Product (ABS, 2018).

No additional costs of extension were included as the K4P project included a high level of industry participation, communication and extension (for more information see Table 5.1.4). Therefore, the existing investment in Table 5.1.5 was assumed to include extension and communication costs.

Impacts

Table 5.1.6 provides a summary of the principal types of impacts from the K4P project. Impacts have been categorised into economic, environmental and social impacts.

Public versus Private Impacts

Both public and private impacts were identified for the project. Private impacts include increased productivity and profitability for the Australian YTK aquaculture industry. Some public impacts may be delivered through environmental and social impacts in the form of improved environmental sustainability, reduced nutrient output, enhanced social licence, regional community spill-overs and increased scientific and industry research capacity.

Distribution of Private Impacts

Private impacts will primarily be captured by individual, commercial YTK aquaculture and aquafeed organisations operating in Australia. There may also be some positive impacts to other parties along the aquaculture input and output supply chains, including other input suppliers, processors and final consumers. Impacts will be distributed according to associated supply and demand elasticities.

Impacts on other Australian industries

There may be some impact on other industries that supply raw materials for YTK aquafeed. For example, reducing reliance on wild derived fishmeal (WD FM) by increasing levels of alternative proteins, such as poultry meal (PM) or soy protein concentrate (SPC), may lead to increased demand, and therefore increased prices, for such alternative aquafeed inputs. New knowledge generated and published by the K4P project may be utilised by other researchers or Australian producers of other carnivorous species farmed in seawater (e.g. Barramundi and Cobia).

Impacts Overseas

No significant impacts to overseas parties are expected. However, there may be some minor impacts on foreign producers and consumers of farmed YTK; for example, reduced demand for domestic Japanese

YTK. Also, new knowledge that was published and presented at international and national conferences by the K4P project team may benefit the international research community and/or foreign producers of other *Seriola* species (Wayne O'Connor, pers. comm., 2019).

Match with National Priorities

The Australian Government's Science and Research Priorities and Rural RD&E priorities are reproduced in Table 5.1.7. The project findings and related impacts will contribute primarily to Rural RD&E Priorities 1 and 4, some contribution to priority 3, and to Science and Research Priority 1.

Valuation of Impacts

Impacts Valued

Analyses were undertaken for total benefits that included future expected benefits. A degree of conservatism was used when finalising assumptions, particularly when some uncertainty was involved. Sensitivity analyses were undertaken for those variables where there was greatest uncertainty or for those that were identified as key drivers of the investment criteria. Three key impacts of the project were valued; 1) Increased productivity for the Australian YTK aquaculture industry driven by improvements in SGR/FCR or other YTK performance measures as a result of improved feed composition and/or adoption of optimal feeding strategies for different water temperatures and/or YTK size-classes, and reduced production losses because of improved management of YTK nutritional health (i.e. reduced incidence of disease); 2) Improved profitability for the Australian YTK industry because of reduced input costs along the supply chain, particularly for aquafeeds made using alternative protein sources, and 3) Maintained social licence to operate for a proportion of the Australian YTK aquaculture industry because of potentially improved environmental sustainability of commercial YTK feed and/or reduced nutrient output from YTK farms.

Impacts Not Valued

Not all impacts identified in Table 5.1.6 could be valued in the assessment. The economic impact identified but not valued included some contribution to increased efficiency of future YTK R&D through the strengthening of YTK R&D networks across regions and between industry participants. The environmental impacts identified but not valued included improved environmental sustainability of YTK feed through reduced use of wild derived fishmeal leading to lower fish in-fish out (FIFO) ratios for commercial feed, and potentially reduced output of nitrogen, phosphorus and carbon from YTK farms as a result of improved feed utilisation and reduced feed wastage through improved feed formulations, feeding strategies and/or use of the improved bioenergetics model. The social impacts identified but not valued included enhanced community well-being as a result of regional spill-overs from a more productive and profitable YTK aquaculture industry and increased scientific and industry research capacity. In general, the impacts identified above were not valued because of a lack of evidence/data, difficulty in quantifying the causal relationships and pathways between the K4P investment and the impacts, and/or the complexity of assigning monetary values to such impacts.

Valuation of Impact 1: Increased Productivity for YTK Producers

Currently, YTK is farmed predominantly by Clean Seas Seafood off the coast of SA in the Spencer Gulf. Trial commercial operations were established by Huon Aquaculture in Providence Bay off Port Stephens (NSW) in 2016 and, in late 2018, the company successfully secured a 2,200 ha farming zone in the waters off the Houtman Abrolhos Islands, WA where they plan to farm YTK (Huon Aquaculture, 2018). There also is an existing 800 ha commercial YTK aquaculture operation in the same region, run by Indian Ocean Fresh Australia (IOFA) Pty Ltd (Department of Primary Industries and Regional Development (DPIRD), 2017a; IOFA, 2018). Approximately 3,000 tonnes of YTK were produced by

the Australian YTK aquaculture industry in 2018. Market research has indicated that demand for YTK exceeds supply (Yeoman, 2013). Also, there is significant scope (per capita) to increase sales domestically and demand from Japan and other export markets (e.g. Europe, China and the USA) is strong (Wright, 2018). R&D is a key driver of the expansion of the Australian YTK aquaculture industry and commercial producers have partnered with key research organisations such as FRDC, SARDI and NSW DPI to improve YTK fish health and productivity across the industry. The valuation of Impact 1 was based on the production trend for YTK observed with the project (increasing sales volume) from 2015 to 2018 and commercial production projections for SA, NSW and WA for 2019 to 2021 and beyond. Given the Australian YTK aquaculture industry's projected growth and expansion, it was assumed that the information generated by the K4P project will contribute to the rate of production growth being 5% higher than it would have been without the project. This increase will be driven by the adoption of improved YTK feeding strategies, enhanced feed formulations for fish of various size/age-classes at different water temperatures, and improved fish health. Some adoption of K4P outputs has already taken place. For example, Clean Seas Seafood implemented a revised winter-feeding strategy for YTK in 2016. Therefore, adoption is assumed to commence from 2016/17, with further outputs implemented over time and reaching maximum output adoption by 2020/21. These drivers are expected to lead to increased productivity through improvements in SGRs and FCRs (or other key performance measures) for farmed YTK as well as through avoided production losses from reduced incidence of disease (e.g. gut enteritis). Further, it was assumed that the additional YTK production would replace some imported white fish in the domestic market and/or would be exported (increasing Australia's world market share for YTK). It was also assumed that the average farm-gate price for YTK of $12.73/kg (past 3-year average) would prevail. Specific assumptions for valuing Impact 1 are provided in Table 5.1.8.

Valuation of Impact 2: Increased Profitability for YTK Producers

Replacement of WD FM by alternative protein and energy sources is considered necessary to ensure the sustainability of the YTK aquaculture industry, both in terms of operational costs and environmental impact. The K4P investment showed that there was considerable flexibility for YTK diet formulation in terms of the substitution of WD FM with various combinations of other protein sources such as fish by-product meal, PM and SPC. All the alternative protein sources investigated as part of the study are lower cost, and more environmentally acceptable, than WD FM. Further, improved, YTK specific feed formulations were shown to improve FCRs for various size/age-classes and different water temperatures. It was assumed that aquafeed companies (such as Skretting Australia and Ridley) will use the K4P project findings to produce new and improved feeds at the request of Australian YTK producers that will improve FCRs and reduce YTK feed input costs by between $60 and $150 per tonne (Michael Salini, pers. comm, 2018). It also was assumed that the aquafeed market is highly competitive and that cost savings would be passed on, in full, to producers. Specific assumptions for valuing Impact 2 are provided in Table 5.1.8.

Valuation of Impact 3: Enhanced Social Licence to Operate

The K4P investment produced new information and improved strategies, methods and models that may improve both the economic and environmental sustainability of the Australian YTK aquaculture industry. Improved feed utilisation through enhanced nutrition of YTK feed, optimal feeding strategies and/or industry adoption of the improved bioenergetic model, may reduce YTK farm output of nutrients (nitrogen, phosphorus, and carbon, all linked to negative potential environmental impacts). Further, reduction of WD FM in YTK aquafeed will lower the FIFO ratio for YTK production and improve the industries overall environmental sustainability. These potential improvements, attributed to the K4P project investment, are likely to contribute to an enhancement of the social licence to operate for a proportion of Australia's YTK aquaculture industry. It was assumed that 50% of the Gross Value of Production (GVP) for Australian YTK aquaculture (approximately $42.0 million in 2017/18 (Clean Seas Seafood, 2018a)) is at risk of some form of loss of social licence. Further, it was assumed that profits are represented by 10% of the GVP. The risk was then assessed as a 10% reduction in the profitability of these marine farms without the K4P investment. Given the availability of the K4P information, tools,

and models, it was assumed that the risk may fall from 10% to a 7.5% reduction in the profitability of the applicable aquaculture farms. Specific assumptions for valuing Impact 3 are provided in Table 5.1.8.

Counterfactual

Both aquafeed companies and YTK producers conduct R&D (independently and jointly) to improve productivity and profitability. For example, Ridley currently engages with fish farms and R&D providers to study various aspects of YTK nutrition (Michael Salini, pers. comm., 2018). However, the appetite and scope for industry driven YTK R&D varies and industry consultation indicated that, without the K4P project, YTK-specific R&D would have progressed but at a slower pace (e.g. one or two research trials every six months) and the level of impact would be reduced because industry findings likely would not have been shared between commercial producers. Therefore, it was assumed that, without the K4P investment, the growth rate for Australian YTK aquaculture production will be 5% lower than the estimated production trend with the project investment. Also, with respect to Impact 2, it was assumed that, without the K4P project, any cost savings associated with new feed formulations that utilise reduced WD FM levels, would have occurred later. For the impact of enhanced social licence to operate (Impact 3), achieved as a result of the improved environmental sustainability of the Australian YTK aquaculture industry, it was assumed to commence in 2018/19 (at the end of the K4P project investment) and run for five years only. Similar to Impact 2, it was assumed that YTK RD&E associated with improved feed utilisation and alternative protein sources for commercial aquafeeds, would have occurred later. Specific assumptions for the valuation of Impacts 1, 2 and 3 are provided in Table 5.1.8.

Summary of Assumptions

A summary of key assumptions made for valuation of the impacts is shown in Table 5.1.8.

Results

All costs and benefits were discounted to the year of evaluation, 2018/19, using a discount rate of 5%. A reinvestment rate of 5% was used for estimating the MIRR. The base analysis used the best available estimates for each variable, notwithstanding a level of uncertainty for many of the estimates. All analyses ran for the length of the project investment period plus 30 years from the last year of investment as per the CRRDC Impact Assessment Guidelines (CRRDC, 2018). The PVB is the discounted sum (present value) of the estimated benefit cash flows and the PVC is the discounted sum of the cost cash flow. The PVB should be interpreted as the present value of expected benefits attributed to the K4P project (based on the assumptions made above) and the PVC is the present value of the RD&E costs for the K4P project. The NPV is equal to the PVB less the PVC. The BCR is equal to the ratio of the PVB to the PVC and represents the estimated expected return to the K4P RD&E investment. The IRR is the rate of discount that produces a NPV of zero (that is, the rate that makes the discounted costs equal to the discounted benefits) (Commonwealth of Australia, 2006). The MIRR is an alternative to the traditional IRR measure and is calculated assuming that the cash inflows from an investment are re-invested at a specified rate representing the cost of capital (the re-investment rate).

Investment Criteria

Table 5.1.9, Table 5.1.10, and Table 5.1.11 show the investment criteria estimated for different periods of benefits for the total investment, the DAWR investment, and the FRDC investment respectively. The PVB attributable to the DAWR investment only (Table 5.1.10) was estimated by multiplying the total PVB by the DAWR proportion of real investment (49.5%). The PVB attributable to the FRDC investment only (Table 5.1.1.11) was estimated by multiplying the total PVB by the FRDC proportion of real investment (12.6%). The annual undiscounted benefit and cost cash flows for the total investment for the duration of the K4P project investment plus 30 years from the last year of investment are shown in Figure 5.1.1. The undiscounted benefit cash flow is positive from the first year of impact (2016/17),

peaks at $27.8 million in 2029/30, then returns to zero by 2033/34. This is due to the counterfactual assumptions for impacts 1, 2 and 3 where the 'without K4P scenario' assumes that YTK specific R&D that would increase productivity and profitability, and enhance the social licence to operate, for the Australian YTK aquaculture industry, would have happened anyway but later and/or at a slower rate of progress. This fact also is reflected in Table 5.1.9 where the discounted benefits do not increase beyond 15 years after the last year of investment. Table 5.1.12 shows the contribution of each impact to the total PVB.

Sensitivity Analyses

A sensitivity analysis was carried out on the discount rate. The analysis was performed for the total investment and with benefits taken over the life of the investment plus 30 years from the last year of investment. All other parameters were held at their base values. Table 5.1.13 presents the results. The results showed a moderate to low sensitivity to the discount rate. This is largely due to the fact that the benefits occur in the first 15 years after the last year of investment.

A sensitivity analysis then was undertaken for the counterfactual assumption that related to the percentage reduction in the current annual YTK production growth rate without the project. This was a key driver of the results and was a variable with relatively high uncertainty. Results of this sensitivity analysis are reported in Table 5.1.14. The results showed a moderate sensitivity to the percentage reduction in production growth without the project.

A sensitivity analysis also was undertaken for the assumption of value of the potential cost saving ($/t) for YTK aquafeed as research and industry personnel indicated that the total value of such costs savings would be dependent on the level of WD FM inclusion and the type and cost of any alternative protein source utilised. Results of this sensitivity analysis are reported in Table 5.1.15. The results showed a low sensitivity to the cost saving assumption; this was largely because Impact 2 contributed only 8.8% to the total PVB (Table 5.1.12).

Given the projected expansion of the YTK aquaculture industry, particularly with respect to production projections for WA, a sensitivity analysis also was undertaken for the assumption on the average YTK farm-gate price. A large increase in the supply of farmed YTK is likely to result in a decrease to the average farm-gate price, particularly if the expansion in production is driven by YTK product being sold at lower prices in the domestic white fish market. Results of this sensitivity analysis are reported in Table 5.1.16. The results showed a moderate sensitivity to the average farm-gate price assumed.

A break-even analysis also was conducted on the assumed farm-gate price for Australian YTK as the price ($/kg) was considered a key driver of the investment criteria estimated. The overall results for the K4P investment remained positive even when the farm-gate price was set to zero. This is because the benefits from Impact 2 (PVB of $8.77 million) which related to feed cost savings would pay for the total costs of the K4P investment (PVC of $7.37 million) on their own.

Finally, a sensitivity analysis was conducted on the probability associated with the likelihood of the projected WA production (48,000 tonnes by 2030) being achieved. Should the projected level of production for WA be achieved, YTK farmed in WA is likely to represent approximately 78% of Australian YTK aquaculture production. Results of this sensitivity analysis are reported in Table 5.1.17. The results showed a high sensitivity to the probability of WA production being achieved. However, it is worth noting that the investment criteria are positive without the projected WA YTK production (0% probability) and, at 3.0 to 1, the BCR is consistent with the results of other fisheries RD&E evaluations carried out by Agtrans over the past five years.

Confidence Ratings and other Findings

The results produced are highly dependent on the assumptions made, some of which are uncertain. There are two factors that warrant recognition. The first factor is the coverage of benefits. Where there are multiple types of benefits it is often not possible to quantify all the benefits that may be linked to the investment. The second factor involves uncertainty regarding the assumptions made, including the

linkage between the research and the assumed outcomes. A confidence rating based on these two factors has been given to the results of the investment analysis (Table 5.1.18). The coverage of benefits was assessed as medium. While the majority of economic impacts identified were taken into account in the K4P quantitative analysis, several of the social and environmental impacts identified were not able to be valued. The impacts valued (increased productivity and profitability for the Australian YTK aquaculture industry and enhanced social licence to operate) were considered the most direct and most significant impacts of the K4P project investment. Confidence in the assumptions, used for valuation of the impact, was assessed as medium. On the one hand, the development of the assumptions included a high-level of researcher and industry consultation. However, the production data underpinning the analysis was based on industry projections and is somewhat uncertain and some data for specific regions was not available due to confidentiality concerns. Some assumptions made therefore were conservative in nature.

Discussion

The K4P project has produced a number of useful, industry relevant results with respect to nutrition, feeding strategies and nutritional health for farmed YTK in Australia. The project had a strong focus on industry participation and collaboration and, as a result, findings were translated to industry throughout the duration of the K4P project and some of the key findings already have been adopted by industry and others are likely to be adopted over the next few years. Also, the cross-industry (multiple YTK producers and aquafeed manufacturers) and cross-region (NSW and SA) collaborations underpinning the K4P project have likely contributed to increased scientific and industry research capacity and supported the training and development of a number of post-graduate students and other aquaculture stakeholders that may contribute to enhanced capability, productivity and profitability of the Australian YTK aquaculture industry. It is worth noting that the results of the current K4P evaluation are highly dependent on the underlying YTK aquaculture production data that includes expected future production for WA. If the outputs of the K4P project are utilised to improve productivity and profitability for YTK production in WA in the future, and the full 48,000 tonnes of projected production is achieved, the estimated investment criteria may be an underestimate of the likely impact of the K4P investment. However, the investment criteria were positive without the inclusion of the WA production data (BCR of 3.0 to 1) and this more conservative result was consistent with the results of previous fisheries RD&E evaluations carried out by Agtrans.

Conclusion

The investment in the K4P project has likely resulted in a more productive and profitable YTK aquaculture industry in Australia. The investment also has likely contributed to improved scientific and industry research capacity and enhanced cross-industry and cross-region YTK R&D collaboration. Funding for the project totalled $7.37 million (present value terms) and produced estimated total expected benefits of $126.63 million (present value terms). This gave a net present value of $119.26 million, an estimated benefit-cost ratio of 17.2 to 1, an internal rate of return of 46.5% and a modified internal rate of return of 16.1%. Several environmental and social impacts identified were not valued. Non-valuation was generally due to a lack of evidence/data, difficulty in quantifying the causal relationships and pathways between the K4P investment and the impacts, and/or the complexity of assigning monetary values to such impacts. Therefore, when taken in conjunction with the conservative assumptions made for the three impacts valued, the investment criteria as provided by the valuation may be an underestimate of the overall impact of the K4P project investment.

Acknowledgments

Agtrans Research and Consulting would like to thank Mr Steven Clarke (Project Executive Officer and Science Initiatives and Support Manager) of the South Australian Research and Development Institute and Wayne Hutchinson (Projects Manager) of the Fisheries Research and Development Corporation for facilitating contact with relevant project personnel and for their guidance and feedback throughout the

Impact Assessment process. Other acknowledgments include: Associate Professor David Stone, Senior Research Scientist, South Australian Research and Development Institute; Dr Mark Booth, Senior Research Scientist, New South Wales Department of Primary Industries, Port Stephens Fisheries Institute; Dr Sarah Catalano, Senior Research Officer (Aquaculture), South Australian Research and Development Institute Aquatic Sciences; Mr Matthew Whittle, Group Development Manager, Huon Aquaculture Group Limited; Dr Michael Salini, Product Developer, Ridley Corporation Limited; Dr Leo Nankervis, Marketing Manager, Skretting Australia; Mr Jay Dent, R&D Manager, Clean Seas Seafood Limited; Mr Dan Fisk, General Manager Aquaculture, Clean Seas Seafood Limited; Dr Wayne O'Conner, Research Leader, New South Wales Department of Primary Industries.

References

Australian Bureau of Statistics, 2018. 5204.0 - Australian System of National Accounts, 2016-17 (January 31). Retrieved from Australian Bureau of Statistics: http://www.abs.gov.au/AUSSTATS/abs@.nsf/DetailsPage/5204.02016-17?OpenDocument

Bureau of Meteorology. (n.d.). Dodge tide. Retrieved from Bureau of Meteorology: http://www.bom.gov.au/oceanography/projects/ntc/dodge/dodge.shtml

Clean Seas Seafood Pty Ltd., 2018a. Annual Report 2017-18. Melbourne: Clean Seas Seafood Pty Ltd.

Clean Seas Seafood Ltd., 2018b. Clean Seas Seafood Limited Annual General Meeting (November 13) (Presentation). Port Lincoln, SA.

Clean Seas Tuna Limited, 2016. Annual General Meeting (November 28, 2016). Port Lincoln, SA: Clean Seas Tuna Ltd.

Commonwealth of Australia, 2006. Handbook of Cost Benefit Analysis. Canberra: Deparment of Finance and Administration (January). Retrieved from https://www.finance.gov.au/sites/default/files/Handbook_of_CB_analysis.pdf

Council of Rural Research and Development Corporations, 2018. Cross-RDC Impact Assessment Program: Guidelines. Canberra: Council of Rural Research and Development Corporations. Retrieved from http://www.ruralrdc.com.au/wp-content/uploads/2018/08/201804_RDC-IA-Guidelines-V.2.pdf

Department of Agriculture and Water Resources, 2015. Agricultural Competitiveness White Paper. Canberra: Commonwealth of Australia. Retrieved from http://agwhitepaper.agriculture.gov.au/SiteCollectionDocuments/ag-competitiveness-white-paper.pdf

Department of Agriculture and Water Resources. (2019, January 17). Rural Research and Development for Profit. Retrieved from Australian Government Department of Agriculture and Water Resources - Agriculture, farming and food: http://www.agriculture.gov.au/ag-farm-food/innovation/rural-research-development-for-profit

Department of Primary Industries and Regional Development, 2017a. Aquaculture Zones. Retrieved from Fisheries (November): http://www.fish.wa.gov.au/Documents/Aquaculture/aquaculture_zones_flyer.pdf

Department of Primary Industries and Regional Development, 2017b. Mid West aquaculture zone (September 28). Retrieved from Fisheries: http://www.fish.wa.gov.au/Fishing-and-Aquaculture/Aquaculture/Aquaculture%20Zones/Pages/Mid-West-Aquaculture-Zone.aspx

Fisheries Research and Development Corporation, 2018. Annual Report. Canberra: Fisheries Research and Development Corporation.

Huon Aquaculture, 2018. Huon Aquaculture secures aquaculture licence in Western Australia (October 12). Media Release. Retrieved from https://www.huonaqua.com.au/wp-content/uploads/2018/10/20181012-HUO-WA-media-release.pdf

Indian Ocean Fresh Australia Pty Ltd., 2018. Yellowtail Kingfish. Retrieved from Indian Ocean Fresh Australia Pty Ltd: https://www.iofa.com.au/

New, M.B., & Wijkstrom, U.N., 2002. Use of Fishmeal and Fish Oil in Aquafeeds: Further Thoughts on the Fishmeal Trap. Rome: Food and Agriculture Organisation of the United Nations. Retrieved from http://www.fao.org/docrep/005/Y3781E/y3781e05.htm

NSW Department of Planning, 2016. Huon Commercial Finfish Lease (MOD 3) (February 19). Retrieved from NSW Government | Planning & Environment: https://majorprojects.accelo.com/public/2519993a1497169c39a80c985c46800b/Development%20Application%20DA%2081-04-01%20MOD%203.pdf

OceanWatch Australia, 2017. Yellowtail Kingfish. Retrieved from OceanWatch Australia: http://www.oceanwatch.org.au/seafood/aquaculture/species/yellowtail-kingfish/

Office of the Chief Scientist, 2015. Strategic Science and Research Priorities. Canberra: Commonwealth of Australia. Retrieved from http://www.chiefscientist.gov.au/wp-content/uploads/STRATEGIC-SCIENCE-AND-RESEARCH-PRIORITIES_181214web.pdf

Wright, R., 2018. Seafood Industry Report. Sydney: EverBlu Capital. Retrieved from http://www.everblucapital.com/wp-content/uploads/2018/02/Seafood_Industry_Report_.pdf

Yeoman, R., 2013. Fish Vol 21 4 - Kingfish star is rising. Retrieved from FRDC: http://frdc.com.au/Media-and-Publications/FISH/FISH-Vol-21-4

Table 5.1.1 Logical Framework for Activity 2 (Theme 1)

Rationale	Research associated with the optimisation of YTK specific nutrition was considered fundamental to the future development of economic and environmentally sustainable improvements to fish feed for commercial YTK producers. A previous analysis of the nutrient profile of YTK production diets compared with information from a literature review of the status of the nutrient requirements of YTK identified potential issues with the conditional deficiency of certain nutrients for commercial YTK production (e.g. taurine, some amino acids and fatty acids, and select vitamins and minerals). Further, understanding the dietary long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) requirements of aquaculture species is vital to sustainably and economically utilise fish oil. However, the dietary LC n-3 PUFA level for YTK (*Seriola lalandi*) was unknown. Under the Rural R&D for Profit Project: *Growing a profitable, innovative and collaborative Australian Yellowtail Kingfish aquaculture industry: bringing 'white' fish to the market*, Activity 2, Theme 1 (Economically sustainable feeds and improved diet formulation) was funded to address these information gaps.
Objectives	Theme 1 addressed the following specific objectives: 1) Evaluate alternative Australian farm protein and oil sources and identify their ideal inclusion levels in juvenile and sub-adult production diets to reduce dependence on fishmeal and fish oil, 2) Investigate the protein sparing effect of using higher energy and lower protein diets, 3) Develop diet formulations that use ideal lipid types and levels for less than two-kilogram YTK during periods of suboptimal water temperatures, 4) Evaluate the cost-benefit of using high versus low energy feeds for juvenile and sub-adult YTK at varying water temperatures, 5) Investigate the cost-benefit of using dietary supplements to improve the production of juvenile and sub-adult YTK, and 6) Determine the dietary requirements of selected essential nutrients for fingerling and sub-adult YTK.
Activities	***Determine choline requirement of sub-adult YTK (NSW DPI: amino-acid experiment 1)*** • Choline (essential B vitamin) is a water-soluble vitamin that has functions in lipid metabolism. Its presence reduces fatty liver disease in fish and may improve tolerance to environmental stressors. • Two six- to eight-week experiments were conducted: 1) A dose-response experiment where juvenile fish (~150 g) were reared at a water temperature of 16.0 ^{o}C and fed specialised, semi-purified diets that were very low in choline. 2) An experiment to evaluate the interaction between choline content and water temperature. In this experiment, juvenile fish were reared at both 16.0 ^{o}C and 24.0 ^{o}C and fed a commercially formulated diet supplemented with either 0 g, 3 g or 6 g choline chloride per kilogram of diet. • At the conclusion of the growth experiment, data on changes in feed intake, weight gain, condition index, hepatosomatic index and viscerosomatic index were collated. • ANOVA[a] and a regression approach were used to examine YTK choline requirements for both groups.

	Determine histidine requirement of sub-adult YTK (NSW DPI: amino-acid experiment 2) • Two histidine experiments were completed: 1) A dose-response trial was designed to quantify the dietary requirements of histidine for sub-adult YTK. Six, semi-purified test diets were formulated with increasing dietary histidine supplemented from 0.0 to 2.0% inclusion. A typical control diet was included to benchmark growth. 2) A second dose-response histidine experiment used common, raw materials to formulate fix experimental feeds containing up to 2% supplemented histidine. The trial was completed at 22 ^{o}C (optimal water temperature). • At the conclusion of the growth experiment, data on changes in feed intake, weight gain, and other biological indicators, including body composition and digestibility of diets were collated. • ANOVA[a] and a regression approach were used to examine YTK histidine requirements. ***Determine taurine/methionine requirement of sub-adult YTK (NSW DPI: amino-acid experiment 3)*** • Taurine is added to commercial aquafeeds for YTK in Australia at a rate of about 10 g per kg of diet (1%). This amount is considered adequate, however the actual requirement of YTK for taurine is unknown (Mark Booth, pers. comm., 2018). • A seven-week factorial dose-response experiment was undertaken to determine the quantitative dietary requirement of taurine and the potential of methionine to spare dietary taurine in sub-adult YTK reared at a water temperature of 23 ^{o}C -24 ^{o}C. • Experimental diets were formulated using a mixture of whole animal proteins (e.g. fish meal (FM), rendered animal meals) and synthetic amino acids. • The dietary concentration of supplemented taurine ranged from 0.2 to 2.0% and the dietary concentration of methionine was held at two levels (approximately 1.0% and 1.8%). • In total, 14 dietary treatments were used in the trial and each dietary treatment was replicated in 3 × 200 L circular research tanks. • Fish were fed to apparent satiation, twice daily, seven days per week. • Changes in biometric measures such as specific growth rate (SGR), feed intake, and feed conversion ratio (FCR) and body composition were measured to determine the YTK requirement for taurine. • Additional responses related to fish health such as enzymatic activity, hepatosomatic and viscera indices, liver composition and whole blood composition (haematology; clinical chemistry; serum taurine) also were measured. ***Determine methionine/cysteine requirement of juvenile YTK (NSW DPI: additional amino acid experiment in lieu of original hydrolysate experiment)*** • Data from the taurine/methionine experiment indicated juvenile YTK may have a higher requirement for methionine • Thus, a factorial dose-response experiment was completed to determine/confirm the methionine requirement of juvenile YTK in the presence of two levels of cysteine. • The eight-week experiment was undertaken at a water temperature of 21 °C. • Experimental diets were formulated using a mixture of whole animal proteins (e.g. FM, rendered animal meals) and synthetic amino acids. • The dietary concentration of methionine ranged from 8-25 g kg^{-1} and the dietary concentration of cysteine was held at two levels (approximately 6 and 14 g kg^{-1}).

	• In total, 10 dietary treatments were used in the experiment and each dietary treatment was replicated in 3 × 200 L circular research tanks stocked with 12 × 53 g juvenile YTK. • Fish were fed to apparent satiation, twice daily, seven days per week. • Changes in biometric measures such as SGR, feed intake, FCR and body composition were measured to determine the YTK requirement for methionine. • Additional responses related to fish health such as enzymatic activity, hepatosomatic and viscera indices, liver composition and whole blood composition (haematology; clinical chemistry; serum taurine) also were measured. ***Evaluate use of commercial bioactives (prebiotics and probiotics) in feeds for sub-adult YTK (NSW DPI and SARDI)*** • A 70-day growth study was undertaken to evaluate the use of four commercially available prebiotic and probiotic products to promote feed intake and growth or boost gut health for juvenile YTK. • Each commercial product was combined with a negative soybean meal (SBM) control diet in order to place YTK under a nutritionally challenging situation. • The four products tested were: 1) Brewer's yeast[b], 2) Inulin powder[c], 3) Protexin® powder[d], and 4) Pro(N8)ure®-IFS powder[e]. • A FM-based control diet also was included in the experimental design resulting in a total of six test diets. • Diets were fed once daily to apparent satiation to groups of juvenile YTK reared in a recirculating aquaculture system (RAS) containing 18 × 500 L tanks. • At the end of the trial, fish were weighed and measured. In addition, blood samples and anal swabs were taken from 3 fish in each tank. • Additional measures also were taken on hepatosomatic index and faecal samples were collected to determine the apparent digestibility of the diets. • Data were analysed using one-way ANOVA[a]. ***Practical dietary long-chain omega-3 polyunsaturated fatty acid requirements for large sub-adult YTK at warm water temperatures (SARDI: N1)*** • An 84-day study was conducted to investigate the growth performance, feed efficiency and health of YTK fed graded dietary fish oil levels, using poultry oil as the replacement. • Experimental work was conducted in the pool-farm facility at the SARDI Aquatic Science Centre. • Fish were between 2.66 and 3.79 kg and held at water temperatures between 15.5 and 24.5 ^{o}C. • Eight experimental diets were formulated to contain 20% FM and graded dietary LC n-3 PUFA levels that ranged from 0.753 to 2.950 g per 100 g of diet. • Pellet kernel, fish oil (FO) and poultry oil (PO) were supplied by Skretting Australia. • Fish were fed to apparent satiation once daily. • Blood samples from three fish per tank (3 fish per tank; 24 tanks; total of 72 fish) were collected at the conclusion of the experiment for blood count and biochemistry analyses. • Growth performance, feed and nutrient utilisation and fish health were measured. • The estimated relationships between dietary LC n-3 PUFA levels and SGR, and between LC n-3 PUFA levels and FCR were reported.

	Evaluation of dietary lipid levels and emulsifiers on growth and feed utilisation in large sub-adult YTK at cold water temperatures (SARDI: N3) • An 84-day study was conducted to investigate the effect of dietary lipid levels and the addition of a dietary emulsifier on the growth, feed utilisation, and gut health of YTK at winter water temperatures. • Fish were between 1.12 and 1.48 kg and held at cold water temperatures between 14.0 and 20.0 ^{o}C. • Experimental diets were formulated to include different lipid levels. • The low lipid diet was formulated at 30% wild derived fish meal (WD FM), 47% crude protein (CP), 20% crude lipid (CL), and 17.7 MJ kg^{-1} (20% lipid). • The high lipid diet was formulated at 42% CP, 30% CL, 20.5 MJ kg^{-1} (30% lipid) • Both diets were manufactured with and without emulsifiers (specifically Kemin LYSOFORTE® Liquid). • Fish were fed to apparent satiation once daily. • Growth performance, feed and nutrient utilisation and fish health were measured. ***Reducing dietary wild derived fish meal inclusion levels in production diets for large sub-adult YTK at warm and cold-water temperatures (SARDI: N5/N2)*** • Apparent digestibility coefficients (ADCs) for a range of FMs, marine meals, rendered animal meals, SBM, legumes and wheat had previously been published for YTK. • However, the data were preliminary and required re-evaluation and further tesing in a growth performance trial with sub-adult YTK. • NSW DPI completed a digestibility experiment with sub-adult YTK to determine ADCs for high priority feed ingredients. • Chemical analysis of ingredients, diets and faecal material was conducted, and the findings were used to prioritise commercial grade feed ingredients for use in the SARDI based YTK trial with respect to reducing dietary fish meal in production diets. • A 36-week study was conducted where up to two-thirds of dietary WD FM was replaced by FM by-product, poultry meal (PM) or soy protein concentrate (SPC) in a series of six diets for YTK. • Fish were between 2.52 and 4.44 kg and were grown from summer to spring at both warm and cold-water temperatures from between 13.0 ^{o}C to 23.5 ^{o}C. • Three experimental diets were formulated by replacing FM content with FM by-product content: (Diet 1) control: 30% WD FM; (Diet 2) 20% WD FM + 10.7% FM by-product; and (Diet 3) 10% WD FM + 21.4% FM by-product. • Three additional experimental diets were formulated by replacing FM content with alternative ingredients: (Diet 4) 20% WD FM + 11.32% PM; (Diet 5) 10% WD FM + 10.7 % FM by-product + 11.32% PM; and (Diet 6) 20% WD FM + 10.88% SPC. • Fish were fed to apparent satiation once daily. • Growth performance, feed and nutrient utilisation and fish health were measured. • Sustainability also was assessed using the fish in-fish out ratio (FIFO = tonne(s) of wild fish it takes to produce a tonne of farmed fish – a measure of the sustainable utilisation of marine resources) to assess the efficiency of WD FM substitution. ***Evaluation of alternative oils for large sub-adult YTK (> 1.5 kg) at cool water temperatures (SARDI: N6)*** • An 84-day study was conducted with two key objectives: 1) To determine the practical dietary LC n-3 PUFA level for optimal growth of large sub-adult YTK at cool water temperatures. 2) To investigate the effect of replacing PO with canola oil (CO) in a series of four diets.

	• Fish were between 1.45 and 2.00 kg and kept at cool water temperatures between 13.0 and 20.0 °C. • All diets contained 25% total lipid; 17% of oils were added to each experimental diet to achieve this level. • Fish oil was added to satisfy the LC n-3 PUFA requirements reported at summer water temperatures (> 2.12 g per 100 g diet), and PO and CO were used to satisfy the remaining lipid/energy requirements at different ratios (100.00 + 0.00%, 66.67 + 33.33%, 33.33 + 66.67% and 0.00 +100.00%, blends of PO and CO, respectively). • Fish were fed to apparent satiation once daily. • Growth performance, feed and nutrient utilisation and fish health were measured. • The relationship between LC n-3 PUFA level and SGR was mapped. • Also, the relationship between CO content and SGR was analysed.
Outputs	***Determine choline requirement of sub-adult YTK (NSW DPI: amino-acid Experiment 1)*** • Results from Experiment 1 indicated that choline deposition and SGR in YTK was optimal when digestible choline intake approached 27 mg per kilogram body weight (BW) per day. • Data from experiment 2 indicated that there were no significant interactions between water temperature and digestible choline content of diets. • Choline retention efficiency declined systematically as the choline content of the FM-based diets increased. • SGR and FCR tended to be slightly better in fish fed commercial formulations containing additional choline chloride. • The results of the study demonstrated that juvenile YTK require no more than approximately 3.0 g of digestible choline per kilogram of diet to ensure performance of YTK is not compromised. ***Determine histidine requirement of sub-adult YTK (NSW DPI: amino-acid experiment 2)*** • Experiment 1 was completed but failed to provide interpretable results. The project team suspected that this may have been due to poor utilisation of the crystalline amino acid mix or unknown interactions between some essential or conditionally essential amino acids. • Results from Experiment 2 showed no difference in SGR and FCR of fish fed diets containing up to 2.0% added histidine. • The strong growth rate and low FCR of all diets suggested that juvenile YTK have no specific requirement for histidine at 20 °C at levels above that contained in standard diet formulations (i.e. minimum requirement of < 0.745% diet) (Mark Booth, pers. comm., 2018). ***Determine taurine/methionine requirement of sub-adult YTK (NSW DPI: amino-acid experiment 3)*** • Results indicated that there was a strong interaction between the dietary level of taurine and methionine when the dietary level of methionine was low. • Breakpoint analyses using SGR as the response variable indicated that the lower threshold of taurine inclusion for rapidly growing YTK was approximately 7.7 g per kilogram of diet when methionine was present at ~1.1% of diet. • YTK fed diets containing approximately 2% methionine exhibited improved growth compared to those fed diets with 1.1% methionine (i.e. the current industry standard). • The SGR of juvenile YTK (reared at 23 °C and fed 2% methionine) did not appear to be dependent on the level of dietary taurine, indicating methionine may spare taurine when the dietary taurine concentration is below 7.7 g per kilogram of diet.

- Fish fed higher levels of methionine had improved SGR and FCR suggesting that their requirement for dietary methionine is greater than 1.1%. This result was found to be contrary to the existing literature on the methionine requirement of other *Seriola* species and may be of benefit to industry (Mark Booth, pers. comm., 2018).

Determine methionine/cysteine requirement of juvenile YTK (NSW DPI: additional amino acid experiment in lieu of original hydrolysates experiment)

- Preliminary evaluation of the data suggested that increasing the dietary level of cysteine from 6 g per kg to 14 g per kg allowed sparing of methionine, but only when the dietary methionine level was at its lowest.
- There was little effect of increasing the methionine content of the diets on the SGR of fish fed diets containing 14 g cysteine per kg of diet; however, there were slight declines in SGR of fish at the highest level of dietary methionine.
- Preliminary analysis of data also indicated that SGR was optimised in juvenile YTK when diets contained between 15.5-17.0 g of methionine per kg.
- There was some evidence of 'opaque eye' in YTK fed the diet having the lowest methionine and cysteine content. This may have been indicative of cataract, but this hypothesis has not yet been confirmed.
- The results from this study supported the conclusions of the taurine trial and confirm that juvenile YTK require higher levels of dietary methionine than previously thought.

Evaluate use of commercial bioactives (prebiotics and probiotics) in feeds for sub-adult YTK (NSW DPI and SARDI)

- Examination of data indicated that none of the commercial prebiotics or probiotic products that were added to the SBM control diet significantly enhanced feed intake, growth rate, condition factor, FCR, protein efficiency ratio (PER) or hepatosomatic index of juvenile YTK when compared to the FM control diet.
- In addition, none of these products significantly affected YTK plasma biochemistry or nutrient digestibility.
- There was also no significant influence of these products on the global level gut microbiome, however, there was some subtle indication of health benefits to YTK with the addition of yeast or Pro(N8)ure® into a soybean meal diet, although this requires further investigation.
- Based on the results of this study there was no clear benefit of adding small amounts of these products to the diets of YTK.

Practical dietary long-chain omega-3 polyunsaturated fatty acids requirements for large sub-adult YTK at warm water temperatures (SARDI: N1)

- Results indicated that there were no treatment related impacts on digestive tract physiology or health related blood or haematological parameters measured for YTK.
- The study found a moderate, positive, significant quadratic relationship between dietary LC n-3 PUFA and SGR.
- The estimated conservative optimal dietary level of LC n-3 PUFA for large sub-adult YTK (based on SGR and FCR) was between 2.12 and 2.26 g per 100 g of diet at summer temperatures.
- This level equates to an intake rate of between 191 and 203 mg LC n-3 PUFA per kg of fish per day at warm water temperatures.
- There was no improvement in SGR by increasing LC n-3 PUFA levels above 2.3 g per 100 g of diet.
- Also, results indicated that there was a moderate, negative, significant quadratic relationship between dietary LC n-3 PUFA level and FCR.
- The FCR of YTK decreased (improved) as dietary LC n-3 PUFA levels increased from 0.75 to 2.14 g per 100 g of diet.

	• Based on the FCR, the optimal level of LC n-3 PUFA was 2.26 g per 100 g of diet. • Also, dietary cholesterol levels need to be considered when fish oil is replaced with alternative lipid sources, including poultry oil. ***Evaluation of dietary lipid levels and emulsifiers on growth and feed utilisation in large sub-adult YTK at cold water temperatures (SARDI: N3)*** • Results indicated that there were no treatment-related impacts on digestive tract physiology or health related blood or haematological parameters measured for YTK. • The SGR and PER of YTK fed the 30% lipid diets were significantly higher than fish fed the 20% lipid diets. • Also, the visceral somatic index and intraperitoneal fat of fish fed the 30% CL diet was significantly higher (by approximately 20%) than for fish fed the lower, 20% CL diet. • Results indicated that LYSOFORTE® Liquid does not significantly influence SGR, PER, or FCR for YTK (consistent with results for other fish species). • Dress-out weight (gutted fish) was not found to be significantly different between treatments. • The extra weight of fish was associated with the viscera and intraperitoneal fat. • Thus, the results suggested that feeding 30% lipid diets may not be beneficial unless fish are sold whole. ***Reducing dietary wild derived fish meal inclusion levels in production diets for large sub-adult YTK at warm and cold-water temperatures (SARDI: N5/N2)*** • The study found no treatment related impacts on digestive tract physiology or health related blood or haematological parameters measured in relation to WD FM substitution. • Results showed that, overall, feed and nutrient utilisation of large sub-adult YTK were not significantly affected by replacing up to two-thirds of WD FM with PM, FM by-product meal or SPC at the levels tested in the study. • SGR was not significantly influenced by diet. Fish fed Diet 2 (20% WD FM + 10.70% FM by-product) and Diet 6 (20% WD FM + 10.88% SPC) tended to grow better than fish fed other diets. • Feed intake rate was not significantly affected by diet; however, those fed Diet 5 (10% WD FM + 10.70 FM by-product + 11.32% PM) and Diet 6 (20% WD FM + 10.88% SPC) tended to have higher feed intake rates than those fed other diets. • FCR of fish was not significantly influenced by diet but tended to be higher (i.e. worse) for fish fed Diet 5 (10% WD FM + 10.70% FM by-product + 11.32% PM) than those fed other diets. • Results from the current study are encouraging to reduce the dietary WD FM inclusions level in production diets for large sub-adult YTK. It was recommended that large sub-adult YTK may be fed a 30% FM (Diet 1), 20% WD FM + 10.7% FM by-product (Diet 2), 10% WD FM + 21.4% FM by-product (Diet 3), 20% WD FM + 11.32% PM (Diet 5), or a 20% WD FM + 10.88% SPC (Diet 6) diet without compromising growth, feed utilisation and fish health. • The sustainable use of marine resources for the production of large sub-adult YTK was improved in all cases of WD FM substitution. • The inclusion of the alternative protein sources resulted in improvements in the FIFO ratios of between 4.8 to 17.9% and 25.4 to 35.1%, respectively, for fish fed diets where WD FM was substituted by 33.3% or 66.7%. • The substitution of WD FM with alternative protein sources resulted in significant reductions in diet ingredient costs.

	• All the alternative protein ingredients used in this study cost less than WD FM. This resulted in approximate savings in diet ingredient costs ranging from $60 to $150 per tonne, depending on the ingredient used and level of WD FM substitution (David Stone, pers. comm., 2018). • Information regarding WD FM substitution with alternative protein sources may improve flexibility in diet formulations for feed manufactures to select raw materials that most economically meet the nutrient criteria. ***Evaluation of alternative oils for large sub-adult YTK (> 1.5 kg) at cool water temperatures (SARDI: N6)*** • In terms of growth, feed utilisation, digestive tract physiology or blood haematology and biochemistry indices measured there was no significant difference between diets. • However, the study found that there was a tendency for fish fed more than 2.34 g LC n-3 PUFA per 100 g of diet diet to perform better than those fed a lower amount. • Results from the current study, combined with previous research at warm water temperatures, conservatively suggest that diets for large sub-adult YTK at cool water temperatures should be formulated to contain 2.12 g LC n-3 PUFA per 100 g of diet. • With regard to CO, it was found that increasing dietary CO inclusion while replacing PO led to decreased fish growth at cool water temperatures. • The project recommended that diets for large sub-adult YTK contain a maximum dietary inclusion of up to ~4% CO (24.13% of the added oil in a 25% total lipid diet). • The project also recommended that PO appears to be a suitable lipid source for high inclusion (73.5% of total added lipid) in diets for YTK at cool water temperatures (David Stone, pers. comm., 2018).
Outcomes	• Adoption of project recommendations will depend on feed manufacturing and retail costs. In most cases, it was recommended that industry conduct pilot scale trials before implementing the recommendations commercially. <u>YTK Feed Formulation Outcomes:</u> • Ridley and Skretting Australia have used the recommendations for LC n-3 PUFA in commercial diets for YTK. Both feed companies also have taken on board the information about the lack of success for the use of the emulsifier tested in this project (David Stone, pers. comm., 2018). • Further, the two participant feed companies have taken on board the information about the recommendations for the maximum CO inclusion levels tested in this project and have taken note of the usefulness of the information to support the use of PO at moderate to high inclusion levels as an energy source for YTK diets (David Stone, pers. comm., 2018). • Also, both feed companies have noted the information regarding WD FM replacement with alternative protein sources and levels of ingredients tested in this study. It is likely that they will use this all this information to improve the current and future commercial diets for YTK production in Australia (David Stone, pers. comm., 2018). • Both YTK producers and feed companies have taken into consideration the data from all the K4P nutrient requirement studies, particularly in terms of assessing current industry practice for inclusion of choline, taurine and methionine (Mark Booth, pers. comm., 2018). There may be additional costs associated with supplementing YTK diets with such nutrients, however, increased production and better food utilisation may more than off-set the increased input costs.

	<u>YTK Production Level Outcomes:</u> • Clean Seas Seafood have used the information derived in the SARDI K4P trials to specify the production diet formulations for YTK production. Clean Seas Seafood specifies to the feed companies that commercial diets for their YTK contain the recommended levels of LC n-3 PUFA derived from results for both trials in the project (David Stone, pers. comm., 2018). • Clean Seas Seafood also now specifies to the feed companies that commercial diets for their YTK contain the recommended maximum levels of CO derived from results from this project (David Stone, pers. comm., 2018). • Based on the results from the emulsifier/high lipid level study, Clean Seas Seafood have begun using high lipid (energy) diets for winter production of YTK (David Stone, pers. comm., 2018).
Impacts/ Potential Impacts	• Increased productivity and profitability for YTK producers through improved YTK diet formulation. This includes improvements from: o Reduced input costs along the supply chain, o Improvements in SGR/FCR/PER or other performance measures as a result of improved feed composition and/or adoption of optimal feeding strategies for different water temperatures and/or YTK size-classes, and o Avoided production losses because of improved management of YTK nutritional health (i.e. reduced incidence of disease). • Improved environmental sustainability of YTK feed through reduced use of wild derived fishmeal leading to lower FIFO ratios for commercial feed. • Potentially, reduced output of nitrogen, phosphorus and carbon from YTK farms as a result of improved feed utilisation and reduced feed wastage through improved feed formulations (Mark Booth, pers. comm., 2019). • Contribution to the enhancement and/or maintenance of the Australian YTK aquaculture industry's social licence to operate. • Enhanced community well-being as a result of regional spill-overs from a more productive and profitable YTK aquaculture industry. • Increased scientific and industry capacity.

[a] Analysis of variance (ANOVA) is a collection of statistical models and their associated estimation procedures used to analyse the differences among group means in a sample. ANOVA was developed by statistician and evolutionary biologist Ronald Fisher.

[b] Farmers Warehouse: http://www.farmerswarehouse.com.au/productDetail/ALL-ANIMAL-PRODUCTS/HORSES/HORSE-SUPPLEMENTS/Item/iO-Brewers-Yeast-4kg/20569

[c] Bulk Powders Pty Ltd; www.bulkpowders.com.au/inulin-powder.html

[d] International Animal Health Products Pty Ltd; www.iahp.com.au/australia/protexin

[e] International Animal Health Products Pty Ltd; http://www.iahp.com.au/australia/feed-additives/pron8ure-ifs

Table 5.1.2: Logical Framework for Activity 3 (Theme 2)

Rationale	Feed and feeding strategies comprise 60% of YTK aquaculture operating costs. A high priority for the Australian YTK industry was to improve feeding practices to increase the sustainability and profitability of YTK aquaculture production. Typical YTK aquaculture practices involve feeding fish once to twice daily to satiation, however, this may result in under- or over-feeding leading to reduced dietary nutrient digestibilities, poor feed conversion efficiencies, reduced growth, and increased effluent discharges. Industry was seeking further information on feeding strategies for YTK production to enhance feed management practices to maximise economic returns. Growth and feed utilisation information would enhance YTK specific growth ration models and enable farmers to determine ideal seasonal feed rates for YTK production to improve profits. Under the Rural R&D for Profit Project: *Growing a profitable, innovative and collaborative Australian Yellowtail Kingfish aquaculture industry: bringing 'white' fish to the market*, Activity 3, Theme 2 (Improved feeding strategies to increase profit) was funded to address these industry R&D priorities.
Objectives	Theme 2 addressed the following specific objectives: 1) Evaluate optimal feeding strategies for juvenile, sub-adult and broodstock YTK, including but not limited to, comparing experimental nutrient-dense and commercially available feeds, floating versus sinking feeds, feed sizes, feed rates and frequencies, water temperature and dissolved oxygen, 2) Develop an improved feed ration model for on-farm YTK feed management, and 3) Evaluate the cost-benefit of high versus low energy feeds for juvenile and sub-adult YTK at varying water temperatures.
Activities	***Refine growth and bioenergetics models for YTK (NSW DPI)*** • Bioenergetic models are useful for predicting growth and predicting feed rate at different water temperatures, thereby giving producers a tool by which to benchmark production performance or plan feeding. • Two oxygen environments were established in separate 10,000 L seawater RASs: (1) hypoxic (60% dissolved oxygen (DO) saturation) (2) normoxic (100% DO saturation) • Ten cylindrical 200 L floating cages were installed in each RAS. Each cage was stocked with 10 juvenile YTK weighing approximately 220 g each. • Cages in each system were fed a high-grade commercial diet at either a maintenance ration (level 0) or at rations that approximated 25%, 50% and 75% of apparent satiation (i.e. 25% of appetite = level 2, up to 100% of appetite = level 5). • At the end of the growing period all fish were weighed and measured. • ANOVA[a] and regression analyses were used to examine the effect of low DO and feed intake on the utilisation of digestible protein and energy. • A second experiment was designed to evaluate the effect of temperature (15 ^{o}C or 25 ^{o}C) on the protein and energy utilisation of sub-adult YTK. • This study followed on from the first bioenergetic experiment.

	Validation of a bioenergetics model using sub-adult YTK fed two diets and held at two temperatures (NSW DPI) • The existing energetic model for YTK was updated with new coefficients that account for the effect of water temperature on growth and protein and energy utilisation. • The new model then was tested on sub-adult YTK grown in research tanks at two temperatures (16.0 ^{o}C and 23.0 ^{o}C). • A series of experimental treatments in which YTK were fed a ration predicted by the model and compared to the performance of YTK fed to satiation were conducted to validate the model. ***Field evaluation of feeds, feeding models and feeding strategies for juvenile and sub-adult YTK (NSW DPI)*** • Two long term field-based experiments were undertaken in a 0.5 ML outdoor pond at PSFI under ambient conditions. • One experiment compared the performance of juvenile YTK fed a commercial-like formulation composed of 55% WD FM to a diet having 15% WD FM and a blend of other protein sources. • A second experiment compared the performance of juvenile YTK fed diets containing prime FM to diets containing fishery by-product meal or a blend of FM and by-product meal sources. • Formulations were based on new information gathered during the project. • All cages within the pond were stocked with 35 fingerlings that were fed once daily for 4 months until they reached approximately 0.8 kg body weight. • Data from the experiments was compared against updated versions of the temperature dependent growth model to examine its validity under real world conditions (Mark Booth, pers. comm., 2018). ***Determine optimum feeding strategies and feed type that boost reproductive performance of NSW YTK broodstock (NSW DPI)*** • The nutrition provided to broodstock is critical in ensuring they are healthy, and that they produce the highest quality milt, eggs and larvae. Production of high-quality larvae from healthy broodstock enhances hatchery and production outcomes through reduced deformities, improved performance and increased on-farm survival. • The Marine Fish Hatchery at the Port Stephens Fisheries Institute (PSFI) (NSW DPI) currently uses natural feeds for their YTK broodstock, predominantly high grade whole Atlantic Squid and Australian Sardines. This regime is known as "best practice" at PSFI. However, such natural food sources are expensive and pose risks to the hatchery in terms of disease (vector) and variability in nutrient composition (Mark Booth, pers. comm., 2018). • A change from natural foods to commercial pelleted diets may have negative impacts on hatchery outcomes and must be tested before changes to current best practice, if any, are made. • Three large-scale broodstock feeding experiments were conducted at PSFI to evaluate the impact of shifting broodstock from current best practice to commercial feed regimes. • Broodstock experiments were undertaken in four, independent 25,000L RAS at PSFI's facility and each tank contained different numbers of broodstock. • Some tanks contained wild caught YTK and others contained first generation fish. • Experiment 1 compared the best practice regime to a feeding regime in which broodstock were fed soft pelletised diets made from proprietary broodstock preparations (i.e. pellet regime: (1) Pelagica sausage, and (2) Breed-M sausage) for 12 months.

	• Fish were fed to apparent satiation once daily on Monday, Wednesday and Friday of each week. • A three-monthly spawning cycle was adopted, aiming for four synchronised spawning events in 12 months, to evaluate the effect of the feeding regimes on broodstock performance. • All tanks of broodstock were induced to spawn naturally in the last week of each cycle by increasing the water temperature from 16 °C to 22 °C within 24-48 h. • The effect of dietary change was assessed by measuring fecundity, egg quality, fertilisation rate, egg composition and larval survival. • The pedigree of eggs was measured in Experiment 1 (as all broodstock had been previously genotyped). • Experiment 2 compared the best practice feed regime to broodstock preconditioned to a 9 mm diameter commercial diet over 4 months. • Experimental protocols and daily routines similar to those utilised in Experiment 1 were adopted and similar measures of broodstock output were evaluated. • In addition, changes to the stocking and final weight of broodstock were measured as well as dietary induced changes to the microbiome of all fish. • Experiment 3 was designed to compare the performance of broodstock reared under the best practice regime to that of fish reared on a regime of commercial pellets (i.e. pellet regime: (1) Huon Select 9 mm diameter, and (2) Breed-M 15 mm diameter hard pellets). • Similar experimental protocols were followed for this trial (short-term trial over one, 3-month cycle); however, no microbiome samples were collected. ***Optimise feeding strategies for YTK in warm and cool water (NSW DPI)*** • Three feeding experiments were conducted with juvenile YTK in RAS at PSFI. • The first experiment examined the performance of juvenile YTK fed a single, commercial aquafeed under various feeding strategies at 24 ^{o}C. • The second experiment used the same commercial diet and feeding strategies but was conducted at a water temperature of 16.0 ^{o}C. • The third experiment compared the performance of juvenile YTK fed a high or low protein diet offered at three different frequencies: 1) 5 days per week, 2) 7 days per week, and 3) at random days during the week (intended to mimic missed feeding events that commonly affect farming operations). • Raw materials, experimental feeds and whole fish carcasses (initial and post-harvest) were analysed for dry matter, crude protein, gross energy, and lipid and ash content. ***Optimising feeding strategies for large sub-adult YTK at cold water temperatures (SARDI: FS1)*** • An 84-day trial was conducted to evaluate the growth performance, feed efficiency, and physiology of YTK fed newly formulated diets. • Two diets and eight treatment combinations were investigated. • A commercial diet formulation (Ridley Clean Seas 2014 Pelagica diet [30% FM; 9% FO]; referred to as formulated diet), was manufactured by Ridley using cooking extrusion technology according to the agreed open formulation and using a least-cost ingredient profile. • Fish with an initial weight of approximately 1.44 kg (+/- 0.13 kg), provided by Clean Seas Seafood, were separated into tanks and fed eight different treatment diets. The eight treatment combinations were: o Treatment 1: Formulated diet fed to apparent satiation six days per week. o Treatment 2: Formulated diet fed to apparent satiation two days per week (Monday and Thursday).

	○ Treatment 3: Formulated diet fed to apparent satiation one day per week (Monday). ○ Treatment 4: Formulated diet fed at 0.1% BW one day per week (Monday). ○ Treatment 5: Formulated diet fed at 0.65% BW two days per week (Monday and Thursday). ○ Treatment 6: Formulated diet fed at 0.35% BW two days per week (Monday and Thursday). ○ Treatment 7: Formulated diet fed at 0.12% BW two days per week (Monday and Thursday). ○ Treatment 8: Sardines fed to apparent satiation every second day. • All trials were conducted at winter water temperatures of between 12.0 ºC and 16.0 ºC. • Diet, and whole initial and final fish were analysed for proximate composition (moisture, protein, lipid, ash, total carbohydrate and energy), taurine and choline, fatty acids and minerals by AsureQuality Laboratories (Auckland, New Zealand). • Diets were also analysed for cholesterol and amino acids profiles. ***Intermittent feed-induced hypoxia (oxygen) effects on the growth and feed utilisation of large sub-adult YTK at warm water temperatures (SARDI: FS2)*** • Hypoxic conditions during and post-feeding are problematic for the YTK sea-cage based industry, particularly during low tidal movement. • An 85-day study was conducted to investigate the effect of DO saturation level and intermittent feed-induced hypoxia oxygen saturation during and post-feeding on the growth, feed utilisation and health of large sub-adult YTK. • Fish were between 2.15 and 3.92 kg and held at water temperatures of between 19.0 ºC and 24.5 ºC. • Fish were exposed to four DO saturation treatments: 1) 100% DO saturation held constant throughout trial, 2) 85% DO saturation held constant, 3) 85% saturation held constant but set to 60% daily before a feed and held at 60% for three hours before returning to 85%, and 4) 85% saturation held constant, on day 13 and 14 of each fortnight during the trial with DO set to 60% daily before feeding and held at 60% for three hours before returning to 85%. This treatment was planned to simulate a feed induced-hypoxic event during twice fortnightly periods of low tidal flow (dodge tide)[b]. • Fish were fed a commercial diet to apparent satiation once daily. • Blood samples were collected from three fish from each of the 12 tanks at the conclusion of the experiment for blood count and biochemistry analyses. ***Optimum dietary protein and lipid levels for large sub-adult YTK (> 1.5kg) at warm water temperatures (SARDI: FS3)*** • The aim of the experiment(s) was to investigate the effects of graded dietary CP (40, 44, and 48%) and dietary CL (25 and 30%) levels on the growth performance, feed utilisation, and health of large sub-adult YTK at warm water temperatures. • Six diets were formulated and then manufactured by Ridley. • Diets were formulated using palatable and digestible ingredients at realistic commercial inclusion levels. • The experiments were conducted in eighteen 5,000 L tanks at the SARDI Aquatic Science Centre with the animals held at ambient sea water temperatures (ranging from 19.0 ºC to 25.5 ºC • Three replicate tanks (n = 3 tanks per treatment) were used per diet with 20 fish per tank.

	• Fish were fed daily to apparent satiation for 84 days. • Growth performance, feed and nutrient utilisation and fish health were measured. ***Effect of feeding frequency on the growth and feed utilisation for large sub-adult YTK at warm water temperatures (SARDI: N4)*** • An 84-day study was conducted to investigate the growth performance and feed utilisation of YTK fed a formulated diet at four different feed frequencies/ration sizes. • Fish were between 3.11 and 4.27 kg and kept at temperatures between 16.0 ^{o}C and 23.0 ^{o}C. • Fish were fed an experimental diet based on the Skretting Australia Pelagica formulation (30% FM; 45% CP; 24% CL; 18.8 MJ kg^{-1}). • Four treatments were applied: 1) fish fed to apparent satiation once daily, 7 days per week, 2) fish fed to apparent satiation twice daily, 7 days per week, 3) fish fed to apparent satiation three times daily, 7 days per week, and 4) fish fed twice daily to the ration provided in treatment (1) (ration split 66.67% and 33.33% morning and afternoon). • Growth performance, feed and nutrient utilisation and fish health were measured.
Outputs	***Refine growth and bioenergetics models for YTK (NSW DPI)*** • Weight gain of YTK responded systematically to feed intake in both the normoxic and hypoxic treatments. • Low DO at 60% saturation negatively affected the nutrient and energy utilisation response in YTK with this response tending to be more pronounced with increasing nutrient and energy intake. • After 38 days fish fed to apparent satiation (level 5) had doubled in weight. • FCR ranged between 1.17 and 1.81 depending on feeding ration, with a better FCR at intermediate rations. • Across all feed rations the SGR of sub-adult YTK reared at 20 °C under the hypoxic regime was 1.18% per day while under the normoxic regime it was 1.25% per day. • With the exception of feeding level 1, SGR of YTK was higher at all feeding levels under the normoxic regime. • Data generated from this study was used to improve growth and feed ration models for YTK facilitating better feed management and formulation through a better understanding of nutrient requirements and therefore dietary specifications for YTK. • Results implied that a restricted feed ration may be more appropriate in a low DO environment. • Temperature was shown to have a varying effect on utilisation responses in YTK with the magnitude of the response dependent on the nutrient examined. • There was little influence of temperature on the utilisation response of methionine and lysine while responses for arginine and taurine utilisation were extremely diverse between temperatures. ***Validation of a bioenergetics model using sub-adult YTK fed two diets and held at two temperatures (NSW DPI)*** • Extreme ambient air temperatures caused significant temperature fluctuations in the cold-water system at the first stages of the trial, however, growth and feed conversion efficiencies of the prescribed ration group were similar to the satiated group. • Results indicated that the model, in its current form, was reliable in predicting growth and feed requirements at cool water temperatures. • At 23 ^{o}C, the model was accurate in predicting growth over the first three weeks of the trial.

	• By the end of the trial, the model tended to overestimate growth. • The project recommended that further refinement of the model be undertaken by using data gathered from the PSFI field evaluation trials and reliable data from farms (Mark Booth, pers. comm., 2018). • Growth and feed demand of YTK based on digestible protein and energy requirements can be modelled with reasonable confidence for YTK grown in cold water of approximately 16 °C. These findings may assist farm managers with on-farm feed management practices. ***Field evaluation of feeds, feeding models and feeding strategies for juvenile and sub-adult YTK (NSW DPI)*** • Results indicated that there was no biological difference between the performances of YTK fed a high WD FM or very low WD FM diet. • However, the raw material cost of the low WD FM diet was about 23% less than the WD FM control. This represents a considerable potential saving on raw material costs (Mark Booth, pers. comm., 2018). • Similarly, the results indicated there was no difference in biological performance of YTK fed diets based on prime FM or fishery by-product meal, indicating that by-product meal is an appropriate substitute meal for feed for YTK. • The experiments demonstrate that there is considerable flexibility in the formulation of diets for YTK, provided these formulations are based on an understanding of nutrient requirements and raw material quality (Mark Booth, pers. comm., 2018). • In the absence of reliable farm data from either NSW or SA, data from the experiments was used to further refine the bioenergetic models for YTK, particularly the temperature dependent growth model (Mark Booth, pers. comm., 2018). ***Determine optimum feeding strategies and feed type that boost reproductive performance of NSW YTK broodstock (NSW DPI)*** • The soft-pelletised diets delivered more dry matter per kg of feed delivered than the natural products that contained more than 70% moisture by weight. • Gross energy content of the soft-pelletised feeds also was significantly higher per unit weight than that available from Australian Sardines or Atlantic Squid. • In terms of feed cost, the Pelagica sausage was cheapest at approximately $1.92 per kg while the Breed-M sausage was about $24.41 per kg. • The feed cost per kilogram of Australian Sardines is about half that of Atlantic Squid. • The data indicated that wild broodfish took more than 200 days to adapt to a soft, pelletised feed. Conversely, the first-generation (F1) broodstock fed soft pelletised feeds were shown to adapt quickly and exhibited a fairly stable intake pattern over all spawning cycles. • Fish held on the natural feed regime spawned more frequently and produced more viable eggs than fish held on soft-pelletised feeds. • There were more hatched larvae originating from the natural feeding regime than from the pellet fed regime. • The study found no statistical difference in the relative cost of feeding broodstock on natural feeds versus the pelletised feeds under the feed management strategy used in the experiment. • Averaged over the life of the trial (experiment 1) it cost approximately $6.97 to feed a kilogram of fish using pelletised feeds and $6.07 to feed a kilogram of fish when using natural feeds. • Based on the enumeration of viable eggs (experiment 1) it appeared that the natural feeding regime was better than the soft-pelletised regime in terms of fecundity.

	• Wild-fish and F1 fish allocated to dry commercial feeds would not accept standard 15 mm diameter floating pellets. Wild fish took almost 50 days to be weaned onto the moist pellets, whereas F1 broodstock weaned immediately. This response has implications for broodstock management and breeding programs reliant on wild fish stocks. • Based on project findings, the broodstock experiments have confirmed that the best-practice feeding regime remains the most viable option for YTK broodstock at the PSFI hatchery (Mark Booth, pers. comm., 2018). ***Optimise feeding strategies for YTK in warm and cool water (NSW DPI)*** • Results indicated that juvenile fish fed 7 days per week consumed significantly more feed than fish fed 5 days per week (Mon-Fri) or randomly. • Feed consumption in YTK fed to apparent satiation 7 days per week was more than 24% higher than YTK fed 5 days per week. This suggests that YTK offered feed 5 days per week (Mon-Fri) cannot physically compensate for missed feeds on Saturday and Sunday. • The trials demonstrated that juvenile fish (150-500 g) are comfortable consuming either 6 mm or 9 mm diameter pellets and that the choice of pellet size had little impact on growth rate or FCR. • The studies also indicated that one carefully fed meal in the morning is probably sufficient to sustain optimum growth while at the same time subtly improving FCR. ***Optimising feeding strategies for large sub-adult YTK at cold water temperatures (SARDI: FS1)*** • Fish (of about 1 kg size class) fed the formulated diet to apparent satiation six days per week exhibited significantly higher growth rates and numerically superior FCR compared to fish fed the same diet at lower feed rates. • The maintenance requirements for YTK (1 kg size class) fed the formulated diet was 0.2047% BW per day. • Based on this feed rate, the initial weight of YTK (1.44 kg) and the dietary gross energy level of the formulated diet (19.1 MJ kg^{-1}), each fish would require 2.95 g of feed or 56.3 kJ per day at an average water temperature of 12.8 °C. • In order to provide slightly above the maintenance rate to ensure positive growth, results indicated that fish may be fed to apparent satiation two days per week. • The project recommended that YTK (1 kg size class) not be fed below this rate under commercial conditions during winter. • The growth rate and FCR of YTK fed Sardines every second day and the formulated diet to apparent satiation six days per week were similar. • However, the FIFO ratio for YTK fed Sardines was 50.1% higher than animals fed the formulated diet. • The project team suggested that feeding Sardines may negatively impact consumer perception and the marketability of YTK. • The project recommended that further research be conducted to investigate the compensatory weight gain post-winter. ***Intermittent feed-induced hypoxia (oxygen) effects on the growth and feed utilisation of large sub-adult YTK at warm water temperatures (SARDI: FS2)*** • Fish exposed to intermittent feed induced-hypoxia (Treatment 4) exhibited significantly reduced SGR and FCR compared to fish held at other oxygen treatments investigated. • No significant differences were observed for SGR and biomass gain for YTK in Treatment 1, 2, and 3. However, there was a tendency for SGR and biomass gain to decrease in fish at 100% to 85%, and 85% dropped to 60% daily. • The findings indicated that dodge tides and crowding for weight checks may cause problems unless sufficient oxygen is provided.

- The trials indicated that YTK may be able to adapt to consistent conditions, including consistent daily hypoxic events as long as they are not too severe.
- Findings indicated that commercial producers of YTK in sea-cages may be able to mitigate exposure to intermittent feed-induced hypoxic events by selecting sites with adequate water flow or by utilising nets with larger mesh sizes to allow high water exchange.

Optimum dietary protein and lipid levels for large sub-adult YTK (> 1.5kg) at warm water temperatures (SARDI: FS3)

- The SGR of YTK fed graded dietary CP (40, 44, and 48%) and dietary CL (25 and 30%) varied between 0.46 and 0.59% per day.
- Feed intake of YTK fed graded dietary CP and dietary CL decreased (on average) as protein increased for the 25% lipid diet in terms of feed intake as a percentage of bodyweight per day.
- Feed intake was highest for the 30% lipid diet at the 48% protein level.
- This indicated that YTK have a reduced feed intake when fed 30% CL diets compared to 25% CL diets.
- Protein intake was higher when fish were fed high CP diets and the effect was more pronounced in the 30% CL series.
- There were no significant differences in the FCR between fish fed the different diets, although there was a tendency for the 44% CP + 25% CL to be best.
- There appeared to be a 'sweet spot' for optimal growth by providing 5.2g of protein per kg BW per day, and 242 kJ per kg of fish per day. The peak CP to energy ratio was 21.6 g per MJ.
- Based on the results of the experiments, the project team recommended that diets for 2.0-3.5 kg YTK at warm temperatures contain:
  - 44% CP,
  - 25% CL,
  - 20.5 MJ kg^{-1} gross energy, and
  - CP to energy ratio of 21.6 g MJ^{-1}.
- The project also found that high dietary lipid (30%) appears to interfere with feed, protein and energy intake, feed utilisation, and growth.

Effect of feeding frequency on the growth and feed utilisation for large sub-adult YTK at warm water temperatures (SARDI: N4)

- Results indicated that there were no treatment related impacts on digestive tract physiology or health related blood or haematological parameters measured for YTK.
- Over the entire experiment, the SGR of YTK was not significantly different between treatments. However, between stocking to the first weight check, fish fed twice and three times daily to apparent satiation exhibited significantly higher SGR compared to those fed to apparent satiation once daily and those fed the equivalent split ration.
- In contrast, between the first weight check to the final harvest, there were no significant differences in SGR between treatments. These response differences may be related to decreasing water temperature throughout the trial.
- The feed intake rate (% BW per day) significantly increased as feeding frequency increased from once to twice to three times a day to apparent satiation daily.
- Overall, fish fed to apparent satiation three times daily exhibited a significantly higher FCR than other treatments investigated.
- Findings suggested that YTK may be fed to apparent satiation twice or three times daily to improve growth and feed utilisation at warmer temperatures (> 20 ^{o}C).

	• Findings also suggested that YTK may be fed to apparent satiation once daily at temperatures below 20 °C to reduce feeding costs and minimise feed wastage as the fish grow at a much slower rate.
Outcomes	• As a result of the research, in 2015 Clean Seas Seafopd adopted a new winter-feeding strategy (> 0.2% BW per day) in the final period of cold water. The R&D Manager of Clean Seas Seafood advised K4P project personnel that the previously used winter feed management strategy (pre-trial) would have resulted in the YTK losing a significant amount of weight over the 2015 'winter' period, equivalent to a $1 million annual loss in profit on a 2,000 tonne operation. • Clean Seas Seafood personnel indicated that utilising the results of the experiment (a higher feeding rate resulting in YTK putting-on biomass over winter) was likely to lead to a $0.35 million net annual profit on a 2,000 tonne operation. • Adoption of other project recommendations will depend on feed manufacturing and retail costs. • Skretting Australia and Ridley have utilised the project's findings to produce improved YTK production diets (David Stone, pers. comm., 2018). • Prior to the current study there was interest by Clean Seas Seafood to oxygenate sea-cages to improve production over summer. However, based on results from the current study, it was found that this practice may be cost prohibitive and logistically impractical. • Based on the findings of the FS2 study, Clean Seas Seafood has utilised the services of the SARDI Oceanography Subprogram to optimise the placement of new sea-cages. • Discussions with Clean Seas Seafood farm management indicated that YTK feed rates are likely to be altered based on tidal movements and dissolved oxygen levels prior to feeding (David Stone, pers. comm., 2018).
Impacts	• Increased productivity and profitability for YTK producers through improved YTK feeding strategies. This includes improvements from: o Reduced input costs along the supply chain, o Improvements in SGR/FCR or other performance measures as a result of improved adoption of optimal feeding strategies for different water temperatures and/or YTK size-classes, and o Avoided production losses because of improved management of YTK nutritional health (i.e. reduced incidence of disease). • Potentially, reduced output of nitrogen, phosphorus and carbon from YTK farms as a result of improved feed utilisation and reduced feed wastage through improved feeding strategies and/or use of the improved bioenergetics model (Mark Booth, pers. comm., 2019). • Contribution to the enhancement and/or maintenance of the Australian YTK aquaculture industry's social licence to operate. • Enhanced community well-being as a result of regional spill-overs from a more productive and profitable YTK aquaculture industry. • Increased scientific and industry capacity.

[a] Analysis of variance (ANOVA) is a collection of statistical models and their associated estimation procedures used to analyse the differences among group means in a sample. ANOVA was developed by statistician and evolutionary biologist Ronald Fisher.

[b] 'Dodge tide' is a local, South Australian term for a 'neap tide'. A neap tide refers to a tide just after the first or third quarters of the moon when there is the least difference between high and low water (Bureau of Meteorology, n.d.). Specifically, a dodge tide is considered a special case where there is almost no tidal movement (Steven Clarke, pers. comm., 2019).

Table 5.1.3: Logical Framework for Activity 4 (Theme 3)

Rationale	A fine line exists between providing the nutritional requirements of YTK and avoiding health problems when substituting alternative ingredients for FM and FO to produce more cost-effective diets. The inclusion of CO and SBM, ingredients commonly used in other animal production sectors, into YTK diets has been demonstrated to negatively impact digestive tract health, feed utilisation, growth and consequently profitability. Nutritional deficiencies and their impact on fish health, including immuno-competency, have been identified as a major long-term impediment of industry development. Under the Rural R&D for Profit Project: *Growing a profitable, innovative and collaborative Australian Yellowtail Kingfish aquaculture industry: bringing 'white' fish to the market*, Activity 4, Theme 3 (Improving nutritional health to boost productivity) was funded to interlink with Theme 1 and 2 to refine knowledge of the interactions between nutrition and health. R&D activities undertaken as part of Theme 3 were split into three main areas (Steven Clarke, pers. comm., 2019): 1) Development of a challenge test (led by Marty Deveney), 2) Histopathology and blood chemistry (completed largely as part of Theme 1 and 2 to additionally access the condition / health of the experimental animals), and 3) Microbiomic studies (led by Andrew Oxley).
Objectives	Theme 3 addressed the following specific objectives: 1) Develop a challenge test method for assessing YTK health associated with tank-based nutrition and feeding strategy R&D, 2) Collect histopathology and blood chemistry data of diseased and healthy YTK to characterise the general health of YTK used in nutrition and feeding strategy R&D, 3) Characterise and understand the microbiome of the digestive system of YTK in particular in relation to different diets and feeding strategies, and how this might be managed to enhance on-farm YTK health, diets or FCRs, and 4) Collect baseline data to differentiate the effects of the environment, YTK growth and farm production cycle, disease and different genetic cohorts on the microbiome.
Activities	***Development of a challenge test for evaluation of the health of YTK in tank nutrition experiments (SARDI: H1)*** • An experiment was designed to develop an improved model for understanding YTK health and how different diets affect their adaptive and innate immunity. • The experiment used sub-adult YTK from the K4P nutritional trials (N5/N2). • 90 YTK were sampled, including 18 fish from the summer component and 72 fish from the winter trial extension. • Fish were held in 5,000 L tanks at the SARDI pool-farm facility and fed the experimental diets where the FM content was reduced and replaced with commercially relevant alternative protein sources. • Half of the fish then were challenged with either an intra-peritoneal injection of killed *Photobacterium damselae piscicida* (a pathogen of YTK) or a saline solution. • Tissues from challenged fish were sampled one day later and then again 250-degree days after the challenge event. • Specific and natural antibody titres of vaccinated and saline injected fish were determined.

- Immune signalling cytokines were measured to assess changes in immune responses.

Objective 1: Elucidating the natural dynamics of YTK gut microbiome over the commercial production cycle in response to alternate (land v sea) farming practices as a reference for downstream health and dietary assessment (SARDI: H2A/ H2B)

Objective 1 included three components:

- Investigation of the dynamics of the gut microbiome for YTK farmed in onshore tanks (land) versus offshore sea-cages,
- Investigation of the dynamics of the gut microbiome for YTK across the commercial production cycle, and
- Comparison of farmed YTK from parts 1 and 2 with wild fish from South Australia.

<u>Component 1 – Onshore (land) vs offshore (sea)</u>

- A total of 20 fish were sampled for this component (11 fish from the Clean Seas Seafood commercial hatchery in Arno Bay [onshore] and 9 fish from a sea-cage site at Point Boston [offshore]).
- Fish were of the same year class (2016) but fed a different diet.
- Three wild fish (caught off Kangaroo Island) were provided by Clean Seas Seafood to act as controls.
- A water sample was taken from both the land-based and sea-cage location to control for the influence the environment may have on the structure and composition of the gut bacterial community.

<u>Component 2 - Commercial production cycle</u>

- A total of 40 fish were sampled across the commercial production cycle (10 at each of four time points between January and March 2016).
- All fish were collected from Arno Bay, South Australia.
- Fish were fed the same commercial diet and were of the same year class (2016).
- Three wild fish (caught off Kangaroo Island) were provided by Clean Seas Seafood to act as controls.
- A water sample was taken from the Arno Bay sea-cage site to control for environmental factors that may affect the composition and/or structure of YTK gut bacterial communities.

<u>Component 3 – Farmed vs wild fish</u>

- A total of 7 wild fish (average weight 6.5 kg) were caught via line fishing at Four Hummocks (SA) in February 2018.
- This dataset was merged and analysed with the previous baseline data, including the land vs sea, commercial production cycle and three ‘wild’ fish components.
- A water sample was taken from the fishing site to control for environmental factors that may affect the composition and/or structure of YTK gut bacterial communities.

Objective 2: Characterise and understand microbiome changes with diet and their potential implications for health and/or performance.

Objective 2 included four experimental components:

- Commercial feed formulations versus ‘natural’ diets (Sardines),
- Fatty acid inclusion nutritional trial (N1),
- Lipid inclusion with and without emulsifiers (N3), and

	• Fish meal replacement nutritional trial (N5/N2). <u>Component 1 - Formulated feeds and natural diets (SARDI: HA1/ H4B, H4G and H4A/D)</u> • For this component, fish were collected from offshore sea-cage sites and land-based tank trials in SA (SARDI pool-farm facility) and WA (Fremantle facility). • Five fish were sampled from three sea-cages in Port Lincoln (15 fish in total) in February 2016. • Fish were fed three different formulated diets (known as feed A, feed B, and feed C). • The sea-cage fish samples were fed: 1) feed A for 12 months prior to sampling (five fish sampled), 2) feed B for 15 months prior to sampling (five fish sampled), and 3) feed C for 3 months prior to sampling (five fish sampled). • A water sample also was taken from the Port Lincoln site. • For the SA tank trial, 18 fish were sampled from the SARDI winter feeding trial (FS1) in September 2015. • Fish were held in 5,000 L tanks at the SARDI pool-farm facility for the duration of the 84-day trial. • Nine fish, collected from three tanks, were fed a formulated diet (known as feed B) to apparent satiation six days per week at winter water temperatures. • Another nine fish were fed a Sardine diet to apparent satiation every second day at winter water temperatures. • For the WA tank trial, five fish were sampled from the Fremantle facility in June 2016. • Three fish were fed feed D and two fish were fed feed C, with fish on these diets for eight months prior to sampling. <u>Component 2 - Fatty acid trial (SARDI: H4B, H4G and H4A/D)</u> • A total of 32 fish were sampled from the SARDI fatty acid trial (N1). • Five fish were sampled pre-trial in March 2016, and 27 fish were sampled post-trial in June 2016. • Fish were held in 5,000 L tanks at the SARDI pool-farm facility and fed different diets with a varying proportion of long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) over the course of an 84-day trial. • Nine fish were collected from three tanks that were fed a diet containing a high level of fatty acid inclusion - 2.95 g LC n-3 PUFA per 100 g (diet 1). • Nine fish were collected that had been fed a diet containing a moderate level of fatty acid inclusion - 2.14 g LC n-3 PUFA per 100 g (diet 3). • Finally, a further nine fish were sampled that had been fed a diet containing a low level of fatty acid inclusion - 0.753 g LC n-3 PUFA per 100 g (diet 8). <u>Component 3 – Lipid inclusion with and without emulsifiers (N3)</u> • A total of 41 fish were sampled from the SARDI emulsifier trial (N3). • Five fish were sampled pre-trial in September 2016, and 36 fish were sampled post-trial in November 2016. • Fish were held in 5,000 L tanks at the SARDI pool-farm facility and fed different diets with a varying percentage of lipid inclusion (low 20% or high 30%) with or without emulsifiers over the course of an 84-day trial. • Nine fish were collected from three tanks fed a diet containing 30% total lipid without emulsifier (Diet 1).

- Nine fish were collected from three tanks fed a diet containing 30% total lipid with emulsifier (Diet 2).
- Nine fish were collected from three tanks fed a diet containing 20% total lipid without emulsifier (Diet 3).
- Nine fish were collected from three tanks fed a diet containing 20% total lipid with emulsifier (Diet 4).
- A water sample was collected and processed from the tank system post-trial to control for the influence of the environment on the structure and composition of the gut bacterial community.

<u>Component 4 – Fish meal replacement (N5/N2)</u>

- A total of 72 fish were sampled from the SARDI FM replacement trial (N5 during summer and N2 during winter).
- Eighteen fish were sampled from two treatments in June 2017 as a modified version of the summer component (N5).
- Fifty-four fish were sampled from six treatments in November 2017 as a 12-week extension of the winter component (N2).
- Fish were held in 5,000 L tanks at the SARDI pool-farm facility and fed different diets where FM content was reduced (to levels of 20% and 10%) and replaced with commercially relevant alternate protein sources, including digestible FM by-product protein, digestible PM and/or digestible SPC.
- Two water samples were collected and processed from the tank system post-trial to control for the influence of the environment on the structure and composition of the gut bacterial community.

Objective 3: Assessing the impacts of changing health status on the microbiome of YTK.

Objective 3 included four components:

- Enteritis: sampling and analysis of the gut microbiome of healthy versus diseased YTK in 2016 (Task H3),
- Investigation of the dynamics of the gut microbiome for YTK with enteritis versus coccidiosis disease (Task H3),
- An enteritis health extension, 2017 sampling from multiple sites, sea-cages and across diet formulations (Task H3); and
- Manipulation of the microbiome (probiotics) trial (Task H4).

<u>Component 1 – Health vs disease 2016 - enteritis</u>

- Thirty-six fish displaying various degrees of a gut enteritis disease were collected in March 2016 from two sea-cage sites.
- Twelve ‘healthy’ fish were collected from a control sea-cage displaying no visible morphological signs of infection or weight loss.
- Twelve ‘disease’ fish displaying visible morphological signs of infection including weight loss and low body condition were collected from the second sea-cage, along with 12 ‘healthy intermediate’ fish displaying no outwards signs of symptoms of disease but forming a mixed cohort with the ‘disease’ sea-cage population.
- Categorisation of the health groups was performed by Clean Seas Seafood veterinarian Dr Matt Landos.
- All fish were fed the same commercial diet prior to being sampled and were of the same year class.

	• A water sample also was taken from the healthy control and disease sea-cage sites to control for the influence of the environment on the structure and composition of the gut bacterial community. <u>Component 2 – Enteritis vs coccidiosis (SARDI: H3)</u> • Twelve fish displaying signs of coccidiosis infection were sampled from a single sea-cage at Arno Bay in July 2016. • Of the 12 fish, six were classed as 'healthy' and six were classed as 'disease' by Clean Seas Seafood health staff based on visual symptomatic features. • All fish were feed the same commercial diet prior to sampling. • A water sample was taken from the sea-cage site to control for the influence of the environment on the structure and composition of the gut bacterial community. • This dataset was merged and analysed with the 2016 enteritis health vs disease component. <u>Component 3 – Health vs disease extension</u> • As an extension to the original 2016 enteritis sampling, 20 fish were sampled in February 2017 from two sea-cages in Arno Bay (10 fish per cage), with a further 40 fish sampled in April 2017 from the same two sea-cages (20 fish per cage) after a controlled feed change. • In February, fish were fed a 4 mm diameter pellet diet (feed B). Then, in April, were transitioned from the 4 mm diameter diet to 6 mm diameter diet pellets. Finally, fish were fed 9 mm diameter pellets. • Fish in February were reported to be of ideal health, whereas in April disease onset had commenced. • In parallel, samples were taken from two sites in Port Lincoln in February 2017 where disease onset had been recorded. • A total of 78 fish were sampled from two sea-cages at Point Boston (20 fish per cage) and two sea-cages at Bickers (one cage with 18 fish sampled, second cage with 20 fish sampled). • Fish from one cage at Point Boston and one at Bickers were fed a 9 mm diameter pellet diet (feed B), whereas fish in the secondary cage at Point Boston were fed a 6 mm diameter pellet diet (feed B) and fish in the secondary cage at Bickers were fed a 9 mm diameter pellet diet (feed C). • Categorisation of the health groups was performed by Clean Seas Seafood veterinarian Dr James Fensham. • The dataset then was merged and analysed with the 2016 enteritis health vs disease component. <u>Component 4 – Manipulation of the microbiome</u> • An on-farm sea-cage was identified containing poor-performing fish (Point Boston) and ten fish were sampled from the sea-cage for pre-trial comparison in May 2018. • A total of 207 fish then were transported from the sea-cage to the SARDI pool-farm facility and held for approximately four weeks. During the initial holding period, health checks and health treatments were performed. • The trial fish were then stocked into twelve 5,000 L tanks in June 2018 with 12 fish per tank. • Inoculum was collected by stripping the faecal material from 102 'healthy' YTK on-farm at a Bickers sea-cage site. • Six treatments then were investigated: o Treatments 1 and 2: water inoculum, with and without antibiotics,

	○ Treatments 3 and 4: gavage[a] inoculum, with and without antibiotics, and ○ Treatments 5 and 6: controls (no inoculum), with and without antibiotics. • Microbiome samples were collected three days after antibiotics were administered but before inoculum was given, then at three subsequent time points: 1) 2 days post-inoculum, 2) 8 days post inoculum, and 3) 15 days post inoculum. • Two microbiome samples were collected per fish, including a hindgut scraping and skin swab, to investigate the effect of the different approaches of administering the inoculum (via gavage or into the water) on the gut and skin microbiome. • Water samples were collected on-farm from the sea-cage site and from the tank system at the commencement and conclusion of the trial to control for the influence of the environment.
Outputs	***Development of a challenge test for evaluation of the health of YTK in tank nutrition experiments (SARDI: H1) and methods for YTK health assessment*** • Substituting two thirds of the wild derived (WD) fishmeal (FM) in a 30% WD FM diet, with either poultry meal or a combination of fish meal by-product and soy protein concentrate in experimental diets had a detrimental effect on the immune responses of sub-adult YTK. • When fed highly substituted diets the natural antibody levels fell in sub-adult YTK when the fish mounted a specific immune response and did not upregulate inflammatory cells. • Vaccination with killed *Photobacterium damselae piscicida* produces a reliable immune response in sub-adult YTK. • Results of the challenge test were consistent with the Nutrition (Theme 1) findings and support the potential future reduction of WD FM in the production diet of large sub-adult YTK. ***Objective 1: Elucidating the natural dynamics of YTK gut microbiome over the commercial production cycle in response to alternate (land v sea) farming practices as a reference for downstream health and dietary assessment (SARDI: H2A/ H2B)*** <u>Components 1, 2 and 3</u> • The study catalogued the active bacterial community in YTK from wild and cultivated fish (onshore, offshore and across the commercial production cycle) along with the global community found in the surrounding environment (water sample), providing important baseline information for future studies. • Differences were observed in the microbiome between the YTK gut and water samples, highlighting that YTK are able to regulate and maintain their own environment-independent bacterial community. • The gut microbiome of wild vs cultivated YTK was distinct, with significant differences in global community structure, bacterial phyla and order composition and relative abundance of the top 15 OTUs. • A general trend of higher species evenness and diversity was observed for wild fish when compared to cultivated fish. • Within cultivated samples, differences in the microbiome were observed based on cultivation strategy (land onshore v sea-cage offshore farming), as well as across the commercial production cycle (different size class/age). • Taxa composition of older fish (1000 and 2000 g) was markedly different to the younger fish (100 to 500 g).

	• Species richness significantly reduced with age, along with greater individual variation in terms of diversity and evenness in larger, older fish. • The results highlight the need for age- and size-specific controls to be included in future sampling events, particularly health and feed surveys. ***Objective 2: Characterise and understand microbiome changes with diet and their potential implications for health and/or performance.*** <u>Components 1, 2, 3 and 4</u> • The results showed that, in general, cultivation effect (offshore tank trials vs on-shore sea-cage farms) and fish size appeared to be important drivers of change in the gut microbiome of YTK. • Gut community structure and dynamics differed between sea-cage and tank trial samples even when diet formulations overlapped (although the project team noted that host genetics may be playing a part due to different fish stocks sampled). • Dominance by a single bacterial taxon was commonly observed in the tank-based trials, while taxa diversity was greater in the sea-cage samples. • Fish fed feed C (from the formulated feeds vs natural Sardine diet component) showed down-regulation of potentially opportunistic pathogens and high diversity at the taxa level observed from both the sea-cage and tank trial samples. • This result indicated that feed C should be further investigated as a diet that promotes good gut health • Results also indicated that the inclusion of LC n-3 PUFA at moderate levels in YTK diets significantly increased gut microbiome species richness, evenness and diversity with greater representation from additional phyla and taxa. It was recommended that LC n-3 PUFA be included into YTK diets at a moderate level of inclusion (2.14 g per 100 g, i.e. diet 3) • High or low levels of dietary lipids, with and without emulsifiers, did not significantly alter the gut microbiome structure or composition. However, the project team noted that all samples (including pre-trial samples) were dominated by a single taxon (with sequence similarity to *Brevinema andersonii*, 0.740) that may have established dominance in the fish prior to commencement of the trial and been able to maintain dominance irrespective of the treatment type. • This organism warrants further investigation as it may be an opportunistic pathogen and had been previously recorded in some of the wild fish from the baseline dataset. • The study also found that reducing or replacing WD FM content in YTK formulated diets can still lead to maintenance of good gut health, with a reduction to 10% WD FM content (diets 3 and 6) or replacing with 11.32% PM (diet 4) recommended (Sarah Catalano, pers. comm., 2019). ***Objective 3: Assessing the impacts of changing health status on the microbiome of YTK.*** <u>Components 1, 2 and 3</u> • General properties of disease (coccidiosis and enteritis) and its influence on the microbiome were established, including a shift in the global community structure, dominance by one or a few taxa, a reduction in species richness, and significant decrease in diversity and evenness; collectively potentially leading to a loss in overall function. • The factors that influence diseased condition appear to be complex, as no single factor was observed to universally change the microbiome composition in enteritis disease fish in the same way. • The study recommended: 1) Further characterisation and elucidation of the involvement of OTU 10950, with

	closest taxonomic similarity to *Photobacterium* sp., as this was the only organism that was found to be enriched in all disease samples across all sites, fish sizes, year classes and feed types, and hence may be specific to the underlying disease condition or an opportunistic pathogen. 2) The classification of *Mycoplasma insons* (OTU 1, 0.420) because of its high abundance (> 90% in some individuals) in the 2016 gut enteritis disease samples. 3) Probiotics that include strains of the down-regulated taxa, particularly the *Bacillus* spp. observed in the coccidiosis disease samples, could be designed and trialled in an effort to manipulate the microbiome to allow for better health management. <u>Component 4</u> • The microbiome manipulation trial found that both the gut and skin microbiome of YTK can be modulated. • Antibiotic therapy, coupled with gavage inoculum, contributed to improved microbiome structure in the gut. • Specifically, at two days post inoculum there was an increase in taxonomic diversity and species evenness within the gut samples of fish where antibiotics and gavage inoculum were administered. This finding was attributed to an increase in the bacterial phyla and decrease in the abundance of potential opportunistic pathogens. • Differences in the global bacterial community structure of the skin samples from antibiotic treated fish administered the inoculum within the seawater also were observed at two days post inoculum. • These differences in the skin microbiome were observed at eight and 15 days post inoculum, highlighting the sensitivity and prolonged effects of such treatments on the skin microbiome of YTK. • Antibiotic therapy also resulted in a decrease in the abundance of a number of key taxa in the gut and skin.
Outcomes	• A challenge model was developed that produces a reliable, measureable immune response in YTK. • Methods for measuring YTK antibody responses were refined. • Assays for a range of immune cytokines were identified as suitable for use in Australian YTK. • Baseline data on the microbiome of wild and cultivated YTK now can be used as a critical reference point to then investigate when conditions change from the norm. • Sampling methods have been refined allowing for more YTK gut samples to be processed for the same cost (a single hindgut scraping is representative rather than a separate fore-, mid- and hindgut scraping per fish). • Adoption of project recommendations will depend on feed manufacturing and retail costs. In most cases, it was recommended that industry conduct pilot scale trials before implementing the recommendations commercially. • Early-detection markers of changing health status for coccidiosis and enteritis infections are proposed and may be used to design cheaper, more rapid molecular assays (e.g. using q-PCR) which can be implemented as part of routine health surveys for the early detection and intervention of disease in YTK. • Probiotics could be trialled that include strains of down-regulated taxa that were identified in disease samples to improve health outcomes. • A catalogue of bacterial taxa associated with disease in YTK has been presented. Confirmation of species identification of these bacterial taxa is suggested to then determine potential pathogenic capabilities which can allow for appropriate control measures to be implemented to improve health outcomes.

	• A catalogue of bacterial taxa from the skin and gut of YTK that were negatively affected by antibiotic treatment also was presented. • Results from the novel microbiome manipulation trial highlighted that both the gut and skin microbiome can be modulated, with antibiotic therapy coupled with gavage inoculum contributing to improved microbiome structure. Further work, using advanced omics-based techniques, is required to correlate increases in microbiome diversity with improved health outcomes. • There is also value of this microbiome data beyond its immediate use, including novel approaches to optimising fish health in the next 10-20 years (as is now being explored for human health issues – e.g. microbiome transplant as an alternative to conventional therapies reliant on antibiotics to treat human diseases).
Impacts	• Potentially, increased productivity and profitability for YTK producers through improved YTK diet formulation. This includes improvements from: o Reduced input costs along the supply chain, o Improvements in SGR/FCR or other performance measures as a result of improved feed composition for different water temperatures and/or YTK size-classes, and o Avoided production losses because of improved management of YTK nutritional health (i.e. reduced incidence of disease). • Improved environmental sustainability of YTK feed through reduced use of wild derived fishmeal leading to lower FIFO ratios for commercial feed. • Potentially, reduced output of nitrogen, phosphorus and carbon from YTK farms as a result of improved feed utilisation and reduced feed wastage through improved feed formulations, feeding strategies and/or use of the improved bioenergetics model (Mark Booth, pers. comm., 2019). • Contribution to the enhancement and/or maintenance of the Australian YTK aquaculture industry's social licence to operate. • Enhanced community well-being as a result of regional spill-overs from a more productive and profitable YTK aquaculture industry. • Increased scientific and industry capacity.

[a] Gavage: the administration of food or drugs by force, especially to an animal, typically through a tube leading down the throat to the stomach.

Table 5.1.4: Logical Framework for K4P Activity 5 (Extending YTK Capacity)

Rational	Communication and extension activities were considered fundamental to K4P R&D quality assurance and technology transfer to industry (Steven Clarke, pers. comm., 2018). Activity 5 (extending YTK capacity) was developed to address the K4P project's communication and extension requirements and to underpin a range of training and capacity building activities targeted at addressing the Rural R&D for Profit Program's three aims: • generating knowledge, technologies, products or processes that benefit primary producers, • strengthening pathways to extend the results of rural R&D, including understanding the barriers to adoption, and • establishing and fostering industry and research collaborations that form the basis for ongoing innovation and growth of Australian agriculture.
Objectives	The specific objectives of Activity 5 included: 1) Conduct annual workshops and provide peer reviewed publications, Project reports and produce regular articles for the FRDC FISH magazine to extend the outputs from the Project to industry participants, and the broader aquaculture industry, scientific community and public in line with Output 1(c). 2) Student training to develop the next generation of industry R&D providers, including up to 3 postdoctoral research fellows, up to 6 PhD students and up to 12 Honours students. 3) Incorporate the outcomes of the Project into the new sub-program established by the FRDC for the development of new and emerging aquaculture growth opportunities to allow the direct extension and translation of outputs to potential wider 'white' fish and other new and emerging aquaculture opportunities.
Activities	• A K4P project Steering Committee (SC) was appointed and met throughout the research program between July 2015 and December 2018. • Regular SC teleconferences and ad-hoc meetings were held throughout the life of the K4P project. • A communication and extension strategy for the K4P project was established. • An internal K4P project titled: '*Aquafeed Manufacturing Company Engagement Plan*' was developed and ratified by the SC. • Regular K4P technical group teleconference meetings were conducted including representatives from each participating research organisation, YTK farm companies and aquafeed manufacturers. • Monthly K4P updates were provided to Clean Seas Seafood by SARDI and NSW DPI researchers and aquaculture managers met fortnightly with Huon Aquaculture management and technical staff. • Regular K4P stakeholder updates were released on the NSW DPI webpage: https://www.dpi.nsw.gov.au/fishing/aquaculture/starting-up/finfish-aquaculture-lease-modification-application • A 'Memorandum of Understanding' was established between FRDC and Ridley, and between FRDC and Skretting Australia, to facilitate collaborative interactions between the K4P project and the two aquafeed companies. • Annual K4P Research Workshops were held in May 2016 (Adelaide), December 2016 (Port Stephens), August 2017 (Adelaide), and September 2018 (Adelaide). The workshops ranged in length from half a day to one and a half days and typically included presentations by the K4P Theme Leaders, researchers and post-graduate students. Guest speakers from the

	participating industry organisations (Skretting Australia, Ridley, Huon Aquaculture, and Clean Seas Seafood) also presented. • The first three Research Workshops were attended by WA YTK researchers and industry representatives from Indian Ocean Fresh Australia Pty Ltd (IOFA) (WA). • Six Honours and Masters student scholarships were undertaken as part of the K4P project. Students were: 1) D. Jackson (Flinders University, SA), 2) C. Candebat (University of Hamburg, Germany in conjunction with Macquarie University, NSW), 3) L. Kuerschner (Flinders University, SA), 4) T. Legrand (Flinders University, SA), 5) M. Rubio (Wageningen University, the Netherlands), and 6) A. Teoh (Flinders University, SA). • Six PhD student scholarships were supported as part of the K4P project. Students were: 1) S. Chown (University of Adelaide, SA), 2) B. Crowe (Flinders University, SA), 3) T. Legrand (University of Adelaide, SA), 4) C. Candebat (James Cook University), 5) T.M. Chinh (University of the Sunshine Coast), and 6) A. Liu (University of New South Wales). • Three Postdoctoral fellows (or equivalent) were employed as part of the K4P project. They were: 1) Dr M. Bansemer (SARDI), 2) Dr S. Catalano (SARDI), and 3) Dr I. Pirozzi (NSW DPI – James Cook University). • In conjunction with the Research Workshops, three separate Student Personal Development Workshops were held for post-graduate students involved with the K4P project. These events also were attended by some junior researchers and technicians, including some from industry. • K4P researchers attended and gave presentations at several national and international conferences and a number of scientific publications, conference posters/abstracts were published. • A large number of university graduate students gained work experience and/or were employed by SARDI as casual technical staff on the K4P project (Steven Clarke, pers. comm., 2019).
Outputs	***University Theses (Published):*** • Jackson, D. (2018). *Effect of variable oxygen levels and feeding frequency on digestive efficiency of large Yellowtail Kingfish (Seriola lalandi) at summer water temperature*. Honours thesis, College of Science and Engineering, Flinders University, South Australia, Australia. 69pp. • Candebat, C. (2017*). Effect of lipid sources and temperature on the critical oxygen level (Pcrit), hypoxia tolerance and routine metabolic rate of juvenile Yellowtail Kingfish (Seriola lalandi).* MSc thesis, Institute of Hydrobiology and Fisheries Science, University of Hamburg in collaboration with Macquarie University, Sydney, Australia and the Port Stephens Fisheries Institute, Port Stephens, Australia. 62pp. • Kuerschner, L. (2016). *Regulation of muscle growth in Yellowtail Kingfish (Seriola lalandi) under fasting and re-feeding conditions*. Honours thesis, Faculty of Science and Engineering, School of Biological Sciences, Flinders University, South Australia, Australia. • Legrand, T. (2016). *Effect of health status on the microbiome of the skin and gill mucosa of Australian Yellowtail Kingfish*. Honours thesis, Faculty of

Science and Engineering, School of Biological Sciences, Flinders University, South Australia, Australia.

- Rubio, M. (2016). *Effect of dissolved oxygen on utilisation of digestible protein and energy in juvenile Yellowtail Kingfish*. MSc thesis, Wageningen University and Research Centre, Holland.
- Teoh, A (2016). *Physiological response to stress in Yellowtail Kingfish (Seriola lalandi) at different oxygen levels under summer temperatures.* Honours thesis, Faculty of Science and Engineering, School of Biological Sciences, Flinders University, South Australia, Australia.

Scientific Publications, and International and National Conference Posters/Abstracts and Presentations (in date order by publication type):

- Bansemer, M.S., Stone, D.A.J., Skordas, P. (2017). *Intermittent feed-induced hypoxia effects the growth and feed utilisation of large Yellowtail Kingfish (Seriola lalandi) at warm water temperatures.* Poster presentation at World Aquaculture 2017, Cape Town, South Africa, 26-30 June 2017.
- Bansemer, M.S., Stone, D.A.J., D'Antignana, T., Skordas, P., Kuerschner, L., Currie, K-L. (2018). *Optimising feeding strategies for Yellowtail Kingfish (Seriola lalandi) at winter water temperatures.* North American Journal of Aquaculture 80, 128-140.
- Bansemer, M., Stone, DAJ; Skordas, P; Nankervis, L., Salini, M. (2018). *Reducing wild derived dietary fish meal inclusion levels in production diets for large Yellowtail Kingfish (Seriola lalandi).* Presentation at Australian Marine Science Association (AMSA) 2018 Conference, Adelaide, South Australia, 1-5 July 2018.
- Candebat, C., Booth, M., Codabaccus, B.M., Pirozzi, I., n.d. Methionine requirement and the sparing effect of cysteine in juvenile Yellowtail Kingfish (*Seriola lalandi*). Aquaculture (in preparation).
- Candebat, C., Pirozzi, I., Codabaccus, M.B., Booth, M. (2018*). Dietary methionine spares taurine in juvenile Yellowtail Kingfish (Seriola lalandi).* Presentation at the 18th International Symposium on Fish Nutrition and Feeding, Las Palmas de Gran Canaria, Spain, 3-7 June 2018.
- Catalano, S.R., Oxley, A.P.A. (2017*). The gut microbiome of Yellowtail Kingfish (YTK) under alternative farming conditions and changing health status.* Poster presentation at the Australian Microbial Ecology Conference, Melbourne, Australia, 13-15 February 2017.
- Chinh, T.M. Dama, Abigail Elizura, Tomer Ventura, Michael Salini, Richard Smullen, Igor Pirozzi and Mark Booth (in review – Nov 2018). *Apparent digestibility of common raw materials by Yellowtail Kingfish (Seriola lalandi).* Aquaculture Journal.
- Chown, S. (2017). *Lipid utilisation in Yellowtail Kingfish (Seriola lalandi).* Presentation at SARDI Aquatic Sciences annual Student Seminars Session, SARDI, Adelaide, South Australia, 21 June 2017.
- Chown, S., McWhorter, T.J., Carragher, J., Bansemer, M., Gibson, R., Stone, D.A.J. (2018). *Reducing long chain omega 3 polyunsaturated fatty acids in formulated diets for harvest size Yellowtail Kingfish (Seriola lalandi) – is there a trade-off between omega 3 and omega 9 in some tissues?* Poster presentation at the 18th International Symposium on Fish Nutrition and Feeding, Las Palmas de Gran Canaria, Spain, 3-7 June 2018.
- Crowe, B. (2017). *Restricted feeding with diets of differing energy levels affects liver structure in cultured Yellowtail Kingfish, Seriola lalandi, at summer temperatures.* Presentation at SARDI Aquatic Sciences annual Student Seminars Session, SARDI, Adelaide, South Australia, 21 June 2017.
- Crowe, B.H., Bansemer, M.S., Harris, J.O., McWhorter, T.J., Stone, D.A.J. (2018). *Effects of partial wild derived fish meal replacement on bile acid production and liver structure in Yellowtail Kingfish, Seriola lalandi*. Poster

	presentation at the 18th International Symposium on Fish Nutrition and Feeding, Las Palmas de Gran Canaria, Spain, 3-7 June 2018. • Dam, C.T.M., Elizura, A., Ventura, T., Salini, M., Smullen, R., Pirozzi, I., Booth, M. Apparent digestibility of common raw materials by Yellowtail Kingfish (*Seriola lalandi*). Aquaculture (under review). • Legrand, T.P.R.A., Camarinha-Silva, A., Wynne, J.W., Weyrich, L.S., Oxley, A.P.A., 2019. Investigating the role of mucosal microbiomes in Yellowtail Kingfish exhibiting different health status using a multi-omics approach. The 5th FRDC Australasian Scientific Conference on Aquatic Animal Health and Biosecurity, Cairns, Queensland, Australia. • Legrand, T.P.R.A., Catalano, S.R., Oxley, A.P.A. (2017). *The inner workings of the outer surface: mucosal barrier bacterial assemblages as indicators of changing health status in Yellowtail Kingfish, Seriola lalandi.* Poster presentation at Australian Microbial Ecology Conference, Melbourne, Australia, 13-15 February 2017. • Legrand, T., Catalano, S., Oxley, A. (2017). *The inner workings of the outer surface: skin and gill mucosal barrier bacterial assemblages as indicators of changing gut health in Yellowtail Kingfish, Seriola lalandi.* Presentation at the Australian Society for Microbiology students award night, University of South Australia, 16 March 2017. • Legrand, T., Catalano, S., Oxley, A. (2018). *Mucosal microbiomes of farmed Yellowtail Kingfish (Seriola lalandi) and markers of changing health status.* Presentation at Australian Marine Science Association (AMSA) 2018 Conference, Adelaide, South Australia, 1-5 July 2018. • Legrand, T.P.R.A., Catalano, S.R., Oxley, A.P.A. (2018*). Mucosal microbiomes of the commercially important species Yellowtail Kingfish (Seriola lalandi) and markers of changing health status.* Poster presentation at the 19th Fish Immunology Workshop, Wageningen University and Research, Wageningen, The Netherlands, 29 April-3 May 2018. • Legrand, T.P.R.A., Catalano, S.R., Wos-Oxley, M.L., Stephens, F., Landos, M., Bansemer, M.S., Stone, D.A., Qin, J.G., Oxley, A.P.A. (2018). *The inner workings of the outer surface: skin and gill microbiota as indicators of changing gut health in Yellowtail Kingfish.* Frontiers in Marine Biology 8, 1-17. DOI: 10.3389/fmicb.2017.02664 • Legrand, T., Oxley, A., Weyrich, L. and Wynne, J. (2018). *The functional role of mucosal microbiomes in fish health.* Presentation at SARDI Aquatic Sciences annual Student Seminars Session. SARDI, Adelaide, South Australia, 25 July 2018. • Legrand, T.P.R.A., Wynne, J.W., Weyrich, L.S., Oxley, A.P.A., n.d. A microbial sea of possibilities: current knowledge and prospects for an improved understanding of the fish microbiome. Reviews in Aquaculture (under review). • Liu, A., Pirozzi, I., Codabaccus, B., Hines, B., Simon, C., Sammut, J., Booth, M., Accepted. Digestible choline requirement of juvenile Yellowtail Kingfish (*Seriola lalandi*). Aquaculture. • Liu, A., Pirozzi, I., Codabaccus, B., Simon, C., Hines, B., Sammut, J., Booth, M. (2018). *Digestible choline requirement for juvenile Yellowtail Kingfish (Seriola lalandi).* Poster presentation at the 18th International Symposium on Fish Nutrition and Feeding, Las Palmas de Gran Canaria, Spain, 3-7 June 2018. • Pirozzi, I., Benito, M.R., Booth, M. (2017*). Low dissolved oxygen affects amino acid utilisation and maintenance requirements in Yellowtail Kingfish Seriola lalandi.* Poster presentation at World Aquaculture 2017, Cape Town, South Africa, 26-30 June 2017.

	• Pirozzi, I., Benito, M.R., Booth, M. n.d. Protein, amino acid and energy utilisation and maintenance requirements of juvenile Yellowtail Kingfish (*Seriola lalandi*): quantifying abiotic influences. Aquaculture (in preparation). • Pirozzi, I., Candebat, C.L., Booth, M. (2017*). The critical oxygen threshold of Yellowtail Kingfish Seriola lalandi acclimated to 15°C and 20°C.* Poster presentation at World Aquaculture 2017, Cape Town, South Africa, 26-30 June 2017. • Stone, D.A.J., Bansemer, M.S., D'Antignana, T., Skordas, P., Kuerschner, L., Currie, K. (2016). *Evaluation of different feeding strategies for the production of Yellowtail Kingfish at winter water temperatures*. Poster presentation at International Symposium on Fish Nutrition and Feeding, Sun Valley, Idaho, USA, 5 - 10 June 2016. • Stone, D.A.J., Bansemer, M.S., Skordas, P., Chown, S., Ruff, N. (2018). *Practical dietary long-chain omega-3 polyunsaturated fatty acid requirements for large Yellowtail Kingfish (Seriola lalandi).* Poster presentation at the 18th International Symposium on Fish Nutrition and Feeding, Las Palmas de Gran Canaria, Spain, 3-7 June 2018.
Outcomes	***Communication and Extension Outcomes:*** Some of the outcomes of the broader K4P communication and extension activities, and capacity building activities, are likely to have included: <u>Undergraduate and Graduate Students:</u> • Improved research capability through participation in applied research and collaboration with K4P research personnel. • Enhanced collaboration, planning, and networking skills. <u>Post-Graduate Students:</u> • Useful exposure to research and industry personnel in alternative activities along the supply chain and hence a better understanding of the industry as a whole, as well as improved networking. • Positive networking with commercial aquaculture companies to discuss areas of common ground, industry needs, and research/adoption constraints. • Improved performance associated with people management; in particular, how to deal with internal and external stakeholders. <u>Industry Participants:</u> • Enhanced capacity to undertake independent and collaborative YTK R&D. • Improved capacity to undertake industry leadership roles including planning activities. • Encouragement of improved cross-industry communication and networking. • Some contribution to increased industry cohesion and collaboration. • Contribution to increased adoption of YTK R&D outputs. ***Other Outcomes:*** • Many additional publications (including up to six PhD theses) are expected to be produced over the next few years as a result of the K4P project (Steven Clarke, pers. comm., 2019). • Between 2016 and 2018, FRDC established several aligned projects with the Challenger Institute of Technology (Western Australia), the WA Department of Fisheries, and IOFA, led by Principal Investigator Dr Gavin Partridge. • The key project was to meet the same three Rural R&D for Profit Program objectives with activities addressing the same YTK industry R&D priority under similar subproject themes (Nutrition, Feeding Strategies, and Health) • Specific objectives of the WA project were to: 1) Benchmark the performance of Yellowtail Kingfish grown in waters representative of the warm waters of the mid-west of WA,

	2) Determine the effect of different commercial diets on the health status of Yellowtail Kingfish, and 3) Compare performance of Yellowtail Kingfish derived from WA and SA broodstock. • A separate YTK Health Training Workshop was held in September 2018, (supported by an additional grant Project No.: CSIRO AAHTS 2018.01). The training workshop arose because of requests from K4P post-graduate students, was organised by participants of the K4P project and was jointly funded by DAWR, FRDC and CSIRO. • Twenty-five participants attended the training workshop (including the trainers). Participants included students, researchers and industry personnel from WA, SA, NSW, TAS and QLD (including from YTK, Barramundi, and Cobia farms). • The aim of the workshop was to increase the capacity of K4P participants (particularly post-graduate students), and participants from other 'white flesh' finfish industry sectors, with respect to YTK health R&D. • The training workshop was considered a success by those that attended and a final project report on the activities and outcomes of the workshop was prepared by Steven Clarke (K4P Executive Officer, SARDI).
Impacts	• Contribution to improved productivity and profitability for the Australian YTK aquaculture industry through increased adoption of YTK R&D outputs. • Potentially, some contribution to increased efficiency of future YTK R&D through the strengthening of YTK R&D networks across regions and between industry participants. • Increased scientific and industry research capacity.

Table 5.1.5: Annual Investment in the K4P project (nominal $)

Year Ended 30 June	**2016**	**2017**	**2018**	**Total**
DAWR ($)	932,679	1,250,653	816,668	3,000,000
FRDC ($)	233,170	312,664	204,167	750,001
Others ($)	724,630	945,418	629,951	2,299,999
Total	**1,890,479**	**2,508,735**	**1,650,786**	**6,050,000**

Table 5.1.6: Triple Bottom Line Categories of Principal Impacts from the K4P Project Investment

Category	**Principal Impacts**
Economic	• Increased productivity and profitability for YTK producers through improved YTK diet formulation and the implementation of optimised feeding strategies. The impact is likely to be driven by: o Reduced input costs along the YTK supply chain, o Improvements in SGR/FCR or other YTK performance measures, and o Reduced production losses because of improved management of YTK nutritional health (i.e. reduced incidence of disease). • Potentially, some contribution to increased efficiency of future YTK R&D through the strengthening of YTK R&D networks across regions and between industry participants.
Environmental	• Improved environmental sustainability of YTK feed through reduced use of wild derived fishmeal leading to lower FIFO ratios for commercial feed. • Potentially, reduced output of nitrogen, phosphorus and carbon from YTK farms as a result of improved feed utilisation and reduced feed wastage

	through improved feed formulations, feeding strategies and/or use of the improved bioenergetics model.
Social	• Contribution to the enhancement and/or maintenance of the Australian YTK aquaculture industry's social licence to operate. • Enhanced regional community well-being as a result of spill-overs from a more productive and profitable YTK aquaculture industry. • Increased scientific and industry research capacity.

Table 5.1.7: Australian Government Research Priorities[a]

Australian Government	
Rural RD&E Priorities (est. 2015)	**Science and Research Priorities (est. 2015)**
1. Advanced technology 2. Biosecurity 3. Soil, water and managing natural resources 4. Adoption of R&D	1. Food 2. Soil and Water 3. Transport 4. Cybersecurity 5. Energy and Resources 6. Manufacturing 7. Environmental Change 8. Health

[a] Sources: (DAWR, 2015) and (Office of the Chief Scientist, 2015).

Table 5.1.8: Summary of Assumptions

Variable	Assumption/Value	Source
General Data		
Maximum potential annual YTK production (sales volume) for SA, NSW, and WA.	61,400 tonnes p.a. by 2030	Based on commercial YTK lease holdings in SA (11,000 tonnes) (Clean Seas Seafood, 2018b) and production projections for NSW (2,400 tonnes) (NSW Department of Planning, 2016) and WA (48,000 tonnes) (DPIRD, 2017b)
Probability of WA production being achieved	20%	Agtrans Research after consultation with K4P project personnel. Note: major commercial production in the Mid West aquaculture zone in WA has not yet commenced but is expected within the next four years (by 2021). A risk factor has been incorporated into the valuation to account for this WA production uncertainty.
Average YTK farm gate price	$12.73/kg	3-year average (2016-2018) (Clean Seas Seafood, 2018b).
Impact 1: Increased Productivity		
WITH K4P project Investment		
Average annual growth of Australian YTK aquaculture production (sales volume) from 2021	1,460 tonnes p.a. from 2021	Based on expected maximum YTK production for SA, NSW and WA by 2030 of 23,000 tonnes (SA 11,000 t + NSW 2,400 t + WA 20% × 48,000 t) and Clean Seas Seafood production of 1,098 tonnes in 2014/15.
WITHOUT K4P project Investment		
Reduction to the annual production growth rate for YTK	5.0%	Agtrans Research based on consultation with industry stakeholders
Average annual growth of Australian YTK aquaculture production (sales volume) – rate that would likely have occurred WITHOUT the K4P project investment	Approximately 1,387 tonnes p.a.	(1 - 0.05) × 1,460 t p.a.
Timing of Impact		
First year of impact	2016/17	Based on Clean Seas Seafood adoption of early K4P project outputs
Year of maximum impact	2020/21	Agtrans Research
Additional Costs		
Additional costs to industry to adopt/implement K4P outputs	Nil	Agtrans Research based on consultation with industry stakeholders

Risk Factors		
Probability of output	100%	Based on successful completion of the K4P project investment
Probability of outcome (usage)	60%	Agtrans Research based on consultation with industry stakeholders
Probability of impact	80%	Agtrans Research based on consultation with industry stakeholders and taking into account environmental and commercial scale factors
Impact 2: Increased Profitability		
WITH K4P project Investment		
Average annual growth of Australian YTK aquaculture production (sales volume) from 2021.	1,460 tonnes p.a. from 2021	As above (see Impact 1)
Average time for YTK grow out (from transition to sea-cage to harvest)	350 days	Based on average time to maturity of 16 months less approximately 100 days pre-sea-cage transition (OceanWatch Australia, 2017)
Average weight of YTK at harvest	4.0 kg per fish	(OceanWatch Australia, 2017)
Estimated economic FCR[a]	2.38	(Clean Seas Tuna Ltd, 2016)
Average YTK aquafeed (pellet) cost saving	$105 per tonne	Mean estimate based on a potential savings range of $60 to $150 per tonne. Saving likely to depend on the quantity of FM substituted and the value of the alternative protein source(s) used (Michael Salini, pers. comm., 2018)
First year of impact	2020/21	Agtrans Research based on consultation with industry stakeholders.
WITHOUT K4P project Investment		
Reduction to the annual production growth rate for YTK	5%	As above (see Impact 1)
Average annual growth of Australian YTK aquaculture production (sales volume) – rate that would likely have occurred WITHOUT the K4P project investment	1,387 tonnes p.a.	
First year of impact	2025/26	5-year delay for private producers to conduct independent feed formulation R&D. Based on consultation with industry stakeholders.
Additional Costs		
Additional costs to industry to adopt/implement K4P outputs	Nil	Agtrans Research based on consultation with industry stakeholders

Risk Factors		
Probability of output	100%	Based on successful completion of the K4P project investment
Probability of outcome (usage)	80%	Agtrans Research based on consultation with industry stakeholders
Probability of impact	80%	Agtrans Research based on consultation with industry stakeholders and taking into account environmental and commercial scale factors
Impact 3: Enhanced Social Licence		
WITH K4P project Investment		
Estimated Gross Value of Production (GVP) for Australian YTK aquaculture	$42 million	Based on Clean Seas Seafood sales revenue 2017/18 (Clean Sea Seafood, 2018a)
Percentage of marine farms assumed to be at risk of loss of social licence	50%	Agtrans Research
GVP for affected farms only	$21.0 million	$42 m × 50%
First year of impact	2018/19	Year of completion of the K4P project investment
Last year of impact	2022/23	Based on counterfactual assumption that relevant R&D would have occurred later
Aquaculture profits as a proportion of GVP	10% ($2.1 million)	Agtrans Research. Estimate based on consultant experience and Clean Seas Seafood annual report information
Risk of reduction in profitability as a result of a loss of social licence – WITH	10%	Agtrans Research
Expected profitability benefit	$52,500 p.a.	($2.1 m × 10%) – ($2.1 m × 7.5%)
WITHOUT K4P project Investment		
Risk of reduction in profitability as a result of a loss of social licence – WITHOUT	7.5%	Agtrans Research

[a] FCR represents the number of units of 'dry' aquafeed required to produce a unit of 'wet' fish or crustacean. However, the economic FCR (or eFCR) takes into account fish mortalities and losses and therefore measures actual feed demand (New and Wijkstrom, 2002).

Table 5.1.9: Investment Criteria for Total Investment in the K4P project

Investment Criteria	**Years after Last Year of Investment**						
	0	**5**	**10**	**15**	**20**	**25**	**30**
PVB ($m)	0.52	18.09	78.00	126.63	126.63	126.63	126.63
PVC ($m)	7.37	7.37	7.37	7.37	7.37	7.37	7.37
NPV ($m)	-6.85	10.72	70.63	119.26	119.26	119.26	119.26
BCR	0.07	2.45	10.58	17.17	17.17	17.17	17.17
IRR (%)	negative	26.5	44.5	46.5	46.5	46.5	46.5
MIRR (%)	negative	32.9	37.5	29.3	22.4	18.6	16.1

Table 5.1.10: Investment Criteria for DAWR Investment in the K4P project

Investment Criteria	Years after Last Year of Investment						
	0	**5**	**10**	**15**	**20**	**25**	**30**
PVB ($m)	0.26	8.95	38.58	62.64	62.64	62.64	62.64
PVC ($m)	3.65	3.65	3.65	3.65	3.65	3.65	3.65
NPV ($m)	-3.39	5.30	34.93	58.99	58.99	58.99	58.99
BCR	0.07	2.45	10.58	17.17	17.17	17.17	17.17
IRR (%)	negative	26.5	44.5	46.5	46.5	46.5	46.5
MIRR (%)	negative	32.9	37.5	29.3	22.4	18.6	16.1

Table 5.1.11: Investment Criteria for FRDC Investment in the K4P project

Investment Criteria	Years after Last Year of Investment						
	0	**5**	**10**	**15**	**20**	**25**	**30**
PVB ($m)	0.07	2.28	9.84	15.97	15.97	15.97	15.97
PVC ($m)	0.93	0.93	0.93	0.93	0.93	0.93	0.93
NPV ($m)	-0.86	1.35	8.91	15.04	15.04	15.04	15.04
BCR	0.07	2.45	10.58	17.17	17.17	17.17	17.17
IRR (%)	negative	26.5	44.5	46.5	46.5	46.5	46.5
MIRR (%)	negative	32.9	37.5	29.3	22.4	18.6	16.1

Table 5.1.12: Contribution of Benefits

Impact	PVB ($m)	% of Total PVB
Impact 1: Increased productivity	117.63	92.9
Impact 2: Increased profitability	8.77	6.9
Impact 3: Enhanced social licence	0.24	0.2
Total	126.63	100.0

Table 5.1.13: Sensitivity to Discount Rate (Total investment, 30 years)

Investment Criteria	Discount rate		
	0%	**5% (base)**	**10%**
PVB ($m)	190.15	126.63	87.77
PVC ($m)	6.67	7.37	8.12
NPV ($m)	183.48	119.26	79.65
BCR	28.50	17.17	10.80

Table 5.1.14: Sensitivity to the Assumed Percentage Reduction in the Annual YTK Production Growth Rate (Total investment, 30 years)

Investment Criteria	**Percentage Reduction in the Annual YTK Production Growth Rate (without K4P project)**		
	2.5%	**5% (base)**	**10%**
PVB ($m)	106.74	126.63	168.93
PVC ($m)	7.37	7.37	7.37
NPV ($m)	99.37	119.26	161.55
BCR	14.47	17.17	22.91

Table 5.1.15: Sensitivity to the Assumed Aquafeed Cost Saving ($/t) (Total investment, 30 years)

Investment Criteria	**Average Cost Saving for YTK Aquafeed ($/t)**		
	$60/t	**$105/t (base)**	**$150/t**
PVB ($m)	122.87	126.63	130.39
PVC ($m)	7.37	7.37	7.37
NPV ($m)	115.50	119.26	123.01
BCR	16.66	17.17	17.68

Table 5.1.16: Sensitivity to the Assumed Average Farm-Gate Price for Australian YTK ($/kg) (Total investment, 30 years)

Investment Criteria	**Average Farm-Gate Price for YTK ($/kg)**		
	$6.37/kg (50% base)	**$9.55/kg (75% base)**	**$12.73/kg (base)**
PVB ($m)	67.82	97.22	126.63
PVC ($m)	7.37	7.37	7.37
NPV ($m)	60.44	89.85	119.26
BCR	9.20	13.18	17.17

Table 5.1.17: Sensitivity to the Assumed Average Farm-Gate Price for Australian YTK ($/kg) (Total investment, 30 years)

Investment Criteria	**Probability that WA YTK Aquaculture Production Achieved**		
	0%	**50% (base)**	**100%**
PVB ($m)	22.10	126.63	616.10
PVC ($m)	7.37	7.37	7.37
NPV ($m)	14.73	119.26	608.72
BCR	3.00	17.17	83.54

Table 5.1.18: Confidence in Analysis of Project[a]

Coverage of Benefits	**Confidence in Assumptions**
Medium	Medium

[a] The rating categories used are High, Medium and Low, where; 1) High denotes a good coverage of benefits or reasonable confidence in the assumptions made; 2) Medium denotes only a reasonable coverage of benefits or some uncertainties in assumptions made; and 3) Low denotes a poor coverage of benefits or many uncertainties in assumptions made.

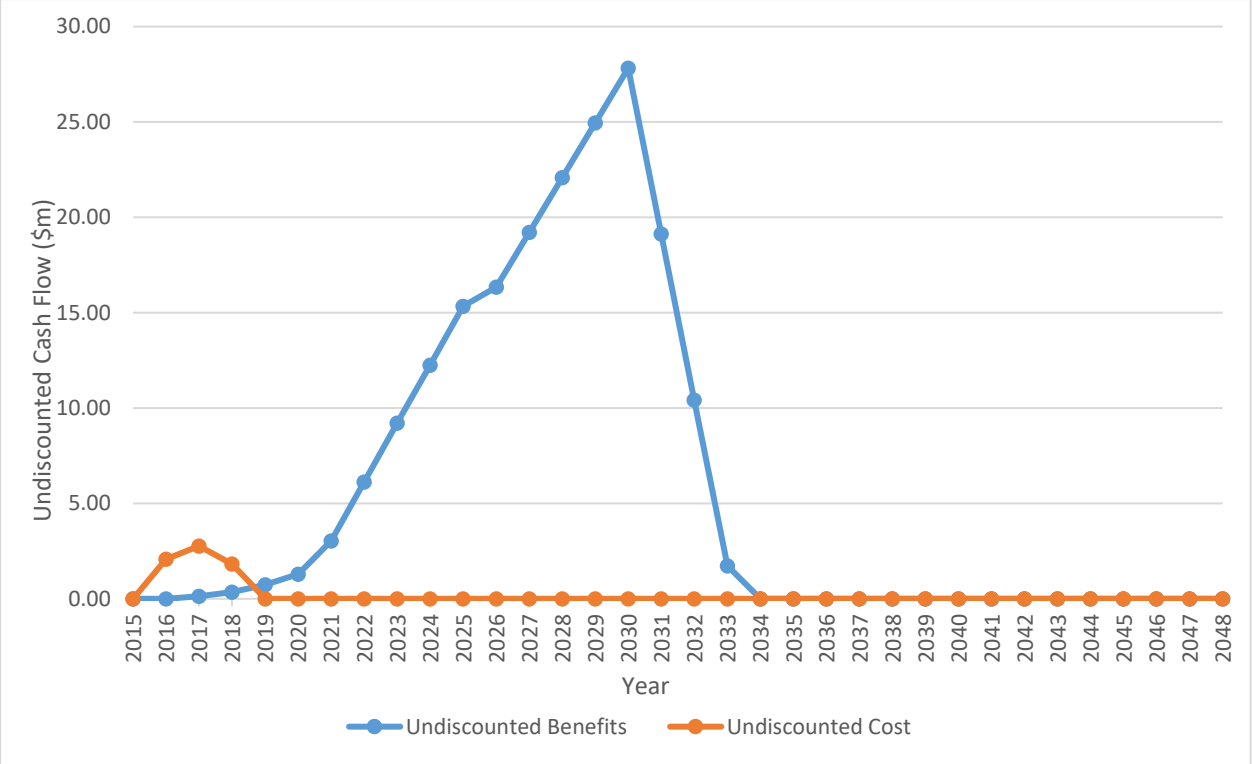


Figure 5.1.1: Annual Cash Flow of Undiscounted Total Benefits and Total Investment Costs

Appendix 1. Glossary of Economic Terms

Economic Term	Definition
Cost-benefit analysis:	A conceptual framework for the economic evaluation of projects and programs in the public sector. It differs from a financial appraisal or evaluation in that it considers all gains (benefits) and losses (costs), regardless of to whom they accrue.
Benefit-cost ratio:	The ratio of the present value of investment benefits to the present value of investment costs.
Discounting:	The process of relating the costs and benefits of an investment to a base year using a stated discount rate.
Internal rate of return:	The discount rate at which an investment has a net present value of zero, i.e. where present value of benefits = present value of costs.
Investment criteria:	Measures of the economic worth of an investment such as Net Present Value, Benefit-Cost Ratio, and Internal Rate of Return.
Modified internal rate of return:	The internal rate of return of an investment that is modified so that the cash inflows from an investment are re-invested at the rate of the cost of capital (the re-investment rate).
Net present value:	The discounted value of the benefits of an investment less the discounted value of the costs, i.e. present value of benefits - present value of costs.
Present value of benefits:	The discounted value of benefits.
Present value of costs:	The discounted value of investment costs.

Appendix 2. Acronyms and Abbreviations

Acronym/Abbreviation	Definition
ANOVA	Analysis of Variance
ADC	Apparent Digestibility Coefficient
ABS	Australian Bureau of Statistics
BCR	Benefit-Cost Ratio
BW	Body Weight
CO	Canola Oil
CSS	Clean Seas Seafood Ltd
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CRRDC	Council of Rural Research and Development Corporations
CL	Crude Lipid
CP	Crude Protein
DAWR	Department of Agriculture and Water Resources (Commonwealth)
DO	Dissolved Oxygen
DPIRD	Department of Primary Industries and Regional Development (WA)
FCR	Feed Conversion Ratio
F1	First Generation
FIFO	Fish In-Fish Out
FO	Fish Oil
FRDC	Fisheries Research and Development Corporation
FM	Fishmeal
GVP	Gross Value of Production
Huon Aquaculture	Huon Aquaculture Group Ltd
IOFA	Indian Ocean Fresh Australia Pty Ltd
IRR	Internal Rate of Return
K4P	Kingfish for Profit Project
LC n-3 PUFA	Long Chain Omega-3 Polyunsaturated Fatty Acids
MIRR	Modified Internal Rate of Return
NPV	Net Present Value
NSW	New South Wales
NSW DPI	Department of Primary Industries (NSW)
OCS	Office of the Chief Scientist
OTU	Operational Taxonomic Unit
PSFI	Port Stephens Fisheries Institute
PM	Poultry Meal
PO	Poultry Oil
PVB	Present Value of Benefits
PVC	Present Value of Costs
PER	Protein Efficiency Ratio
QLD	Queensland
RAS	Recirculating Aquaculture System
R&D	Research and Development
RDC	Research and Development Corporation
RD&E	Research, Development and Extension
Ridley	Ridley Corporation Ltd
SA	South Australia
SARDI	South Australian Research and Development Institute
SPC	Soy Protein Concentrate
SBM	Soybean Meal
SGR	Specific Growth Rate
SC	Steering Committee
TAS	Tasmania
WA	Western Australia

WD FM	Wild Derived Fishmeal
YTK	Yellowtail Kingfish

6. General Discussion and Conclusions

This project (Growing a profitable, innovative and collaborative Australian Yellowtail Kingfish Aquaculture Industry: bringing 'white' fish to market. RnD4Profit-14-01-027) ran from 2015 to 2019 and was focused on growing the production and profitability of the key existing Australian Yellowtail Kingfish (YTK) industry participants, as well as the industry as a whole, and directly addresses FRDC's new strategic plan to build Australian sustainable aquaculture development through the activities of the 'New and Emerging Aquaculture Opportunities' (NEAO) Subprogram. The project also aligns with the National Marine Science Plan to grow the blue economy, the National Aquaculture Statement and Strategy to grow Australian aquaculture production, and the National Research Providers Network to better coordinate fisheries and aquaculture R&D resources nationally.

In the overall project, there were three main research themes that were aimed at developing more cost effective, sustainable feeds and feeding strategies to enhance growth and health of cultured juvenile and sub-adult YTK; which was identified as the YTK industry's highest common R&D priorities as feed and feeding strategies comprise 60% of operating costs.

The key performance indicators for the scientific and technical activities of the project were based on Australia's leading YTK producers, identifying that a move from the sashimi to 'white' fish market required meeting the following criteria:

- A fingerling equivalent of 3.0 kg weight per fingerling within 2 years;
- A feed conversion ratio (FCR) of ≤ 1.5 and ≤ 2.2 for fish between 0.01 - 1.5 kg and 1.5 - 3.5 kg, respectively; and
- Survival of >90% from the stocking of fingerlings until harvest.

This project was part of the Rural R&D for Profit Programme, Department of Agriculture and Water Resources, Australian Government. It aligned with the Round 1 Programmes priorities:

1. Increase the profitability and productivity of primary industries
    - Help producers increase yields and/ or reduce costs by applying innovative technologies and/ or technologies from other industries.
    - Help producers manage natural resources in an integrated way at enterprise or regional level for long-term use and profit.
2. Strengthen primary producers' ability to adapt to opportunities and threats
    - Integrate data and deliver information to help producers manage risk, benchmark performance and make production decisions for greatest profit.
3. Strengthen on-farm adoption and improve information flows
    - Consolidate knowledge of extension and adoption to better deliver practical results to primary producers, founded on what producers want from extension services.
    - Identify practical proposals to stimulate private sector extension services, particularly to fill current gaps.
    - Identify practical means to co-ordinate extension services for producers, including the development of tools and/or platforms.

To meet with these Rural R&D for Profit Programme priorities, the project addressed the Australian YTK industry's key common R&D priorities, both at conception during 2014/15, and throughout the course of the project, through three key themes and their associated activities and outputs. The three key themes were:

Theme 1 Nutrition: identify economically sustainable feeds and improved diet formulations;

Theme 2 Feeding Strategies: improve feeding strategies to increase profit;

Theme 3 Health: Improve nutritional health to boost productivity.

The key activities of this project central to the efficient and effective delivery of its objectives/outputs were:

1. Project initiation and management;
2. Identify economically sustainable feeds and improve diet formulation;
3. Improve feeding strategies to increase profit;
4. Improve nutritional health to boost productivity; and
5. Extending YTK capability.

Project conclusions are presented in this section in order of the following themes, activities and outputs:

6.1. Activity 1. Project initiation and management

Output 1(a) Establish steering and research advisory committees and provide their terms of reference

Output 1(b) Execute agreements and contracts with partner organisations and service delivery agents as needed

Output 1(c) Finalise an extension and communication strategy. The strategy must include communications and extension activities including, but not limited to publications, workshops and newsletters

Output 1(d) Create a monitoring and evaluation plan for the project

Output 1(e) Undergo end of project evaluation in accordance with output 1(d) and provide a report to the department. The evaluation must report on the projects outcomes against the program objective, including quantitative information on the outcomes achieved and independent expert analysis of expected and/or demonstrated quantifiable returns on investment

All outputs associated with this activity were achieved.

The designated project governance committees were established and operated effectively for the duration of the project. An additional Technical Group was also established and met monthly to optimise coordination and scientific and technical discussion.

Agreements and contracts were executed between partner organisations and service delivery agents as needed. The project 'head' agreement was between the Department of Agriculture and Water Resources and FRDC. FRDC then established agreements with the two key government R&D agencies, SARDI and NSW DPI, which respectively established agreements with the YTK farming companies in their state, Clean Seas Seafood (South Australia) and Huon Aquaculture (NSW). After initiation of the project the two major Australian aquafeed companies, Ridley and Skretting Australia, joined the core project group, FRDC establishing agreements with both. The research agencies established a range of further agreements with universities as required in relation to student participation in the project and collaborative research. FRDC also subcontracted Agtrans to produce the independent Impact Assessment report of the project.

Relevant documentation was developed and monitored; this included 'Terms of Reference' for each committee, 'student engagement guidelines', an 'extension and communication plan' and a 'monitoring and evaluation' plan. A project activity matrix was also developed as the key tool for the Steering Committee to monitor project progress, which incorporated the necessary cross-linkages between the Department of Agriculture and Water Resources and FRDC 'head' agreement and FRDC - Research participant agreements.

Agtrans has delivered an independent Impact Assessment report for the project, reporting on project outcomes against project objectives, including presenting quantitative information on the outcomes achieved and expert analysis of expected and/or demonstrated quantifiable returns on investment and where this was feasible.

The following addresses the priorities (1. Increase the profitability and productivity of primary industries) of the Rural R&D for Profit Programme as outlined at the start of this section. Agtrans Research, the company contracted to do the independent Impact Assessment of this project, identified the total funding from all sources for the project was $7.37 million (present value terms) with the Department of Agriculture and Water Resources investment totalling $3.65 million (Section 5 Impact Assessment and Industry Implications). Their analysis indicated that this investment produced an estimated total expected benefits of $126.63 million (present value terms). This gave a net present value of $119.26 million, an estimated benefit-cost ratio of 17.2 to 1, an internal rate of return of 46.5 % and a modified internal rate of return of 16.1%.

6.2. Activity 2. (Nutrition theme) Identify economically sustainable feeds and improved diet formulations

Output 2(a) Evaluate alternative Australian farm protein and oil sources and identify their ideal inclusion levels in juvenile and sub-adult production diets to reduce dependence on wild derived (WD) fishmeal (FM) and WD fish oil (FO)

This output was achieved.

The apparent digestibility of 14 common raw materials by YTK was examined using the diet substitution method and yttrium oxide as the inert marker. Ingredients examined included different fish meal (FM) sources, poultry meals (PM), meat meal, soy protein concentrates (SPC), blood meal, legumes and cereals such as wheat. The results from the study (Manuscript 3.1.4.1) indicate that YTK are generally efficient at digesting nutrients and energy from marine and land animal protein sources. Plant proteins such as faba bean meal and lupin kernel meals appear to have a relatively high protein and energy digestibility for YTK and may prove useful as secondary protein and energy sources in aquafeeds. The poor digestibility of products such as blood meal and corn gluten meal used in this study suggests these products interfere with digestibility in YTK or there was some form of interaction between these raw materials and other raw materials in the reference diet. The apparent digestibility coefficients (ADCs) derived for the raw materials examined in this study will assist in the more accurate formulation of research and commercial aquafeeds for this developing aquaculture species. The data presented here will also serve as an extremely useful starting point for constructing a larger data base of raw material digestibility coefficients for this developing species. Please refer to Manuscript 3.1.4.1 for a complete description of raw materials, their composition and apparent digestibility to YTK.

With regard to wild derived (WD) FM replacement for large sub-adult YTK, results from the study (Manuscript 3.1.3.1) provided valuable commercially relevant information to reduce the dependence on WD FM inclusion in production diets at summer and winter water temperatures. Reducing dietary WD FM inclusions in current commercial diets with alternative ingredients derived from cheaper sustainable sources may lead to improved diet sustainability and diet cost savings. Sustainability, as measured by the Fish-in Fish-out ratio (FIFO), was improved by up to ~35% by the incorporation of a combination of PM and FM by-product. This may provide Australian YTK producers with major advantages in terms of market access and improved consumer perception. Diet cost were reduced considerably, which in turn, may lead to reductions in production costs for the industry. In addition, information pertaining to the replacement of WD FM with alternative protein sources will improve flexibility for feed manufactures to select raw materials that most economically meet the nutrient criteria in diet formulations for YTK. This is particularly advantageous, as availability and prices for fish feed ingredients vary greatly, especially in periods of drought. Based on results from the current study, we may recommend that when using SPC that diets contain no less than 20% WD FM. When using PM, we may recommend that diets contain 20% FM (derived from a combination of FM from wild stocks and seafood by-products). When using FM by-product, we may recommend that diets contain a total of 30%

FM, where 10% is derived from wild stocks, and 20% is derived from seafood by-products. These results are for large sub-adult YTK of the size range investigated in the current study and these recommendations are dependent on the changing cost of raw materials. We recommend that WD FM substitution with SPC, PM and FM by-product in diets be followed up with further pilot scale commercial trials before full diet formulation flexibility is realised.

With regard to WD fish oil (FO) replacement for large sub-adult YTK, results from this study (Manuscript 3.1.1.2 and Manuscript 3.1.2.1) provided valuable commercially relevant information to reduce the dependence of WD FO inclusion in production diets at winter water temperatures. We may recommend that canola oil (CO) dietary inclusion in sub-adult YTK production diets should be limited to ≤ 4% in a 25% total lipid diet during winter water temperatures. Feed conversion ratio (FCR) tended to increase (worsen) as dietary inclusion of CO increased above 4%. In contrast, FCR tended to decrease (improve) as dietary inclusion of poultry oil (PO) replaced CO, suggesting PO is suitable as an energy source at high inclusions (up to ~18% total added oil) in production diets for large sub-adult YTK at winter water temperatures. Increased PO inclusion, to reduce the reliance on WD FO will lead to immediate diet cost reductions and provide feed manufacturers with greater feed formulating flexibility.

Output 2(b) Investigate protein sparing effect of using higher energy and lower protein diets

This output was achieved.

With regard to for large sub-adult (2.0-3.5 kg) YTK at summer water temperatures, results from this study (Manuscript 3.1.2.2), on a practical basis we recommend that diets contain a crude protein (CP) level of 43% (digestible protein [DP] 37%), a crude lipid (CL) level of 25% (digestible lipid [DL] 24%), a gross energy (GE) level of 20 MJ kg^{-1} (digestible energy [DE] 17 MJ kg^{-1}) with a CP:GE ratio of 21.6 g CP MJ^{-1} GE (21.8 g DP MJ^{-1} DE). Based on feed intake rates, this provided fish with 5.2 g CP^{-1} kg BW^{-1} d^{-1} (4.5 g DP^{-1} kg BW^{-1} d^{-1}) and 242 KJ^{-1} GE kg BW^{-1} d^{-1} (207 KJ^{-1} DE kg BW^{-1} d^{-1}). We do not recommend the use of high lipid levels (30% CL) in commercial diets for large sub-adult (>2 kg) YTK at warm water temperatures. There were no diet related alterations to digestive tract histology, or the majority of blood biochemical and haematological parameters measured. High dietary lipid level (30% CL) did not affect visceral mass, intraperitoneal fat levels or product yield of large sub-adult YTK at warm water temperatures. However, high dietary lipid levels (30% CL) appeared to interfere with daily feed, protein and energy intake rates and feed utilisation and ultimately growth, especially at lower dietary protein levels. It is also commercially impractical to formulate and manufacture diets containing high CP and CL levels (~48% CP, 30% CL). With regard to winter water temperatures (Manuscript 3.1.2.1), large sub-adult YTK may be fed a diet containing 30% lipid to improve growth rates and feed utilisation, compared to feeding a 20% dietary lipid level. However, in contrast to feeding high lipid diets (30% CL) at warm water temperatures, sub-adult YTK fed a 30% CL diet at winter water temperatures exhibited significantly increased visceral mass and intraperitoneal fat levels which reduced product yield of processed fish. Therefore, in terms of production and dietary lipid levels, target market needs to be considered on a seasonal basis as the weight increase during winter was in-part related to increase in visceral and intraperitoneal fat weights and not dress-out yield (gutted, head on and gills in). This information will assist feed companies in providing YTK producers with more cost effective and sustainable diets.

Output 2(c) Develop summer and winter diet formulations that use ideal lipid types and levels for less than two kilogram YTK during periods of suboptimal water temperatures

This output was achieved.

Based on results for growth performance and feed intake rates from this study (Manuscript 3.1.1.1) it was estimated the optimal dietary ∑LC n-3 PUFA daily intake level for large sub-adult YTK at warm summer water temperatures ranged from 191 to 203 mg kg^{-1} d^{-1}. To provide this to fish in practical terms, it is estimated diets for large sub-adult YTK at warm summer water temperatures should be formulated to contain between 2.12 and 2.26 g ∑LC n-3 PUFA 100 g $diet^{-1}$. The 95% CI for each response variable ranged between 1.90 to 2.33 g 100 g^{-1} and 1.93 to 2.58 g 100 g^{-1} for SGR and FCR, respectively. These levels compare to the reported requirement for ∑LC n-3 PUFA of the closely related Japanese Yellowtail

(*Seriola quinqueradiata*; 45-80 g) of 2.00 g 100 g^{-1} (Deshimaru et al., 1982). It is important to recognise the aforementioned estimates were made by fitting second order polynomial regression models to the data and that other dose-response models may provide different values.

Based on results for growth performance and feed intake rates from this study (Manuscript 3.1.1.1 and 3.1.1.2), it was estimated the optimal dietary ∑LC n-3 PUFA daily intake level for large sub-adult YTK at cool winter water temperatures ranges from 164-233 mg kg^{-1} d^{-1}. To provide this, it is estimated diets for large sub-adult YTK at cool winter water temperatures should be formulated to contain levels similar to those used at summer water temperatures (~2.12 g ∑LC n-3 PUFA 100 g $diet^{-1}$ [95% CI, 1.90 to 2.33 g 100 g^{-1}]).

Please refer to Output 2(a) in this section for recommendations for optimal levels of CO and PO for sub-adult fish.

Output 2(d) Determine dietary requirements of selected essential nutrients for juvenile and sub-adult YTK

- *Determine the choline requirement of juvenile YTK*

This output was achieved

A dose-response experiment was employed to determine the digestible choline requirement of juvenile YTK at 16 °C. The requirement was found to be 27.3 mg kg BW^{-1} d^{-1} when using choline deposition rate as the response variable or 26.1 mg kg BW^{-1} d^{-1} when using specific growth rate (SGR) as the response variable. The 95% CI for each response ranged between 20.9 to 36.1 mg kg BW^{-1} d^{-1} and 21.6 to 31.5 mg kg BW^{-1} d^{-1}, respectively. On a dietary basis, the break-point in choline deposition rate and SGR were reached when diets provided 1.94 g and 1.93 g digestible choline kg^{-1}, respectively. The 95% CI for each response ranged between 1.55 to 2.48 g digestible choline kg^{-1} diet and 1.73 to 2.23 g digestible choline kg^{-1} diet, respectively. It is important to recognise the aforementioned estimates were made by fitting a segmental linear regression model to the data and that other dose-response models may provide different values. It should also be noted that these requirements are derived from test diets containing AMP, a known inhibitor of de novo choline synthesis. Based on comparison to a diet devoid of AMP, the de novo rate of choline synthesis in juvenile YTK reared at 16 °C was estimated to be 4.2 mg choline kg BW^{-1} d^{-1}. The magnitude of the estimate compared to the requirement value suggests juvenile YTK have a limited capacity for de novo synthesis of choline (Manuscript 3.5.1.1).

Analysis of a commercial diet similar to that being used by industry was found to have a residual choline concentration of 2.05 g kg^{-1} diet. Adding 3 g (i.e. standard industry practice) and 6 g CC kg^{-1} (CC = choline chloride; i.e. double industry practice) to the basal formula elevated the dietary choline concentrations to 3.87 and 5.44 g choline kg^{-1}, respectively. The digestible choline concentration of the same diets was found to be, on average, 1.77, 3.54 and 4.66 g kg^{-1} diet, respectively. Absolute weight gain and FCR of juvenile YTK reared at 16 °C and 24 °C tended to improve when they were fed a the commercial formulation supplemented with 3 g CC kg^{-1}, but these parameters did not improve further when the mash was supplemented with 6 g CC kg^{-1}, indicating the current industry practice is probably satisfactory under most production conditions. Based on the results from both experiments we recommend formulating diets for YTK based on the upper limit of the 95% CI's for choline deposition rate and SGR. This conservative approach will ensure all fish within a population receive an adequate amount of digestible choline. All relevant data from these studies is provided in Manuscript 3.1.5.1 along with the specific mathematical models used to derive requirement estimates. However, the choice of the mathematical model applied to dose response data can significantly influence requirement values.

- *Determine the histidine requirements of juvenile YTK*

This output was achieved

Prior to this project amino acid research on YTK was virtually non-existent. Histidine proved to be a difficult amino acid to quantify, mostly because it was difficult to reduce the dietary level of histidine to the point where it was deficient. Therefore a pragmatic approach to quantifying the minimum histidine

requirement of juvenile YTK was employed (see Manuscript 3.1.5.2). This study identified that the minimum requirement for histidine in rapidly growing juvenile YTK is < 7.45 g kg^{-1} diet. Although an absolute histidine requirement was not quantified, current industry feeds available for YTK should easily meet this specification. The use of research diets with YTK that contain very high levels of synthetic amino acids raised questions about the efficacy of these amino acids when they are used in excess and requires further investigation. The results of this study indicate that as long as commercial formulations contain at least 7.45 g histidine kg^{-1} diet, then the normal growth and performance of YTK will be sustained.

- *Determine the taurine and methionine requirement of juvenile YTK*

This output was achieved

Results presented in this study (Manuscript 3.1.5.3) indicate that the recommended levels of taurine, cysteine and methionine in aquafeeds for YTK need to be reassessed. Based on the combined results of each study, we recommend a higher inclusion of methionine in commercial aquafeeds for YTK which exceeds current industry practice (i.e. ≈11 g methionine kg^{-1} diet), but does not exceed 21.1 g methionine kg^{-1} diet when the cysteine content of diets is approximately 5.6 g kg^{-1} diet. The minimum dietary methionine specification that meets the methionine requirement of rapidly growing juvenile YTK is approximately 13.9 g methionine kg^{-1} diet. If diets contain approximately 11.0 g methionine kg^{-1} diet we recommend a minimum of 7.7 g taurine kg^{-1} diet in order to optimise growth rate. These values are on a crude basis and therefore appropriate specifications may change depending on the digestibility of raw materials and diets. Cysteine can spare methionine up to at least 50.7%; however, high levels of cysteine may depress growth. Further investigation into the interactive relationships among the sulphur containing amino acids and their impact on overall requirements is therefore required. These recommendations are relevant for the size and culture conditions undertaken in this study. We further recommend investigation of the impacts of ontogenetic and abiotic factors on total sulphur amino acid requirements in YTK. All relevant data from these studies is provided in Manuscript 3.1.5.3 along with the specific mathematical models used to derive requirement estimates. However, the choice of the mathematical model applied to dose response data can significantly influence requirement values.

Output 2(e) Investigate the cost-benefit of using dietary supplements to improve the production of juvenile and sub-adult YTK

- *Investigate the use of prebiotic and probiotic bioactive supplements on growth, digestibility and gut health in sub-adult YTK*

This output was achieved

Four commercially available bioactive products were added to a soy-based control diet to examine whether they could improve the weight gain, feed intake and FCR of juvenile YTK (see Manuscript 3.1.6.1). The products were spent brewer's yeast (2.0% diet), inulin powder (1.0% diet), Protexin® powder (0.1% diet) and Pro(N8)ure®-IFS powder (0.1% diet). A positive control diet composed of prime FM (55.0%) and FO (15.9% diet) was used for comparative purposes. The digestibility of diets, plasma biochemistry and impacts on the gut microbiome were also examined. After 70 days there were no significant differences among soy-based diets with respect to SGR, relative fed intake, FCR, condition factor, PER or HSI. There were also no significant differences among diets with respect to nutrient digestibility and levels of plasma cholesterol, triglycerides, total protein, glucose, lactate or aspartate aminotransferase (AST). There was also no significant effect on gut microbiome. Based on these results there is no clear benefit of adding small amounts of spent brewer's yeast, inulin powder, Protexin® powder or Pro(N8)ure®-IFS powder to a soy-based control diet for YTK. A large variety of proprietary bioactive supplements are commercially available in the animal feed sector (prebiotics, probiotics etc.) and there may be merit in evaluating others. Of additional interest in this study was the exceptional growth and FCR performance of YTK fed high soy diets. These carefully formulated diets also contained optimal levels of methionine as per recommendations made in Manuscript 3.1.5.3. We encourage further investigation of the use of high quality soy products in diets for YTK.

Based on results from this study (Manuscript 3.1.2.1), we determined the dietary inclusion of LYSOFORTE® Liquid at a concentration of 40 mg kg lipid^{-1} did not significantly influence the growth or feed utilisation parameters at both lipid levels (30 and 20%) investigated with large sub-adult YTK at winter water temperatures. After discussions with project participants, we do not recommend any further investigation of LYSOFORTE® Liquid for YTK at winter water temperatures. However, further investigation of the potential use of other emulsifiers to improve lipid utilisation at optimal growth rates at summer water temperatures may be warranted.

6.3. Activity 3. (Feeding strategies theme) Improve feeding strategies to increase profit)

Output 3(a) Evaluate optimal feeding strategies for juvenile and sub-adult YTK, including but not limited to comparing experimental nutrient-dense and commercially available feeds, floating versus sinking feeds, feed sizes and feeding strategies

- *Complete a pond based trial to asses growth and FCR on newly developed feeds and feeding strategies for juvenile and sub-adult YTK (FM origin)*
- *Complete a pond cage based benchmarking study of a commercial diet and feeding strategies for YTK on the NSW DPI MARL (FM reduction)*

This output was achieved.

These two milestones are presented jointly in Manuscript 3.2.5.1 which presents encouraging results on FM reduction and the use of different FM sources in aquafeeds for juvenile YTK reared under fluctuating field conditions at PSFI. The results demonstrated that the dietary level of prime FM can be reduced from 55% to 15% without short term productivity being affected when FM reduction is offset by inclusion of other high quality proteins (including brewer's yeast and feather meal). The economic (measured as reduction in raw material cost) and environmental benefits of feeding a low FM diet were reflected in a 24% reduction in raw material cost and a 46% reduction in the FIFO of the low FM diet, respectively. A second experiment demonstrated that 30% fishery by-product meal can be used to wholly replace an equivalent amount of prime FM in diets for juvenile YTK without significantly affecting short term production outcomes. While there was little economic benefit (measured as reduction in raw material cost) in using 30% fishery by-product meal to replace an equivalent amount of prime FM in diets for YTK, there was a 45% reduction in the FIFO of the fishery by-product meal diet. These results confirm there is enormous scope in not only the choice of alternative protein sources for YTK but also a high degree of formulation flexibility. The incremental changes in body weight of YTK during experiments closely matched the predicted body weight of YTK according to an NSW DPI updated temperature-dependent growth model for this species. Models such as these will require constant updating; however they will remain highly beneficial for benchmarking growth in laboratories and field situations. We recommend follow up research to test other alternative protein sources for YTK and field experiments that test even lower amounts of dietary FM.

As hypoxia may be defined as any level of dissolved oxygen low enough to negatively impact the behaviour and physiology, results from this study (Manuscript 3.2.1.1) indicate large sub-adult YTK are relatively susceptible to this condition. Feed utilisation, oxidative stress and ultimately growth of large sub-adult YTK were negatively impacted by reductions in dissolved oxygen saturation levels, more so when exposed to hypoxic events on an irregular basis. This has important implications for site selection and farm management practices such as feeding leading up to weight checks, disease treatments and feeding during periods of low water movement (dodge/neap tides), especially as water temperatures exceed 24 ºC during summer. Prior to the current study there was interest to oxygenate sea-cages by Clean Seas Seafood to improve production over summer (personal communication, Dr T. D'Antignana; former R&D manager, Clean Seas Seafood). Subsequently, based on results from the current study, combined with an in-depth economic evaluation conducted by Clean Seas Seafood management, it was decided this practice was considered to be cost prohibitive and logistically impractical (personal communication, Dr T. D'Antignana; former R&D manager, Clean Seas Seafood). In terms of feed management, however, commercial producers of YTK may be able to mitigate exposure to feed-induced

hypoxic events by adopting a number of different feeding strategies. For example, commercial producers may be able to monitor tidal movements and dissolved oxygen levels prior to feeding, and withhold feed or reduce feed rates during periods of low water movement (dodge/neap tides). However, careful consideration needs to be given to this approach, as restricting feed rates also results in reduction in large sub-adult YTK growth rate. Other strategies that may be adopted to improve dissolved oxygen levels in sea-cages include careful site selection, utilising computer modelling, such as those recently developed by the Oceanography group at SARDI SAASC, to ensure adequate water flow, or by utilising nets with larger mesh sizes to reduce fouling effects and allow higher water exchange. Further research in pilot scale commercial trials are needed to validate these hypotheses before implementing these strategies on-farm.

Output 3(b) Evaluate the cost-benefit of using high versus low energy feeds for juvenile and sub-adult YTK at varying water temperatures

- *Complete a warm water (24 °C) study with sub-adult YTK to determine optimum feeding frequency.*
- *Complete a cool water (16 °C) feeding frequency study with sub-adult YTK to determine optimum feeding frequency.*

This output was achieved

Two experiments are presented in Manuscript 3.2.4.1 that demonstrate the growth rate and FCR of YTK are better in fish reared at 24 °C as opposed to 16 °C. The results also provide strong evidence that feeding sub-adult YTK a single meal to apparent satiety once per day supports acceptable growth rate and feed utilisation, irrespective of water temperature. However, industry should continue to feed at least twice daily in farm situations to ensure the average fish has the opportunity to consume enough feed to support their growth potential. There appears to be no added benefit of splitting meals into equal sized portions during the day, however studies that investigate similar meal frequencies, but where fish are fed to apparent satiation, may be worthwhile. The apparent proximate digestibility of a commercial diet determined using stripping methods was relatively unaffected by water temperature. However, lipid digestibility was slightly depressed at 16 °C. This may relate to gastric evacuation rate (GER), the lipid content and composition of the feed or the activity of specific digestive enzymes (see Manuscript 3.2.4.1). Ideally, more research should be done to understand the impact of water temperature on the digestibility of commercial diets and raw materials. Until better techniques are developed, such studies should be done using stripping methods. The GER of sub-adult YTK is slower at 16 °C than at 24 °C. In addition, regardless of the temperatures investigated here, fish between 150-500 g appear unable to consume more than about 3% of their body weight on a dry weight basis in a single meal. Refed fish appear to consume only as much food as has been evacuated. This wide ranging study provides an extensive data set that will assist YTK farm managers improve their on-farm feeding practices. Importantly it demonstrates the biological plasticity of YTK with respect to selected feed regime and pellet size across a conservative range in environmental temperature; factors that can be manipulated to improve the economic and environmental outcomes of farm raised fish.

- *Complete experiment to evaluate the effects of feeding strategy and diet specification on performance of sub-adult YTK*

This output was achieved.

Manuscript 3.2.4.2 examined the interactive effects of three satiation feeding regimes (i.e. once daily 7 days per week; once daily 5 days per week and once daily at random days each week) and two diet specifications (std. spec vs high spec.) on the performance of 1 kg sub-adult YTK reared at optimal water temperature for eight weeks. With respect to the most advantageous combination of diet and feeding regime, the results (premised on results of two-way ANOVA), indicate unequivocally that 1 kg sub-adult YTK should be fed a high quality diet at least once daily to apparent satiation seven days per week. Relative feed intake, SGR and FCR were all numerically better in groups of YTK fed to apparent satiation once per day, seven days per week and there was no biological benefit in feeding YTK once

per day, five days per week. Irrespective of feed specification, the groups of fish fed five days per week or randomly were generally unable to increase their feed intake (i.e. nutrient and energy intake) to such an extent that it compensated for the lack of feeding opportunity.

Direct comparisons of performance and digestible nutrient and energy intake between YTK fed the standard and high specification diet on a daily basis with recent data from bioenergetic models on YTK suggests YTK were eating primarily to satisfy their DP requirements. As a consequence, fish reared on the standard specification diet may have indirectly limited their DE intake (fat) to levels that inhibit optimal growth and feed utilisation. As such they were unable to meet their genetically programmed growth potential due to a subtle but chronic state of energy deficiency, perhaps related to a slight imbalance in the optimum DP:DE ratio of the diet for this size animal. These results demonstrate that the performance of 1 kg sub-adult YTK is extremely sensitive to the nutrient and energy composition of aquafeeds.

The biological conclusions of this study are clear. However, there may be some economic benefit to be derived from feeding YTK a high specification diet to about 80% of a satiety ration five days per week; at least in terms of economic FCR and labour savings (see tables in Manuscript 3.2.4.2). However these decisions also need to be made with reference to optimal growth rates of YTK, which proved to be between 15-19% higher in YTK fed the high specification diet than in YTK fed the same diet 5 days per week or randomly, respectively.

- *Report on 3 feeding experiments designed to evaluate impacts of dietary shift on reproductive output and health of YTK broodstock*

This output was achieved

Three long term experiments were completed with YTK broodstock held at the NSW DPI Marine Fish Breeding Hatchery. Broodstock feeding and nutrition is one of the least studied and most poorly understood areas in aquaculture and research into broodstock husbandry, with care and wellbeing often neglected due to the focus of farmers and industries on the grow-out stage. However the implications of farming progeny of poorly maintained and nourished broodstock are profound, having negative ramifications across the whole nursery and production cycle.

The results from the first experiment (Manuscript 3.2.6.1) shows that the fecundity and diversity of offspring from YTK is higher in broodstock fed Squid and Sardines as opposed to the commercial feeds selected for comparison. In addition regular three month spawning cycles adopted in this experiment may have placed undue reproductive stress on the YTK broodstock, impacting on their ability to recover physically and sexually after each spawning event. The number of offspring groups identified from select spawning events (i.e. heredity testing) was also higher in broodstock fed Sardines and Squid compared to broodstock fed commercial pellet preparations. These results will be useful in planning commercial hatchery operations for industry and to guide the YTK hatchery development program at PSFI.

Manuscript 3.2.6.2 presents results of a second study which examined differences in growth FCR, fecundity and gut microbiome of YTK broodstock brought about by the choice of different feed types (i.e. a commercial diet vs a ration of Sardines and Squid). However, wild and F1 broodstock failed to spawn in this trial following thermal-photoperiod manipulation. Reasons broodstock did not spawn are unclear, but they could relate to the sexual naivety of the wild and F1 stock, or the additional stress placed on stock at the beginning of the experiment as a result of weighing and microbiome sampling.

This manuscript also presents, for the first time, extremely detailed accounts of the microbiome of YTK broodstock, brought about by a project collaboration between NSW DPI researchers and SARDI microbiologists studying the microbiome of wild and farmed YTK from South Australian waters. This analysis found there were significant differences in the global community structure of the tank water and broodstock swabs, indicating that YTK broodstock are able to select, regulate and maintain their own environmentally independent microbiome (for more detail see Manuscript 3.2.6.2). Groups of wild fish fed Sardines and Squid and sampled four months prior to and after attempted spawning also recorded differences in their global community structures and relative percent abundances of the top 15 operational taxonomic unit (OTUs) for these groups, suggesting other factors aside from diet have an

influence on the gut community structure and dynamics of these broodstock. F1 broodstock held exclusively on commercial pellets (Huon 9 mm diameter) and sampled four months prior to and after attempted spawning had significant differences in their global community structures. At the bacterial phyla and taxa level, similarities were observed across tanks of broodstock before and after attempted spawning. However, clear differences were recorded at the bacterial phyla and taxa level between the pre and post spawning samples from the same tank, even though fish from different isolated tanks were fed the same commercial diet. Again this suggests other factors aside from diet have an influence on the gut community structure and dynamics of broodfish at PSFI. Understanding the impact of feed type on the structure and diversity of the gut microbiome will provide valuable insights into fish health and an enable, where possible, reproductive output to be correlated with changes in the microbiome.

Manuscript 3.2.6.3 presents the results on final broodstock trial at PSFI. Groups of YTK broodstock were continued from Experiment 2 in the same groups and on the same experimental feeding regime (Sardines and Squid vs Huon 9 mm diameter commercial feed). At the end of October two-groups of broodstock were switched to a dry pellet (15 mm) made from a commercially available booster feed (Breed-M) to see if this "nutritional boost" enhanced spawning and fecundity outcomes. This approach allowed the integration of data from the second and third experiment providing a broader overview on the use of pelletised feeds, feed costs, growth and bio-security implications for broodstock at PSFI. Unfortunately, none of the broodstock spawned in this experiment and this may be related to the sexual naivety of the wild caught and F1 stock, although this is not certain. This outcome means the impact of the pelletised feeding regime could not be assessed against the best-practice regime with respect to investigations of fecundity and egg quality. Further long term trials will be necessary to evaluate the efficacy of feeding dry pelletised rations and booster feeds to broodstock.

With regard to feeding rates and large sub-adult YTK at warm summer and autumn water temperatures (Manuscript 3.2.3.2), there is scope to improve commercial productivity for large sub-adult YTK production by altering the frequency of feeding practices in response to seasonal fluctuations in water temperatures. With regard to SGR and FCR, it is recommend that large sub-adult YTK are fed to apparent satiation twice daily at water temperatures > 20 °C, and fed to apparent satiation once daily as water temperatures drop from 20 to 16 °C and possibly lower. There did not appear to be any benefit in adopting a split ration feeding strategy for large YTK. However, further research in pilot scale commercial trials are needed to validate results from the current study before implementing these altered feeding strategies under commercial conditions. The extent of the cost benefit of adopting this research needs to be determined on a case by case basis by YTK producers taking into account logistical costs and on-farm productivity.

With regard to feeding rates and large sub-adult YTK at cool winter water temperatures (Manuscript 3.2.3.1), if commercial producers aim to capitalise on limited fish growth during periods of cooler sub-optimal water temperatures, it is recommended that fish (~1.5 kg) are fed a formulated diet to apparent satiation six days week^{-1}. In contrast, if the primary aim of farms is to reduce feed and feeding costs, and maintain fish weight, YTK require a maintenance ration of 0.2047% BW d^{-1}·(provided an estimated energy maintenance ration of~ 56.3 kJ fish^{-1} d^{-1}), which was achieved, albeit slightly to excess, by feeding fish the formulated diet to apparent satiation two days week^{-1}. If large sub-adult YTK are fed a maintenance ration, attention to the essential dietary nutrients levels are needed. We do not recommend feeding below this maintenance rate as fish lost weight during the study. With regard to feeding Sardines, the growth performance of large sub-adult YTK fed Sardines every second day was similar to fish fed the formulated diet to apparent satiation six days week^{-1}. However, large sub-adult YTK fed Sardines had a FIFO ratio that was 50.1% higher than those fed the formulated diet. The higher FIFO associated with feeding Sardines may have a negative impact on consumer perception, with regard to sustainably, and may present market access problems. The use of Sardines is a decision to be made by YTK producers. With regards to cost benefits of this research (Manuscript 3.2.3.1), by adopting the new winter feeding strategies of feeding six times per week, a saving of ~$350,000 each winter (annum) for the production of 2,000 tonnes of YTK may be achieved (personal communication, Dr C. Foster; former CEO, Clean Seas Seafood). When this practice is extrapolated and applied to the future targeted annual production levels of 34,000 tonnes of Australian YTK, a saving of $5,950,000 per annum would be achieved (Manuscript 3.2.3.1).

Please refer to Output 2(b) in this section for other recommendations for high versus low energy diets for sub-adult fish.

Output 3(c) Develop an improved feed ration model for on-farm YTK feed management

- *Utilisation and maintenance requirements of juvenile YTK; quantifying abiotic factors (temperature and dissolved oxygen)*

This output was achieved.

Manuscript 3.2.2.1 presents the results of two experiments that evaluated the effect of abiotic factors on the digestible nutrient and energy utilisation of sub-adult YTK. These trials were undertaken specifically to provide new data to improve an existing, but limited bioenergetic model for this species. One trial considered the effect of temperature (15 ºC vs 24 ºC. and the other considered the effect of dissolved oxygen (DO) saturation (60% vs 100%). Temperature proved to have a significant effect on model parameters, however, the magnitude of the effect varied depending on the nutrient assessed. Protein and energy utilisation efficiencies were not statistically different at different temperatures as determined by linear regression analyses; however, it is remains important to integrate a temperature function into the new models to ensure predictive accuracy. Low levels of DO (60% saturation) negatively affected the nutrient and energy utilisation response in YTK, with this response tending to be more pronounced with increasing nutrient and energy intake. The underlying mechanisms for this are unknown and warrant further investigation to provide greater insight on the nutritional physiology of YTK. The results from these experiments will facilitate the integration of abiotic parameters into existing bioenergetic models for YTK resulting in more accurate predictive tools for nutrient requirements and feed management with changing aquaculture conditions.

- *Refine bioenergetic model for YTK and develop a predictive farm-based management tool for YTK*

This output was achieved

Specific data from Manuscript 3.2.2.1 has been combined with other reliable data from the majority of recent experiments done at PSFI including some historical data from previous YTK projects (e.g. ASCRC) to improve and update the published bioenergetic model for YTK (detailed in Manuscript 3.2.2.2). Growth information from large sub-adult YTK from Manuscript 3.1.3.1 carried out at SARDI was provided to NSW DPI for inclusion in the bioenergetics model development. This was achieved by determining the impact of changing water temperature and dissolved oxygen concentration on important model coefficients related to utilisation of nutrients (including amino acids) and energy for maintenance and growth. New models have been refined and validated against tank and field based trials at PSFI. The new model will be extremely useful in benchmarking performance of YTK reared on-farm as well as in research trials (as seen in Manuscript 3.2.4.2) and will be further improved by integrating reliable seasonal data from YTK farms (see Manuscript 3.2.2.2 for an overview of the development of the model). The goal of constructing a bioenergetic model for YTK is ongoing and will be assisted by the provision of growth and temperature data from farms. We recommend the development of a desk-top or phone based application making it readily available and accessible to farm managers. It will also be a useful tool for feed manufacturers, allowing forecasting of feed demand from customers.

- *Critical oxygen threshold and hypoxia tolerance in juvenile YTK*

This output was achieved

Manuscript 3.2.2.3 presents valuable industry applicable results on the critical dissolved oxygen threshold of sub-adult YTK. YTK are hypoxia sensitive, especially when held at warmer water temperatures. For example at a temperature of 20 °C a DO concentration below 2.6 mg O_2 L^{-1} (~38% saturation) will induce hypoxia. YTK have an elevated resting metabolic rate in warm water compared to YTK held in cool water. The time taken to deplete normoxic saturated (100%) water to $[O_2]_{crit}$ levels at 15 °C is more than double that of YTK at 20 °C; this has significant implications on the reaction time

to implement re-oxygenation of a rearing system should a system failure occur. Concomitant with the critical oxygen threshold is the onset of a sequence of behavioural responses to a hypoxic environment including exaggerated movement of the operculum and mouth-gulping and surface swimming followed by loss of equilibrium. Standard management practices should ensure aquaculture systems remain saturated (100% DO) at all times; however, if any of these behavioural responses is observed in the culture environment then we recommend rapid re-oxygenation of the system (tank, cage etc.) to prevent the potential loss of stock.

6.4. Activity 4. (Health theme) Improve nutritional health to boost productivity;

Output 4(a) Develop a challenge test method for fish health evaluations associated with tank based nutrition and feeding strategy R&D

- *Further refine the challenge model by better understanding the YTK immune system*

This output was achieved

The challenge model was developed and validated. During the validation phase, results showed detrimental effects on the immunity of sub-adult YTK fed select experimental diets formulated to replace 66.7% of WD FM with alternative protein sources, compared to the control commercial diet. These dietary treatments were removed from the nine month study in Manuscript 3.1.3.1 after three months due to poor performance. It is important to understand the level of substitution at which the diminished immune response is initiated, or the relationship between the amount of substitution and the changes in the immune system. It is also important to consider that industry improvements in profitability from lowering feed costs and improving growth could potentially be negated by greater losses to reduced health and disease.

Output 4(b) Collect histopathology and blood chemistry data of diseased and healthy YTK to characterise the general health of YTK used in tank based nutrition and feeding strategy R&D

- *H2B - Further refine the role of the gut microbiome in YTK gastrointestinal health by sampling additional wild fish in South Australia for subsequent histological and microbiomic evaluations*

This output was achieved.

In general the digestive tract histology, blood haematology and biochemistry of large sub-adult YTK was not significantly impacted by dietary treatments in relation to WD FM and WD FO replacement, and other changing nutritional and environmental factors (Output 4(b); across a range of Manuscripts).

For the YTK health activity in farmed fish (Manuscript 3.3.1.3), the general properties of disease (coccidiosis and enteritis) and its influence on the microbiome were established, including that a shift in the global community structure occurs and is associated with a significant reduction in species richness, diversity and evenness and is accompanied by the dominance of one or more select taxa. Such diminished microbiomes are likely to be associated with a loss of overall functionality which may have consequences to the health and fitness of the animal and requires further investigation. A general trend of loss of mucous cells, decrease in villi length and thinner submucosa, muscle and serosa was observed in the histology specimens from fish with enteritis, along with an outer surface change in the skin and gill microbiota with changing gut health status. This suggests that underlying diseases of the gut are likely to cause body-wide microbiome changes in the outer surfaces, the consequences of which are unknown, though may led to weakened barrier functions that may make the fish more susceptible to secondary infections. The factors that influence these disease conditions appear to be complex and multifactorial, as no single factor was observed to account for the changes in the microbiome composition in fish with enteritis, with further studies needed to elucidate this in greater detail using age/size class appropriate controls. Further characterisation and elucidation of the involvement of

specific taxa universally identified across the disease samples (namely an unidentified *Photobacterium* species) is recommended, as this was the only organism that was found to be enriched in all samples irrespective of site, fish size, year class or feed type, and hence may be specific to the disease condition or represent an opportunistic pathogen. Collectively, the results outlined in Manuscript 3.3.1.3 serve as a resource for further improving our understanding of disease in farmed YTK.

For the wild fish component, the enrichment of environmentally-independent bacterial taxa was apparent, highlighting that YTK are able to select, regulate and maintain their own gut-specific communities. Significantly distinct differences were also observed in the global community structure, bacterial phyla and order compositions and relative abundances of the top 15 taxa between wild and farmed fish, along with increased levels of species diversity and evenness in the wild fish, indicating the possible influence of natural diets on the gut microbiome, while formulated feeds may contribute to the reduced diversity and/or enrichment of select taxa in farmed fish. Baseline data (presented in Manuscript 3.3.1.1) on the microbiome of wild and farmed YTK can now be used as a critical reference point for downstream health and dietary assessments.

Output 4(c) Characterise and understand the microbiome of the digestive system of YTK in particular in relation to different diets and feeding strategies, and how this might be managed to enhance YTK health, diets or food conversion ratios

- *H4 - New health theme activity - manipulation of the microbiome of diseased YTK*

This output was achieved.

From the dietary assessments (Manuscript 3.3.1.2), considerable variation in the gut microbiome was observed to arise in the use of different commercial feeds within and between individual farms, with some formulations appearing to increase microbial diversity even over more 'natural' diets, while others promoted the enrichment of potentially opportunistic species. In some instances, increased abundance of potentially opportunistic taxa occurred irrespective of diet, raising the notion that other factors such as host size class/age, environment/seasonality, cultivation practice and host genetics are likely to contribute to the emergence of these organisms within the gut of farmed YTK. While it is not clear whether these enriched taxa represent specific pathogens or opportunistic species, their occurrence as a dominant feature is indicative of a microbiome with depleted diversity (and likely diminished gut functionality). Despite a clear role for diet in their emergence, for some amended diets comprising optimal levels of select feed ingredients, these taxa were found to occur in lower abundances and were likely displaced from improved diversity associated with the enrichment of potentially beneficial taxa. While such feeds may thus represent interesting prospects for potentially promoting diversity (and possibly gut health), in some instances, select additives used in promoting bioavailability and nutritional uptake of specific feed ingredients were found to enrich for (albeit in a low abundances) typically environmental organisms. In the absence of clear knowledge for their role/s in the host, the broader effects that diet exerts on both the major and minor components of the gut microbiome needs to be considered in the manufacture and testing of new dietary formulations.

From the novel microbiome manipulation experimental trial (Manuscript 3.3.1.4), it was shown that both the gut and skin microbiome (bacterial assemblages) of YTK can be modulated, with antibiotic therapy coupled with gavage inoculum contributing to improved gut microbiome structure, as observed from an increase in diversity and evenness and a decrease of potentially opportunistic pathogens. This was likely due to the enrichment of the varied (albeit less active) constituents from the donor inoculum. Differences in the global bacterial community structure of the skin samples from antibiotic treated fish administered the inoculum within the seawater were also observed at two days post inoculum. A further finding from this work was that while a more prolonged effect of the therapy was evident in the skin, only temporary effects were observed in the gut, thus highlighting that future studies should include repeat dosages of the inoculum (and/or at a higher concentrations) in order to sustain potential beneficial outcomes. From comparisons of fish where no inoculum was given (with and without antibiotics), it was also possible to establish a catalogue of taxa that were effected by antibiotic treatment (i.e. a single dose of 200 mg kg^{-1} oxytetracycline and 50 mg kg^{-1} erythromycin and metronidazole). While a broad variety of taxa were influenced, this treatment appeared to have limited effect on an organism with closest sequence identity to *Mycoplasma insons*, which was also previously observed as a dominant constituent

in YTK with an underlying gut enteritis in the earlier work components (see Chapter 3.3.1) and may be specific to disease in YTK or an opportunistic pathogen that is able to dominate the gut community when other taxa are eliminated. This presents an important consideration if treatment with antibiotics for YTK disease is to be used on-farm in the future. Specifically, our results show that if the combination and dosage of antibiotics used in this trial was applied to treat YTK with underlying gut enteritis disease on-farm, the issue may be exacerbated by allowing the proliferation of other potentially opportunistic species.

Output 4(d) Collect baseline data to differentiate the effects of the environment, YTK growth and farm production cycle, disease and different genetic cohorts on the microbiome

This output was achieved.

From these baseline activities (Manuscript 3.1.1.1), differences were observed in the microbiome (bacterial assemblages) between the gut and water samples, highlighting that YTK are able to select, regulate and maintain their own environmentally-independent communities. Cultivation strategy appears to influence the microbiome composition, with lower levels of diversity and the enrichment of potentially opportunistic bacterial species occurring in onshore (tank-based) compared to offshore (sea-cage) systems. Differences in the microbiome structure were also observed across the commercial production cycle (i.e. between different size classes/ages), with smaller, 'younger' fish primarily dominated by *Proteobacteria* and *Cyanobacteria*, whereas larger, 'older' fish were characterised by *Proteobacteria* and *Firmicutes*, with lower levels of *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes*, *Spirochaetae* and *Fusobacteria*. Species richness significantly reduced with size class/age, along with increased inter-individual variation (in terms of diversity and evenness) in larger, 'older' fish. While this likely reflects the natural 'maturation' processes that occur between the microbiome and its host throughout growth and development, other factors (e.g. diet) may also be contributing features and require further elucidation. This work (Manuscript 3.1.1.1) provides critical baseline information for future studies seeking to elucidate changes in the health and nutrition of farmed YTK.

6.5. Activity 5. Extending YTK capability

Output 5(a) Conduct workshops and provide publications to extend the outputs from the project to industry participants, and the broader aquaculture industry, scientific community and public in line with output 1(c)

Output 5(b) Student training to develop the next generation of industry R&D providers including up to three postdoctoral research fellows, up to six PhD students and up to 12 Honours students

Output 5(c) Incorporate the outcomes of the project into the new subprogram established by the FRDC or the development of new and emerging aquaculture growth opportunities to allow the direct extension and translation of outputs to potential 'white' fish and other new and emerging aquaculture opportunities

These outputs were achieved.

As outlined in Section 7. Communication and Extension, R&D progress and outcomes were extended to project participants, both researchers and industry, through ad-hoc communications, fortnightly meetings (NSW DPI and Huon Aquaculture), monthly update reports (SARDI and Clean Seas Seafood), monthly Technical Group meetings, occasional Research Advisory Committee, and quarterly Steering Committee meetings (representatives of all participants), and four annual project Research Workshops. Project outcomes were disseminated more broadly by 21 presentations at national and at international conferences, 51 presentations at workshops, and two publications in peer-reviewed scientific journal papers in addition to three popular articles for inclusion in the FRDC FISH magazine.

As outlined in Section 4. Student Activities, people capability was built through the project's employment of three postdoctoral positions (the targeted number), and six PhD (the targeted number) and two Masters/Master Intern students and four Honours students (the target was up to 12 Honours students), A PhD student professional development program was delivered which included an invite to

participate in project Technical Group meetings attendance at three annual workshops, and national and international conferences (Section 4 Student Activities).

The project Executive Officer worked closely with FRDC's NEAO Subprogram Leader. He attended all FRDC NEAO Subprogram meetings, contributed to grant submissions, provided project updates and comments on forwarded documentation. He also obtained advice and support from the FRDC NEAO Subprogram Leader in developing the YTK Health Training Workshop that had broader participation than just this project (e.g. included participants from the Barramundi and Cobia industries).

6.6. Overall Conclusion

Overall, results from this project were positive, and provided information that directly addressed the project themes, activities and outputs. Information from this project has been rapidly extended to YTK producers and feed manufacturers, and has been adopted into commercial dietary formulations and on-farm feeding practices which have led to significant improvements in growth feed efficiency and survival of fingerling and sub-adult YTK throughout the production cycle. Further research in pilot scale commercial trials are needed to validate results from the current project before implementing these altered feed formulations and feeding strategies across farm under commercial conditions.

We also now have an improved understanding of how diets and farm practices may both positively and negatively influence the health of YTK through our investigations of the microbiome. Its role in supporting host health and nutrition is paramount, and we have established that an optimal microbiome most likely needed to support this, is one that is diverse and has the capacity to displace potentially opportunistic pathogens. From our investigations we have also provided new insights into the possible involvement of the microbiome (or select constituents) in previously poorly understood conditions of disease in YTK and have identified new strategies that could be used for improved monitoring and early detection of changing health status. Though still in its infancy, as the first of its kind we have also begun to trial new whole microbiome therapies for improving the health of YTK which are likely to become relevant for the farming of fish more broadly in the forthcoming decades as an alternative to conventional (antimicrobial) treatments.

There has been a large student training component to this project. A major benefit of the student training component is the output of new industry ready entrants, trained with relevant skills that will contribute to future industry development.

The YTK industry has continued to build over the duration of this project, that is, during the financial years 2015-2018. Over this time, Clean Seas Seafood, based in SA, has significantly grown its production sales volume each year from about 1,000 to 2,500 tonnes and improved its profitability (based on annual reports on-line, http://www.cleanseas.com.au/investors/asx-releases/A), primarily as a result of advancing its farm management practices and processing infrastructure, growing its markets nationally and internationally, and increasing its average annual sale price of YTK per kilogram. Recently, Clean Seas Seafood has also increased its lease area available for YTK farming by 283 ha; it now has a total lease capacity across all lease sites with the potential to increase production to 11,000 tonnes per annum.

During the time of this project, Huon Aquaculture also started production on a trial basis on the Marine Aquaculture Research Lease (MARL) offshore of Port Stephens, NSW and despite a set-back due to the loss of most YTK from one of three stocked sea-cages in January 2018, progressed with harvesting and marketing of the remaining fish, estimated at a few hundred tonnes, later in 2018. The results of the MARL trial to-date, have demonstrated that its farmed YTK grew exceptionally well with good survival and that there was significant market demand (https://www.huonaqua.com.au/huon-aquaculture-port-stephens/), Based on results from the MARL trial, Huon Aquaculture has announced that it is now not only going to continue to expand production on commercial leases in NSW (a media article in the Newcastle Herald, 12th March 2016 refers to two leases, 62 ha and 12 sea-cages), but is also going to undertake the development of a YTK farm on two leases of a total of 281 ha within an about 3,000 ha aquaculture zone near the Abrolhos Islands, WA (https://www.huonaqua.com.au/about/truth/western-australia-kingfish-lease/). A media article (https://www.abc.net.au/news/rural/2018-10-12/huon-reveals-wa-fish-farm-plans/10366240) has suggested that production from this aquaculture zone, which

also includes the lease area of an existing YTK farming company, Indian Ocean Fresh Australia, will reach 24,000 tonnes of YTK per annum within a decade, employing about 3,000 people. Agtrans, the company contracted to do the independent Impact Assessment of this project, has liaised with research project participants and has come up with a more conservative average annual production (sales) growth figure for the Australian YTK industry of 490 tonnes (Section 5 Impact Assessment and Industry Implications).

7. Communication and Extension

7.4. Activities

7.1.1. Governance

This project (K4P) was managed through three levels: a Steering Committee, a Research Advisory Committee and a Technical Group. In consultation with the FRDC New and Emerging Aquaculture Opportunity (NEAO) Subprogram Leader, a K4P Extension and Communication Strategy was developed. A K4P Monitoring and Evaluation Plan was also produced.

Steering Committee

The Steering Committee was established at the start of the project and a 'Terms of Reference' developed, which included that minutes of the meetings be produced. Its initial composition was:

- Two members from FRDC (Dr Patrick Hone, Managing Director, FRDC, who was the Committee Chair and Mr Joshua Fielding then Dr Jennifer Cobcroft, who were leaders of the FRDC NEAO Subprogram).
- A single member of each Yellowtail Kingfish (YTK) producer involved in the project (Dr Craig Foster, Chief Executive Officer, Clean Seas Seafood; Mr David Whyte, Group Technical Manager, Huon Aquaculture; and Ms Erica Starling, Director and Owner, Indian Ocean Fresh Australia).
- A single member of each key research organisation (Prof Gavin Begg, Research Director, SARDI Aquatic Sciences and deputy Committee Chair; Dr Wayne O'Connor, Research Leader, NSW DPI; and Mr Greg Jenkins, Director, South Metropolitan TAFE, WA).
- The Executive Officer appointed by the Steering Committee (Mr Steven Clarke, Senior Research Scientist, SARDI).

During the project a number of changes occurred to the membership of the Steering Committee with its final composition:

- Two members from FRDC (Dr Patrick Hone, Managing Director, FRDC, who was the Committee Chair and Mr Wayne Hutchinson, Portfolio Manager, FRDC and the leader of the FRDC NEAO Subprogram).
- Two members of each YTK producer involved in the project (Mr David Head, Chief Executive Officer, and Mr Dan Fisk, General Manager Aquaculture, Clean Seas Seafood and Mr Matthew Whittle, Group Development Manager, Huon Aquaculture with the second position not filled).
- Two members of each key research organisation (Prof Gavin Begg, Research Director, SARDI and deputy Committee Chair, and Mr Steven Clarke; SARDI Aquatic Sciences; and Dr Wayne O'Connor, Research Leader and Dr Mark Booth, Senior Research Scientist, NSW DPI).
- One member of each of the participating Australian aquafeed manufacturing companies (Dr Richard Smullen, Manager, with proxy Dr Michael Salini, Product Development Manager, Ridley and Dr Leo Nankervis, Marketing Manager, Skretting Australia)
- An Executive Officer appointed by the Steering Committee (Mr Steven Clarke, Senior Research Scientist, SARDI).

Theme leaders also participated in the first session of each Steering Committee meeting, where they provided a brief progress report and identified any issues.

Research Advisory Committee

The Research Advisory Committee was established at the start of the project and a 'Terms of Reference' developed, which included that minutes of the meetings be produced. Its initial composition was:

- The Executive Officer appointed by the Steering Committee (Mr Steven Clarke, Senior Research Scientist, SARDI Aquatic Sciences and Committee Chair).
- A FRDC representative (Mr Wayne Hutchinson, Portfolio Manager, FRDC and the leader of the FRDC NEAO Subprogram).
- The Theme Leaders (Assoc. Prof. David Stone, Senior Research Scientist, SARDI - Nutrition; Dr Mark Booth, Senior Research Scientist, NSW DPI - Feeding Strategy; Dr Marty Deveney, Senior Research Scientist, SARDI (Challenge Test and Surveillance) and Dr Andrew Oxley, Senior Research Scientist, SARDI (Microbiomics) - Nutritional Health).
- One member of each YTK producer involved in the project (Dr Trent D'Antignana and then Mr Jay Dent, Research and Development Manager, Clean Seas Seafood; Mr David Whyte Group, Technical Manager then Mr Matthew Whittle, Group Development Manager, Huon Aquaculture; and Dr Gavin Partridge, Research Scientist, South Metropolitan TAFE, WA for the first half of the project).
- One member of each of the participating Australian aquafeed manufacturing companies once they had joined the project (Dr Michael Salini, Product Development Manager, Ridley and Dr Leo Nankervis, Marketing Manager, Skretting Australia).

The Research and Advisory Committee only met occasionally when confidentiality to this specific group was required. At all other times it undertook its activities during Technical Group meetings.

Technical Group

Initially SARDI established a monthly project teleconference with Clean Seas Seafood to facilitate collaboration and coordination between researchers and technical staff, but within 1-2 meetings this had been expanded to include all project participants as the process was working well. At this time a 'Terms of Reference' was developed and the Committee comprised:

- The Executive Officer appointed by the Steering Committee (Mr Steven Clarke, Senior Research Scientist, SARDI).
- A FRDC representative (Mr Wayne Hutchinson, Portfolio Manager, FRDC and the leader of the FRDC NEAO Subprogram).
- The Theme Leaders (Assoc. Prof. David Stone, Senior Research Scientist, SARDI - Nutrition; Dr Mark Booth, Senior Research Scientist, NSW DPI - Feeding Strategy; Dr Marty Deveney, Senior Research Scientist, SARDI (Challenge Test and Surveillance) and Dr Andrew Oxley, Senior Research Scientist, SARDI (Microbiomics) - Nutritional Health).
- One member of each YTK producer involved in the project (Dr Trent D'Antignana and then Mr Jay Dent, Research and Development Manager, Clean Seas Seafood; Mr David Whyte Group, Technical Manager then Mr Matthew Whittle, Group Development Manager, Huon Aquaculture; and Dr Gavin Partridge, Research Scientist, South Metropolitan TAFE, WA for the first half of the project).
- One member of each of the participating Australian aquafeed manufacturing companies once they had joined the project (Dr Michael Salini, Product Development Manager, Ridley and Dr Leo Nankervis, Marketing Manager, Skretting Australia).
- The Postdoctoral Fellows or their equivalent (Dr Matthew Bansemer and Dr Sarah Catalano, Research Scientists, SARDI; and Dr Igor Pirozzi, Senior Research Fellow, NSW DPI and James Cook University).

A standing invitation also existed for other project researchers and PhD students to participate in these meetings, the latter as a component of their 'personal development' activities.

7.1.2. General Communication

Ad-hoc Communications

SARDI and NSW DPI researchers, in particular Theme leaders, communicated by e-mail, telephone and face-to-face meetings, on a frequent ad-hoc basis between themselves and with research and technical representatives of the YTK producers, Clean Seas Seafood and Huon Aquaculture, and the aquafeed companies, Ridley and Skretting Australia. The primary purpose of these communications were to engage industry in the development and design of research, coordinate sampling and the supply of materials, provide updates on the progress of each individual research component undertaken and discuss the interpretation and use of research results.

Fortnightly / Monthly Reporting

SARDI and NSW DPI both developed systems of reporting to meet the respective requirements of Clean Seas Seafood and Huon Aquaculture. As Huon Aquaculture's operation was geographically located close to Port Stephens Fisheries Institute (PSFI) fortnightly meetings were held; whereas where Clean Seas Seafood were geographically remote from SARDI, monthly written progress reports were provided by SARDI to Clean Seas Seafood and feedback responded to as required.

Annual Research Workshops

K4P Research Workshop 1, 16th May 2016 (half day)

This K4P Research Workshop was held at SARDI Aquatic Sciences, West Beach, SA and was attended by 33 people, from SA, NSW and WA, including researchers (a number of veterinarians and aquatic animal health officers; scientists; postdoctoral fellows and equivalents; Honours and PhD students; and technicians) and industry (YTK farmers – Clean Seas Seafood, SA; Huon Aquaculture, NSW; and Indian Ocean Fresh Australia, WA). The workshop addressed research progress to-date, relevance of outcomes to industry, future planned research, and at the request of all industry participants, a specific afternoon session on *Photobacterium*, a disease impacting YTK aquaculture in SA and WA. A tour was provided of SARDI's South Australian Aquatic Sciences Centre. In total 21 presentations were given.

All students gave short presentations and responded to questions on the work they were planning to undertake or had initiated.

Following the workshop all technical presentations were provided as Adobe files to K4P project participants.

K4P Research Workshop 2, 1st December 2016 (1 day)

This K4P Research Workshop was held at the PSFI, Port Stephens, NSW, and was attended by 31 people, including researchers (scientists; postdoctoral fellows and equivalents; Honours, MSc and PhD students; and technicians), YTK farmers from SA, NSW and WA. The two major Australian aquaculture feed manufacturing companies, Ridley and Skretting Australia, also attended and presented. The Research Workshop and a tour of the PSFI facilities focused on disseminating research progress-to-date and its relevance to industry, as well as facilitating networking between researchers and researchers and industry. In total 20 presentations were given.

All students gave presentations and responded to questions on the work they were undertaking and/or planning for the future.

Following the workshop all technical presentations were provided as pdf files to K4P project participants.

K4P Research Workshop 3, 28-29th August 2017 (1 day)

This K4P Research Workshop was held at SARDI Aquatic Sciences, West Beach, SA and was attended by 26 project participants, including most K4P researchers and all K4P industry participants (Clean Seas Seafood, Huon Aquaculture, Ridley and Skretting Australia). All presentations, including from PhD students, focused on providing an overview of the research that had been undertaken since the start of the K4P project and its benefits to industry. In total 21 presentations were given.

Following the workshop all technical presentations were provided as pdf files to K4P project participants.

K4P Research Workshop 4, 12-13th September 2018 (1.5 days)

This K4P Research Workshop was held at SARDI Aquatic Sciences, West Beach, SA and was attended by 26 project participants, including most K4P researchers and all K4P industry participants (Clean Seas Seafood, Huon Aquaculture, Ridley and Skretting Australia). All presentations, including from PhD students, focused on providing an overview of the research that had been undertaken since the start of the K4P project and its benefits to industry. A total of 23 presentations were given by participants.

Following the workshop all technical presentations were provided as pdf files to K4P project participants.

National and International Conferences and Workshops

The K4P Executive Officer attended the national FRDC NEAO Workshops held over the life of this project and provided project updates at these. He also liaised with the FRDC NEAO Leader to develop each of the K4P student personal development workshops.

Most project Theme Leaders and Postdoctoral Fellows (or equivalents) attended at least one national Australian conferences and one overseas conference to communicate, benchmark and have peer reviewed the research undertaken. Five of the PhD students, as part of their personal development activities have attended an international conference and the other will in early 2019. Some have also attended a national conference. All have presented either orally or by way of a conference poster (the references to these are listed below).

International conference attendance was approved by the Steering Committee in consultation with the FRDC and Department of Agriculture and Water Resources, with benefits and outcomes being presented by way of a short written report and/or presentations at the K4P Research Workshops.

7.5. Communications

7.2.1. Scientific Papers Published

Bansemer, M.S., Stone, D.A.J., D'Antignana, T., Skordas, P., Kuerschner, L., Currie, K-L., 2018. Optimizing feeding strategies for Yellowtail Jack at winter water temperatures. North American Journal of Aquaculture 80, 128-140.

Legrand, T.P.R.A., Catalano, S.R., Wos-Oxley, M.L., Stephens, F., Landos, M., Bansemer, M.S., Stone, D.A.J., Qin, J.G., Oxley, A.P.A., 2018. The inner workings of the outer surface: skin and gill microbiota as indicators of changing gut health in Yellowtail Kingfish. Frontiers in Marine Biology 8, 1-17.

7.2.2. Scientific Publications Submitted or In-Review

Candebat, C., Booth, M., Codabaccus, B.M., Pirozzi, I., n.d. Methionine requirement and the sparing effect of cysteine in juvenile Yellowtail Kingfish (*Seriola lalandi*). Aquaculture (in preparation).

Chinh, T.M., Elizura, D.A., Ventura, T., Salini, M., Smullen, R., Pirozzi, I., Booth, M., Submitted. Apparent digestibility of common raw materials by Yellowtail Kingfish (*Seriola lalandi*). Aquaculture (under review, November 2018).

Chown, S., Bansemer, M.S., McWhorter, T., Carragher, J.F., Gibson, R., Stone D., Submitted. Optimising long chain omega 3 polyunsaturated fatty acids in formulated diets for harvest size Yellowtail Kingfish (*Seriola lalandi*) - is there a trade-off between omega 3 and omega 9 fatty acid deposition in red and white muscle tissues? Aquaculture (under review, April 2019).

Crowe, B.H., Harris, J.O., Bansemer, M.S., Stone, D.A.J., n.d. Restricted feeding and dietary energy levels affects liver structure in cultured Yellowtail Kingfish (*Seriola lalandi*) at summer water temperatures. Aquaculture (in preparation).

Dam, C.T.M., Elizura, A., Ventura, T., Salini, M., Smullen, R., Pirozzi, I., Booth, M. Apparent digestibility of common raw materials by Yellowtail Kingfish (*Seriola lalandi*). Aquaculture (under review).

Legrand, T.P.R.A., Wynne, J.W., Weyrich, L.S., Oxley, A.P.A., n.d. A microbial sea of possibilities: current knowledge and prospects for an improved understanding of the fish microbiome. Reviews in Aquaculture (under review).

Liu, A., Pirozzi, I., Codabaccus, B., Hines, B., Simon, C., Sammut, J., Booth, M., Accepted. Digestible choline requirement of juvenile Yellowtail Kingfish (*Seriola lalandi*). Aquaculture.

Malik, A., Kuerschner, L., Stone, D.A.J., Shuller, K., A., n.d. Expression of PGC-1α, citrate synthase and cytochrome c oxidase in response to restricted feeding in Yellowtail Kingfish (*Seriola lalandi*). Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology (in preparation).

Pirozzi, I., Benito, M.R., Booth, M. n.d. Protein, amino acid and energy utilisation and maintenance requirements of juvenile Yellowtail Kingfish (*Seriola lalandi*): quantifying abiotic influences. Aquaculture (in preparation).

7.2.3. Published University Theses

Candebat, C., 2017. Effect of lipid sources and temperature on the critical oxygen level (Pcrit), hypoxia tolerance and routine metabolic rate of juvenile Yellowtail Kingfish (*Seriola lalandi*). MSc thesis. Institute of Hydrobiology and Fisheries Science, University of Hamburg, Germany and Macquarie University, New South Wales, Australia. 62 pp.

Doherty, J., 2018. Effect of variable oxygen levels and feeding frequency on digestive efficiency of large Yellowtail Kingfish (*Seriola lalandi*) at summer water temperature. Honours thesis. College of Science & Engineering. Flinders University, South Australia, Australia. 69 pp.

Kuerschner, L., 2016. Regulation of muscle growth in Yellowtail Kingfish (*Seriola lalandi*) under fasting and re-feeding conditions. Honours thesis. College of Science & Engineering. Flinders University, South Australia, Australia. 74 pp.

Legrand, T., 2016. Effect of health status on the microbiome of the skin and gill mucosa of Australian Yellowtail Kingfish. Honours thesis. College of Science & Engineering. Flinders University, South Australia, Australia.

Rubio, M., 2016. Effect of dissolved oxygen on utilisation of digestible protein and energy in juvenile Yellowtail Kingfish. MSc intern university report. Wageningen University and Research Centre, Holland.

Teoh, A., 2016. Physiological response to stress in Yellowtail Kingfish (*Seriola lalandi*) at different oxygen levels under summer temperatures. Honours thesis. College of Science & Engineering. Flinders University, South Australia, Australia. 86 pp.

7.2.4. Science Conference Posters / Abstracts

Bansemer, M.S., Stone, D.A.J., Skordas, P., 2017. Intermittent feed-induced hypoxia effects the growth and feed utilisation of large Yellowtail Kingfish (*Seriola lalandi*) at warm water temperatures. The World Aquaculture Conference, Cape Town, South Africa, 26-30th June 2017 (Poster).

Catalano, S.R., Oxley, A.P.A., 2017. The gut microbiome of Yellowtail Kingfish (YTK) under alternative farming conditions and changing health status. Australian Microbial Ecology Conference, Melbourne, Victoria, 13-15th February 2017 (Poster).

Chinh, D.T.M., Booth, M., Pirozzi, I., Salini, M., Smullen, R., Ventura, T., Elizur, A. 2018. The impact of raw material selection on apparent digestibility, the gut microbiome and the expression of digestive enzyme-encoding genes in Yellowtail Kingfish Seriola lalandi. The World Aquaculture Conference, New Orleans, USA 7-11th March 2019 (Abstract).

Chown, S., McWhorter, T.J., Carragher, J., Bansemer, M., Gibson, R., Stone, D.A.J., 2018. Reducing long chain omega 3 polyunsaturated fatty acids in formulated diets for harvest size Yellowtail Kingfish (*Seriola lalandi*) - is there a trade-off between omega 3 and omega 9 in some tissues? The 18th International Symposium on Fish Nutrition and Feeding, Las Palmas de Gran Canaria, Spain, 3-7th June 2018 (Poster).

Crowe, B.H., Bansemer, M.S., Harris, J.O., McWhorter, T.J., Stone, D.A.J., 2018. Effects of partial wild derived fish meal replacement on bile acid production and liver structure in Yellowtail Kingfish, *Seriola lalandi*. The 18th International Symposium on Fish Nutrition and Feeding, Las Palmas de Gran Canaria, Spain, 3-7th June 2018 (Poster).

Legrand, T.P.R.A., Camarinha-Silva, A., Wynne, J.W., Weyrich, L.S., Oxley, A.P.A., 2019. Investigating the role of mucosal microbiomes in Yellowtail Kingfish exhibiting different health status using a multi-omics approach. The 5th FRDC Australasian Scientific Conference on Aquatic Animal Health and Biosecurity, Cairns, Queensland, Australia.

Legrand, T.P.R.A., Catalano, S.R., Oxley, A.P.A., 2017. The inner workings of the outer surface: mucosal barrier bacterial assemblages as indicators of changing health status in Yellowtail Kingfish. Australian Microbial Ecology Conference, Melbourne, Victoria, 13-15th February 2017 (Poster).

Legrand, T.P.R.A., Catalano, S.R., Oxley, A.P.A., 2018. Mucosal microbiomes of the commercially important species Yellowtail Kingfish (*Seriola lalandi*) and markers of changing health status. The 19th Fish Immunology Workshop, Wageningen University and Research, Wageningen, The Netherlands, 29th April-3rd May 2018 (Poster).

Liu, A., Pirozzi, I., Codabaccus, B., Simon, C., Hines, B., Sammut, J., Booth, M., 2018. Digestible choline requirement for juvenile Yellowtail Kingfish (*Seriola lalandi*). The 18th International Symposium on Fish Nutrition and Feeding, Las Palmas de Gran Canaria, Spain, 3-7th June 2018 (Poster).

Pirozzi, I., Benito, M.R., Booth, M., 2017. Low dissolved oxygen affects amino acid utilisation and maintenance requirements in Yellowtail Kingfish *Seriola lalandi*. World Aquaculture Conference, Cape Town, South Africa, 26-30th June 2017 (Poster).

Pirozzi, I., Candebat, C.L., Booth, M., 2017. The critical oxygen threshold of Yellowtail Kingfish Seriola lalandi acclimated to 15 °C and 20 °C. World Aquaculture Conference, Cape Town, South Africa, 26-30th June 2017 (Poster).

Stone, D.A.J., Bansemer, M.S., D'Antignana, T., Skordas, P., Kuerschner, L. Currie, K-L., 2016. Evaluation of different feeding strategies for the production of Yellowtail Kingfish at winter water temperatures. International Symposium on Fish Nutrition and Feeding, Sun Valley, Idaho, USA, 5-10th June 2016 (Poster).

Stone, D.A.J., Bansemer, M.S., Skordas, P., Chown, S. Ruff, N., 2018. Practical dietary long-chain omega-3 polyunsaturated fatty acid requirements for large Yellowtial Kingfish (*Seriola lalandi*). The 18th International Symposium on Fish Nutrition and Feeding, Las Palmas de Gran Canaria, Spain, 3-7th June 2018 (Poster).

7.2.5. Scientific Conference Presentations (excluding at K4P Research Workshops and Internal Organisation Events)

Bansemer, M., Stone, D.A.J., Skordas, P., Nankervis, L., Salini, M., 2018. Reducing wild derived dietary fish meal inclusion levels in production diets for large Yellowtail Kingfish (*Seriola lalandi*). Australian Marine Science Association Conference, Adelaide, South Australia, 1-5th July 2018 (Oral presentation).

Candebat, C., Pirozzi, I., Codabaccus, M.B., Booth, M., 2018. Dietary methionine spares taurine in juvenile Yellowtail Kingfish (*Seriola lalandi*). The 18th International Symposium on Fish Nutrition and Feeding, Las Palmas de Gran Canaria, Spain, 3-7th June 2018 (Oral presentation).

Chown, S., 2017. Lipid utilisation in Yellowtail Kingfish (*Seriola lalandi*). SARDI Aquatic Sciences annual Student Seminars Session, West Beach, Adelaide, South Australia, 21st June 2017 (Oral presentation).

Clarke, S.M., Stone, D., Booth, M., Hutchinson, W., Fisk, D., Whittle, M., Salini, M., Nankervis, L., 2019. Kingfish - Australia's new potential white fleshed salmon? ABARES Outlook 2019 Conference, Canberra, Australia, 5-6th March 2019

Crowe, B.H., Bansemer, M.S., Harris, J.O., McWhorter, T.J., Stone, D.A.J. (2019). Histological observations of dietary energy and protein influences on liver structure in Yellowtail Kingfish, *Seriola lalandi*. 5th FRDC Australasian Scientific Conference on Aquatic Animal Health & Biosecurity, July 8th-12th, Cairns, Queensland, Australia, 2019 (Oral presentation).

Crowe, B., 2017. Restricted feeding with diets of differing energy levels affects liver structure in cultured Yellowtail Kingfish *Seriola lalandi* at summer temperatures. Presentation at SARDI Aquatic Sciences annual Student Seminars Session, West Beach, Adelaide, South Australia, 21st June 2017 (Oral presentation).

Jackson, J., 2017. The digestive enzyme activity and physiological responses of large Yellowtail Kingfish (*Seriola lalandi*) to variable feeding strategies at summer and autumn water temperatures. SARDI Aquatic Sciences annual Student Seminars Session, West Beach, Adelaide, South Australia, 21st June 2017 (Oral presentation).

Legrand, T., Catalano, S., Oxley, A., 2017. The inner workings of the outer surface: skin and gill mucosal barrier bacterial assemblages as indicators of changing gut health in Yellowtail Kingfish, *Seriola lalandi*. The Australian Society for Microbiology students award night, University of South Australia, Adelaide, South Australia, 16th March 2017 (Oral presentation).

Legrand, T., Catalano, S., Oxley, A., 2018. Mucosal microbiomes of farmed Yellowtail Kingfish (*Seriola lalandi*) and markers of changing health status. Presentation at Australian Marine Science Association (AMSA) 2018 Conference, Adelaide, South Australia, 1-5th July 2018 (Oral presentation).

Legrand, T., Oxley, A., Weyrich, L., Wynne, J., 2018. The functional role of mucosal microbiomes in fish health. SARDI Aquatic Sciences annual Student Seminars Session, West Beach, Adelaide, South Australia, 25th July 2018 (Oral presentation).

7.2.6. Popular Publications Published

Norwood, C., 2017. 'Kingfish research gathers momentum'. Fisheries Research & Development Corporation News'. Fish 25 (3), 26-28. http://www.fishfiles.com.au/Media/FISH-Magazine/FISH-Vol-25-3/Kingfish-research-gathers-momentum.

Norwood, C., 2018. 'Research reveals fishmeal alternatives. Fisheries Research & Development Corporation News'. Fish 26 (4), 9. http://www.frdc.com.au/media-and-publications/fish/fish-vol-26-4/research-reveals-fishmeal-alternatives.

Norwood, C., 2019. 'Yellowtail Kingfish growing availibility for customers'. Fish 27 (1), 12-14. http://www.frdc.com.au/Media-and-Publications/FISH/FISH-Vol-27-1/Yellowtail-Kingfish-growing-availability-for-consumers

7.2.7. Industry Conference Communications (e.g. K4P Research Workshops)

Bansemer, M., 2017. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Bansemer, M., 2018. Protein and energy requirements for larger YTK at summer water temperatures. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Booth, M., 2016. Brief update of K4P progress against milestones; introduction of students and supervisors. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November, 2016 (Oral presentation).

Booth, M., 2017. NSW feeding frequency trials with YTK. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Booth, M., 2017. Overview of NSW R&D on Nutrition and Feeding Strategy themes. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Booth, M., 2018. Overview and update of current R&D at NSW DPI. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Candebat, C., 2016. Effect of temperature and lipid source on the critical oxygen demand in juvenile YTK. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November, 2016 (Oral presentation).

Candebat, C., 2017. Critical oxygen level and amino acid studies on juvenile YTK. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Candebat, C., 2018. Update on choline requirements in juvenile YTK. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Catalano, S., 2016. The inner workings of the outer surface: mucosal barrier bacterial assemblages as indicators of changing health status in YTK. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November, 2016 (Oral presentation).

Catalano, S., 2017. Extension of health vs disease task: assessing the effect of temporal variation, sea-cage site locations and changes in on-farm diets on YTK health outcomes. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Catalano, S., 2018. Probiotics trial and wild fish critical reference point. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Chinh, D.T.M., 2016. Overview of PhD plan and links to K4P research. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November, 2016 (Oral presentation).

Chinh, D.T.M., 2017. Raw material digestibility, microbiomes and gene expression. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Chinh, D.T.M., 2018. Update on digestibility of raw materials and their impact on the gut microbiome of YTK. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Chown, S., 2016. Seasonal variation in dietary lipid utilisation in Yellowtail Kingfish. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, South Australia, 30th November, 2016 (Oral presentation).

Chown, S., 2017. Seasonal variation in dietary lipid utilisation in YTK. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Chown, S., 2018. Fatty acid metabolism in YTK; an update. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Crowe, B., 2016. Bile and taurine metabolism in relation to fishmeal and fish oil replacement in cultured YTK. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November, 2016 (Oral presentation).

Crowe, B., 2017. Dietary fish oil and fish meal replacement may affect bile and cholesterol metabolism in cultured YTK. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Crowe, B., 2018. Bile metabolism of YTK; an update. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Deveney, M., 2016. A challenge model for assessing kingfish immune status. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November, 2016 (Oral presentation).

Deveney, M., 2017. Health surveillance: lessons from what happens while waiting for other things to happen. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Deveney, M., 2018. A challenge model for assessing kingfish health. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Deveney, M., 2018. SARDI YTK R&D health surveillance program and improved skin, gill and blood fluke treatment options. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Doherty, J., 2017. The digestive physiology of large YTK under different feeding strategies at warm water temperatures. Update on YTK winter lipid and alternative protein studies K4P research workshop.

South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Fielder, S., 2016. Effect of natural vs commercial feeding regimes on spawning outputs from wild and first generation YTK brood fish. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November, 2016 (Oral presentation).

Hardaker, T., 2018. Impact assessment of investment in the Kingfish for profit R&D program. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Legrand, T., 2016. Progress of K4P project activities at SARDI Aquatic Sciences. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, 30th November, 2016 (Oral presentation).

Legrand, T., 2017. The functional role of mucosal microbiomes in changing health and nutrition of YTK. K4P research workshop. West Beach, South Australia, South Australia, 28th August 2017 (oral presentation).

Legrand, T., 2018. The functional role of mucosal microbiomes in YTK health. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Liu, A., 2016. Effect of temperature and choline supplementation on the performance of juvenile YTK. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November 2016 (Oral presentation).

Liu, A., 2017. Effects of dietary choline on YTK. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Nankervis, L., 2016. Research for implementable benefits to industry. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November 2016 (Oral presentation).

Nankervis, L., 2017. Fish growth and appetite. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Nankervis, L., 2018. K4P lessons learned from a feed company perspective. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

O'Connor, W., Whyte, D., 2017. Marine Aquaculture Research Lease (MARL) & Huon Aquaculture Update. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation)

Oxley, A., 2016. Brief update on the progress of the microbiomics component of the K4P Health theme; and introduction of the themes postdoc and student. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November 2016 (Oral presentation).

Oxley, A., 2017 Nutritional Health theme update: summary and key implications of microbiome findings to date from all sampled experiments. K4P research workshop. West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Oxley, A., 2018. Summary and implications of microbiomic YTK nutritional health findings. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Partridge, G., 2016. The WA YTK situation: health, challenge trials, immunology and blood chemistry. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November 2016 (Oral presentation).

Pirozzi, I., 2016. Effect of temperature and oxygen saturation on nutrient retention in juvenile YTK. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November 2016 (Oral presentation).

Pirozzi, I., 2017. Review of progress on bioenergetic and histidine studies. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Pirozzi, I., 2018. Validation and implications of bioenergetic models for YTK. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Salini, M., 2016. YTK research strategy for Ridley. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November 2016 (Oral presentation).

Salini, M., 2017. Lipid, fat or oil - or whatever you want to call it. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Salini, M., 2018. Putting the ccience into practice or the practice into science: industry adoption of the K4P practice. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Stone. D.A.J., 2017. Overview of SA R&D on Nutrition and Feeding Strategy themes. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 28th August 2017 (oral presentation).

Stone, D.A.J., 2018. Overview of K4P nutrition and feed strategy R&D at SARDI. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Whittle, M., 2018. Challengers of farming YTK offshore duration. K4P research workshop. South Australian Research and Development Institute (Aquatic Sciences), West Beach, Adelaide, South Australia, 12-13th September 2018 (oral presentation).

Whyte, D., 2016. Update on MARL; set-up, stocking and progress over the first 50 days: options for sea-cage R&D. K4P Research Workshop. Port Stephens Fisheries Institute, Taylors Beach, Sydney, New South Wales, 30th November 2016 (Oral presentation).

7.2.8. Media Communications

Articles (with author)

Hansen, A., 2018. 'Kingfish feed findings win symposium award'. Fisheries Research & Development Corporation News. Fish 26 (3). http://www.frdc.com.au/Media-and-Publications/FISH/FISH-Vol-26-3/Kingfish-feed-findings-win-symposium-award**.**

Neindorf, B., 2016. 'Potential $2 million boost for Yellowtail Kingfish industry with new feeding regime'. ABC Rural News, 18th April 2016. https://www.abc.net.au/news/rural/2016-04-18/kingfish-feeding-regime-to-boost-industry/7334294.

Norris, S., 2016. 'Port Stephens Fisheries Research Institute works on Yellowtail Kingfish project'. Port Stephens Examiner, 5 February 2016. https://www.portstephensexaminer.com.au/story/3709686/hopes-for-farm-raised-fish/.

PIRSA., 2016. '$2 million boost for Yellowtail Kingfish industry'. 14th April 2016. http://www.pir.sa.gov.au/alerts_news_events/news/sardi/$2_million_boost_for_yellowtail_kingfish_industry.

Towers, L., 2016. 'New Feeding Strategy a Boost for Yellowtail Kingfish Industry'. The fish Site. 18th April 2016. https://thefishsite.com/articles/new-feeding-strategy-a-boost-for-yellowtail-kingfish-industry.

Articles (no author)

2016. 'Hopes for farm-raised fish'. Port Stephens Examiner, 18th February 2016.

2016. 'NSW Planning & Environment - Exhibition of Modification Requests - Marine Finfish Research & Commercial Aquaculture - Providence Bay'. Port Stephens Examiner, 10th March 2016.

2016. 'NSW DPI and Huon Aquaculture plan to breathe new life into defunct Pisces fish farm off Port Stephens'. Port Stephens Examiner, 5th April 2016 https://www.portstephensexaminer.com.au/story/3831222/new-life-for-pisces-fish-farm/.

2016. 'New life for Pisces fish farm'. Port Stephens Examiner, 7th April 2016.

2016. 'Better results for kingfish'. Port Lincoln Times, 16th April 2016.

2016. 'Boost for Yellowtail Kingfish industry as feeding regime altered news", NewsUnited.com. 16th April 2016

2016. 'Boost for Yellowtail Kingfish industry as feeding regime altered news'. World News, 16th April 2016.

2017. 'Study rejects ocean fears'. Newcastle Herald, 6th October 2017,

2017. 'A future feeding frenzy'. The Land, 6th October 2017.

Interviews

2016. 'Winter feeding strategy experiment', Country Hour, ABC 639 (interview with David Stone), 18th April 2016.

2016. 'Winter feeding strategy experiment', 8.00am Bulletin, ABC 639 (interview with David Stone), 18th April 2016.

8. Collaborations

Significant project collaborations were formalised by agreements executed between partner organisations and service delivery agents as needed. The project 'head' agreement was between the Department of Agriculture and Water Resources and the Fisheries Research and Development Corporation (FRDC), but included all key initial project participants. FRDC then established agreements with the two key government research and development (R&D) agencies, the South Australian Research and Development Institute (SARDI) and New South Wales Department of Primary Industries (NSW DPI), which respectively established agreements with the Yellowtail Kingfish (YTK) farming companies in their state, Clean Seas Seafood (South Australia - SA) and Huon Aquaculture (New South Wales - NSW). After initiation of the project the two major Australian aquafeed companies, Ridley and Skretting Australia, joined the core project group, FRDC establishing agreements with both. The research agencies established a range of further agreements with universities as required in relation to student participation in the project and collaborative research. FRDC also subcontracted Agtrans to produce the independent, impact assessment report on this project (Section 5).

As a national, multidisciplinary project, the R&D undertaken comprised an array of collaborators that included participants from a number of private companies and organisations, universities and government departments (see full list in Section 7. Communication and Extension). Of particular importance to this work was the strong collaborative network that was forged with the relevant industry participants and the feed companies. Their continued support throughout the project and in generating the R&D within this report was paramount and, as entitled, has fostered the project's success in "Growing a profitable, innovative and collaborative Australian Yellowtail Kingfish aquaculture industry: bringing 'white' fish to the market".

9. Further Development

The current project has provided industry stakeholders with valuable and information to assist the Australian Yellowtail Kingfish (YTK) industry improve productivity and profits. Overall, results from this project bode well for the future development of the YTK aquaculture industry in Australia However, dietary development work for this industry should not remain static, as important advancements in our knowledge of nutrient requirements and feeding practices will need to be ongoing to ensure the economically sustainable and healthy production of Australian YTK and a flourishing industry. Throughout the project, opportunities for further research and development within each relevant activity were identified:

Activity 2. Nutrition: Identify economically sustainable feeds and improved diet formulations;

- Nutrient requirement work must take into consideration seasonal water temperatures and fish sizes during different stages of production.
- It should be acknowledged that recommended dietary nutrient levels in commercial diets may be further reduced by optimising dietary amino acid profiles (e.g. methionine, lysine and histidine) based on new information as it comes to hand.
- Further work evaluating the dietary requirements for essential amino acids, vitamin and minerals should be undertaken for fingerling and sub-adult YTK to advance the sustainable performance.
- Currently, the recommended minimum inclusion levels of fish oil (FO) in commercial diets for sub-adult YTK is restricted by the ∑LC n-3 PUFA requirement of the fish and ranges between 5-10%, depending on the ∑LC n-3 PUFA content of the FO. This has implication on the Fish-in Fish-out (FIFO) ratio and sustainability. There is a pressing need to evaluate new oils rich in ∑LC n-3 PUFA with YTK as they come to hand.
- In order to be able to tailor fish for specific markets, further research is warranted to understand the kinetics associated with the uptake of ∑LC n-3 PUFA from finishing diets rich in FO, prior to harvest.
- LC n-3 PUFA levels and ratios in red blood cells are considered to be a good biomarker for inflammatory responses in humans and other animals. Given the importance of red blood cells in oxygen transport, fatty acid modifications in relation to saturated and unsaturated fatty acids may contribute to alterations in metabolic function. Further research is warranted to understand this aspect of YTK metabolism.
- In relation to dietary lipid levels and biometric measurements, the targeted processing method and markets should be taken into consideration when assessing growth performance to account for differences in fat partitioning.
- Developing functional feeds, including those that include pre- and pro-biotics and enzymes, to enhance YTK performance.
- The use of commercially available bioactives in soy-based feeds for YTK did not enhance growth or feed utilisation. However it would be prudent to explore the use of other similar products. The preliminary evidence gathered in this experiment suggests there is no major benefit in adding any of the selected bioactives into diets for juvenile YTK, at least at the levels tested. Diets for YTK that contain soybean meal and soy protein concentrate (SPC) and optimal levels of methionine are worthy of further investigation.
- Further investigation of the potential use of emulsifiers to improve lipid utilisation at optimal growth rates at summer water temperatures may be warranted.
- Due to the slow growth rate of large sub-adult compared to fingerling YTK at suboptimal water temperatures, it should be noted that attempting to gain an insight into the growth performance and feed utilisation of sub-adult YTK at winter water temperatures is inherently difficult. It may

be beneficial to run trials with sub-adult YTK for a longer period from winter into spring to assess any dietary deficiencies or benefits that may become apparent once growth rates accelerate.

- As the aquaculture industry is tending to reduce the use of high FM and FO diets due to economic and sustainability issues, further consideration to sustainability and customer perception are needed before YTK are fed Sardines under commercial conditions.

Activity 3. Feeding strategies: Improve feeding strategies to increase profit;

- The goal of constructing a bioenergetic model for YTK is ongoing and will be assisted by the provision of growth and temperature data from farms. We recommend the development of a desk-top or phone based application making it readily available and accessible to farm managers. It will also be a useful tool for feed manufacturers, allowing forecasting of feed demand from their customers.
- Fingerling and sub-adult YTK should be fed daily to ensure optimum growth and productivity.
- There did not appear to be any advantage in adopting split ration feeding to fingerling or larger sub adult YTK.
- Further research into the effectiveness of bioactive markers, such as the digestive enzyme dipeptidyl peptidase-4 (DPP4), as indicators of feed intake and digestion regulators.
- Given the reduced growth rates associated with oxygen deficiency, further research in relation to hypoxic stress is warranted for harvest sized YTK.
- Selective breeding targeted at YTK growth, efficiency of assimilation of feed nutrients and disease resistance/ health.

Activity 4. Health: Improve nutritional health to boost productivity;

- Use of the challenge model to understand the effect of WD FM and WD FO substitution on the health and immune system of YTK.
- Improving strategic approaches to skin and gill fluke management based on understanding which treatments are best in which circumstances.
- Approaches or management strategies which aim to enhance gut microbiome diversity in onshore systems is recommended for optimising fish robustness and may improve the natural adaptive processes of the fish to local environmental microbial communities when transferred offshore to sea-cages for grow-out.
- With changes in microbiome composition and diversity observed among major size classes associated with the commercial production cycle, there is also a need to ensure that appropriate size/age-specific controls are taken when surveying the relevance of the microbiome in changing health and nutrition in future surveys.
- Future investigations should be directed to developing dietary formulations that select for ‘optimal’ (diverse) gut microbiomes by conducting more detailed assessments of the underlying (gene) functions contributing to varied health and/or performance in YTK.
- Confirming the identification and determining the involvement of potentially opportunistic pathogens (namely *Mycoplasma insons*, *Brevinema andersonii*, *Photobacterium* sp.) that were found to occur at high levels in association with fish fed certain diets or that were suffering from conditions like enteritis, is recommended. With this additional information, appropriate control measures could then be implemented to improve health outcomes.
- We also recommend directing further efforts to the involvement and replenishment of organisms that may be of benefit to the host (e.g. *Bacillus* species), which were otherwise diminished in the microbiomes of diseased fish.

- Biomarkers of changing health status for coccidiosis and enteritis conditions could be established using more targeted, rapid and cost-effective tools (e.g. q-PCR), with the potential to implement non-invasive testing through the collection of swabs from the skin, which could foreseeably be implemented as part of routine health surveys for the early detection of disease.
- Strategies which promote broader microbial diversity in the gut of YTK should be investigated (e.g. probiotics, prebiotics, whole microbiome therapies), as they are most likely to improve the robustness of the fish to potentially opportunistic pathogens, ultimately improving health outcomes.
- From the findings of the microbiome manipulation trial, we believe additional experimental work is warranted. In particular, future studies should include repeat dosages of the whole microbiome inoculum (and/or at a higher concentrations) in order to sustain potential beneficial outcomes. Trialling administration of inoculum on-feed is recommended, which would allow for easier repeated dosing and would also prove to be more applicable on-farm compared to gavage. Including more varied microbiomes or individual strains that have known therapeutic potential (or that were observed to be depleted in diseased individuals in the early work) is suggested in a refined trial.
- Further work is required to elucidate whether increases in diversity support improved health through the displacement of pathogens and the potential occurrence of more diversified functions. The use of more advanced omics-based techniques is recommended to investigate this further.
- As there was no difference in the global bacterial community structure between the three gut regions, sampling methods were refined and future work should be directed at taking a single hindgut scraping (instead of separate fore-, mid- and hindgut), allowing for more samples to be processed at the same cost. This would increase the capacity to sample across multiple sea-cages, seasons and sites and provide a greater overview of farm-wide changes.

Activity 5: Extending YTK capability;

- Ensure that the results of the ongoing PhD projects for this project are captured and disseminated to industry as planned.
- Discuss with industry the most appropriate manner to continue YTK industry networking; this might be in the form of a new dedicated association or as part of an existing networking group (e.g. FRDC's NEAO Subprogram).
- Hold a workshop involving all YTK industry participants following completion of the project to identify and prioritise future needs to further drive the development of this industry.

10. Project Assets and Intellectual Property Developed

10.1. Infrastructure and Equipment

No capital expenditure of an amount requiring approval from the Fisheries Research and Development Corporation (FRDC) or the Department of Agriculture and Water Resources (DAWR) occurred in this project. As such, no project assets have been identified.

It is worth noting, however, that the South Australian Research and Development Institute (SARDI) and News South Wales Department of Primary Industries (NSW DPI) both expended significant funds of their own to upgrade select infrastructure at their sites to specifically undertake research and development (R&D) associated with this project. The benefits of this infrastructure will continue beyond this project and likely benefit future Yellowtail Kingfish (YTK) and other finfish R&D.

10.2. Intellectual Property

No project intellectual property (IP) needing protection has been identified. R&D was transferred directly to industry participants as it occurred, initially in draft form through verbal and preliminary written communications and then later in final form through written reports and scientific presentations and publications. Only the latter have been used to disseminate information to broader beneficiaries of the project.

10.3. Data

As required by the FRDC and DAWR project agreements, all project data is held appropriately backed and locatable by the Theme Leaders on their organisation's (SARDI and NSW DPI), computer systems.

11. Lessons Learned

11.1. Start-Up

While the Department of Agriculture and Water Resources (DAWR) agreement with the Fisheries Research and Development Corporation (FRDC) was completed close to the planned start date for the project, many months were involved in finalising the agreements between the FRDC and other participants, which delayed the actual start of the project. This was primarily because of the much greater level of detail required in these agreements (essentially every task/experiment/trial detailed), but also because state government research agencies can require agreements for such significant funding levels to be inspected by legal entities such as Crown Law and signed by such persons as Chief Executives or Minsters, a time consuming process.

Most research organisations now also have a policy where they are unable to start activity on a project, including the employment process, until the project agreement has been signed by all parties. For this project, the employment of the postdoctoral fellows was fundamental to the planned research and development and like all appointments took about three months to go through the approval, advertising and appointment stages. This again delayed the start-up of this project.

The suggested solution is to ensure that multi-level agreements associated with large projects are finalised six months prior to the planned start date of the project.

11.2. Governance

The composition of the project Steering Committee had been identified in the funding application to the DAWR and membership was finalised immediately the project was approved. The Steering Committee rapidly formalised who were the Theme Leaders, appointed an Executive Officer, and established Terms of Reference for each committee, a Principles of Funding Projects Document, a Dissemination, Extension and Commercialisation Plan, a Student Expectations Document and a Student Promotional Flyer. Subsequently an Overseas Travel Application Form and Overseas Travel Report Form were also developed.

As envisaged in the funding application, a number of changes were made to the Steering Committee overtime to build useful collaboration (i.e. representatives from each of the key Australian aquafeed manufacturing companies Ridley and Skretting Australia joined and the representatives associated with Indian Ocean Fresh Australia left).

The comprehensive governance structure outlined above and the members commitment to the project, demonstrated by the frequency of meetings and the attendance level at them, was considered fundamental to the success of the project.

11.3. Research and Development

R&D by its nature often involves innovation and results in unexpected outcomes. The necessity to apply rigorous methodology in R&D means that small biological or technical challenges that arise can also affect outcomes. When the subjects of the R&D are live animals, particularly finfish held in confined containers where environmental control is critical to their health and survival, issues can arise on occasion due to technical systems faults and/or extreme weather events despite comprehensive risk assessments and management systems.

This project experienced a small number of biological and technical issues that affected R&D outcomes, but in each instance the work was repeated, sometimes with necessary modification. Industry R&D priorities also changed to a degree during the project as an outcome of research results, and the program was adapted to address this. DAWR on occasion expressed dismay at the number of variations sought by the project, but it was pleasing that with due explanation they accepted the reasons for these and approved them. A reasonable level of flexibility is considered essential to achieve industry outcomes through innovative applied R&D.

11.4. Reporting

This project was one within Round 1 of the Rural R&D for Profit Programme, Department of Agriculture and Water Resources, Australian Government. An initial absence of some project templates (e.g. Milestone Report, Extension and Communication Strategy, and Management and Evaluation Plan) created some challenges for the project and the use of FRDC substitutes did not always provide what was sought. Also, as Rural R&D for Profit Programme templates were developed and provided, additional work was created in converting information to these new templates.

Milestone Reports Section 5. Technical Progress Report within each Milestone Report, while considered unnecessary by some, was appreciated by the project's Executive Officer and the members of various governance committees. The comprehensive information included in this section not only ensured that research was collated, analysed and written-up to draft publication standard as it was undertaken, but that all project participants had an excellent appreciation of the research undertaken and could readily assess progress.

Milestone Reports, Section 3.3. Achieving Future Milestones was used by the project to clearly articulate what slippage had occurred and what variations were sought, which was considered useful for governance purposes. However, the time involved in collating and having these variations signed in formal Deeds of Variation was excessive, the time sometimes exceeding the time between milestone reports leaving project participants to progress R&D when they were uncertain whether it would be approved or not.

11.5. Communication

The establishment of a Technical Group by the Steering Committee to meet frequently by teleconferencing to facilitate collaboration, coordination and technology exchange between researchers and with industry, was considered a major project success.

Project media communications proved challenging, because both YTK producers participating in the project, Clean Seas Seafood and Huon Aquaculture were ASX listed companies. As much of the outcomes of the applied research from the project had the potential to influence share trading, there was considerable reluctance to produce media communications. This was unfortunate as much of the R&D did have positive outcomes and would likely have been of general interest to the public.

11.6. Students

While the students that were engaged in this project were well selected and supervised, two challenges arose with their involvement. Firstly, attracting the numbers desired to work on a particular project and topic did not prove possible despite advertising and seeking students from many Australian universities. Secondly, that as Australian PhD students take an average of about 3.5 years to complete their thesis and not all can be recruited in the first year, it is likely that they will not complete their thesis by the end of a three year project such as this one. Most would not have been able to participate without FRDC agreeing to underwrite them over the lengthy time DAWR took to make a decision as to whether the project could support them or not.

This project achieved meeting its target of involving three postdoctoral fellows or equivalent and up to six PhD students, as six were recruited. It also achieved the equivalent of two thirds of its target of up to 12 Honours students, with four recruited as well as two MSc students, each which is reasonable to equate to two Honours students. The Honours students have all completed (three finishing with a 1st Class grade and one with a 2A) as have the MSc students. Alternatively, all PhD students have yet to complete, although their progress is satisfactory and they are meeting their designated timelines and most of their applied research results have been incorporated into the relevant sections of this report (Section 3. Research).

It would be beneficial for the Rural R&D for Profit Programme to include within its guidelines clearer instruction as to the participation of students within its projects.