Final Report

Sustainable Feeds and Feed Management for Yellowtail Kingfish

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Project No. 2009/728

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Project Objectives

The four objectives corresponding to the four Research Priorities identified by Clean Seas Tuna Ltd (CST) for this project are listed below with those relating to each subproject. Note that some objectives were subsequently removed from the project prior to its early termination, on the request of CST:

Objective 1: Improve on-farm feeding strategies: develop and validate a growth-feed intake model for Yellowtail Kingfish cultured in sea cages (removed from project).

- Subproject 1: Run a technology transfer facilitation framework meeting with CST staff, and project research and development (R & D) participants (remained in the project).

- Subproject 2: Develop a growth-feed intake model specific to Yellowtail Kingfish. Due to an Australian Seafood Cooperative Research Centre (AS CRC) approved variation this subproject was removed from the project.

- Subproject 3: Test and validate the growth-feed intake model developed in Subproject 2 with Yellowtail Kingfish cultured in replicated sea cages on the CST R & D farm at summer water temperatures. Due to an AS CRC approved variation this subproject was removed from the project.

Objective 2: Implement new, less labour intensive, more accurate methods for the determination of sea cage biomass (removed from project).

- Subproject 4: Review current fish counting methods used by CST to establish a more accurate on-farm fish counting system. Due to an AS CRC approved variation this subproject was removed from the project.

- Subproject 5: Calibrate, validate and implement two electronic fish biomass counter systems to CST Yellowtail Kingfish sea cages for the determination of Yellowtail Kingfish biomass. Due to an AS CRC approved variation this subproject was removed from the project.

Objective 3: Determine the protein to energy ratios of 1 to 4 kg Yellowtail Kingfish (removed from project).

- Subproject 6: Test the effects of varying protein to energy ratios on the growth performance, and feed efficiency of ~1 to 4 kg Yellowtail Kingfish cultured in sea cages

in the CST research farm. Due to an AS CRC approved variation this subproject was removed from the project.

Objective 4: Improve the sustainability of Yellowtail Kingfish farming by investigating the maximum dietary inclusion levels of alternative protein and lipid sources to replace fish meal and fish oil, at optimal (22 °C) and suboptimal (18 °C) water temperatures (remained in the project).

- Subproject 7: Investigate the suitability of canola oil, and poultry oil as alternative sources to fish oil for Yellowtail Kingfish when cultured at optimal and suboptimal temperatures.

- Subproject 8A and 8B: Explore the suitability of soybean products as alternative protein sources (8A, Soy 48, a solvent extracted soybean meal (SESBM); and 8B, soy protein concentrate (SPC)) in juvenile diets at optimal and suboptimal temperatures.

- Subproject 9: Test the effects of alternative protein sources on the growth performance of ~4 kg Yellowtail Kingfish cultured in the CST research farm. Due to an AS CRC approved project variation this subproject was removed from the project.

- Subproject 10: Evaluate the enzyme activity of Yellowtail Kingfish under a range of temperatures (8-28 °C) (remained in the project).

- Subproject 11: A literature review: current status of knowledge of the nutritional requirements of Yellowtail Kingfish (*Seriola lalandi*). Due to an AS CRC approved project variation the literature review was an AS CRC approved addition to the original project that was requested by CST in September 2012.

Outcomes Achieved

Planned Outcomes

Yellowtail Kingfish, *Seriola lalandi*, is the main closed cycle finfish cultured in South Australia (SA) and this industry has great potential to expand in other states of Australia. One of the major inefficiencies identified by industry was feeds and feed management in CST's Yellowtail Kingfish production. Henceforth, the newly formed CST Research Management Advisory Group (RMAG) identified improving feed conversion ratios (FCR) in Yellowtail Kingfish operations as an urgent priority. The production efficiency of Yellowtail Kingfish by CST has been hindered by the poor understanding of feeding strategies, sea cage biomass determination, growth performance and nutrient requirements of Yellowtail Kingfish at different life stages and water temperatures. It was also apparent that in order for CST to gain market acceptance for their Yellowtail Kingfish products on a global basis, the issue of sustainable use of marine ingredients, such as fish meal and fish oil, for the production of Yellowtail Kingfish needed to be addressed. In order to address the issue of sustainability detailed changes to current dietary formulations for Yellowtail Kingfish, which contained high levels of fish meal and fish oil, had to be made. Species specific information regarding nutrient availability, growth performance, fish health and maximum dietary inclusion levels of alternative sustainable ingredients for Yellowtail Kingfish cultured at fluctuating water temperatures were required to make these changes. Overall, the outcomes that should arise from the commercialisation of research outputs from this project will be an improved feed management system contributing to a reduction in FCRs from above 2:1 to 1.7:1, and revised and more sustainable Yellowtail Kingfish diet formulations (i.e. 25-30% lower proportions of marine based proteins and lipids).

Outcomes Achieved or Anticipated

Due to AS CRC approved variations, this project specifically addressed the topics of improving the sustainable use of marine ingredients, and growth performance and health of Yellowtail Kingfish cultured at optimal (22 °C) and suboptimal water temperatures (18 °C).

The research carried out in this project, while not completely conclusive, has achieved the planned outcomes of improving the feed conversion efficiencies and sustainable production of Yellowtail Kingfish. We have improved our ability to formulate commercial diets with nutrient specifications more suited to the seasonal production of Yellowtail Kingfish. The project also provided new information for the inclusion of two practical alternative oil sources to replace fish oil (poultry and canola oil), and two alternative plant protein sources, solvent extracted soybean meal (SESBM) and soy protein concentrate (SPC), to replace fish meal protein at optimal and suboptimal water temperatures.

- Steps were taken throughout this project to ensure the rapid extension of knowledge and recommendations to CST management, feed company participants and fish health groups (Chapter 2).

- A major outcome of this project is that CST management have acted on the knowledge and recommendations provided within this report, in collaborations with feed companies, to formulate and manufacture improved diets for the seasonal production of Yellowtail Kingfish in their sea cage operations. The production diets have been formulated to:

    - Contain nutrient specifications to meet the animal's nutritional requirements to ensure improved health, growth and FCRs.

    - Safely utilise greater levels of two dietary ingredients to produce cost effective and sustainable diets:

        1. .SPC at dietary inclusion levels of up to 20%, to replace up to 20% of dietary fish meal; and
        2. Poultry oil at dietary inclusion level of up to10-15%, to replace up to 50% to 75% of dietary fish oil.

    - Improve market access by a 33% improvement in the sustainable production of Yellowtail Kingfish by reducing the inclusion levels of dietary marine ingredients from ~60% down to 40%.

    - Exclude or limit the inclusion of dietary SESBM and canola oil. Both of which may be potentially detrimental to the health, growth and FCRs of cultured Yellowtail Kingfish, especially at the lower suboptimal water temperature of 18 °C.

    - Be used in conjunction with information pertaining to Yellowtail Kingfish growth and feeding behaviour at optimal and suboptimal temperatures in normoxic and hypoxic conditions (Chapters 3 to 7) to improve on-farm feeding practices, and attempt to reduce on-farm whole cycle FCRs from above 2:1 to 1.7:1 for Yellowtail Kingfish.

A comprehensive literature review was prepared reporting on the current status of knowledge of the nutritional requirements of Yellowtail Kingfish. The review points out many shortfalls in our knowledge base for this species, but it also provides a large body of information gleaned from research with other closely related *Seriola* spp. and other marine fish. This comprehensive review contains nutrient requirement information that is now being used by other producers, feed companies and research organisations, both domestically and

internationally, to improve the nutritional profile of their production diets, with the potential outcome of improvements in feed efficiency and fish health. These groups are also using this information to formulate diets that reduce the reliance on fish meal and fish oil from the marine environment for the healthy production of fish. This equates to a significant public benefit outcome, as it will directly reduce our reliance and impact on the marine environment for the production of Yellowtail Kingfish and related *Seriola* spp.

A comprehensive list of recommendations has also been provided in Chapter 10 that may lead to of the development of an improved research strategy for future nutrition and health projects by CST.

Another potential outcome from this project was the construction of CST's experimental Yellowtail Kingfish sea cage farm in Boston Bay, Port Lincoln. Unfortunately, due to CST altered production and R & D plans, this facility never became operational. However, if pressed into service, this facility would enable CST to test new technologies in a well replicated pilot scale sea cage setting prior to implementation into production systems and ultimately improve farm productivity.

The project has also achieved a significant education and training outcome (Refer to Public Benefits section of report and Appendix 1).

List of Outputs Produced

The outputs from this project address the following AS CRC Outputs and Milestones:

Outputs 1.3 - Removal or reduction of key production constraints in selected aquaculture systems

Milestones 1.3.4 - New low-cost aquaculture diets targeting improved feed conversion developed and evaluated

Milestones 1.3.5 - Production efficiency gains from genetic, health management and nutritional interventions quantified to inform long-term strategies and estimate commercial benefits

The projects outputs are listed below:

1. Project extension. Information arising from this project has been disseminated at domestic and international scientific conferences, AS CRC and industry workshops, directly to feed company representatives, government departments, and the general public. Thirteen scientific publications, seven theses, three reports and eleven conference abstracts, and numerous presentations containing information specifically targeting ways to enhance the sustainable production of Yellowtail Kingfish, arose from this project. Specific details are presented in Appendix 1. All information from Chapters 3 to 7 have been published in international peer reviewed scientific journals, and has been well received when presented, both domestically and internationally, to the broader scientific community. Numerous presentations were also given by project participants to extend the information arising from this project to the CST staff at the technology transfer meeting (Chapter 2; Appendix 2) and on numerous other occasions.
2. A literature review reporting on "the current status of knowledge of the nutritional requirements of Yellowtail Kingfish" (Chapter 8).

3. A range of Standard Operating Procedures (SOP) to improve fish weighing and stocktaking in sea cages developed by CST in cooperation with R & D partners (Chapter 2).

4. Education and Training. The project has produced a large number of undergraduate and post graduate students who have been provided with high quality relevant training in the field of fish nutrition, feed technology, physiology and health. The project directly trained one visiting Post Doctoral Research Fellow, one PhD student, six Honours students and several undergraduate students. The majority of these students have been either employed directly by companies within the aquaculture industry, both domestically and internationally, or have gone on to do post graduate studies on other CRC or industry related projects. The students and their course outcomes are listed in Appendix 1.

Background and report structure

CST still faces many challenges with feeds and feed management for Yellowtail Kingfish production. The nutritional requirements for this species have not yet been established. CST management, in collaboration, with R & D providers, identified four high priority research topics to build on information acquired from the previous AS CRC Project 2008/903 and other related projects that addressed Yellowtail Kingfish nutrition. The four key areas were:

1. Improve on-farm feeding strategies;
2. Implement less labour intensive, more accurate methods for the determination of sea cage biomass;
3. Determine the protein to energy ratios of 3 to 5 kg Yellowtail Kingfish; and
4. Improve the sustainability of Yellowtail Kingfish farming by determining the maximum dietary inclusion levels of alternative protein and lipid sources to replace fish meal and fish oil, at optimal (22 °C) and suboptimal (18 °C) water temperatures.

The project was developed in consultation with CST management, Dr Craig Foster (Research Consultant to CST), representatives from Ridley Aquafeeds and Skretting Australia and Dr Clive Talbot (independent nutrition and feed management consultant) to address these four research priorities. The report is comprised of 8 chapters, briefly described below. The majority of information in this report (Chapters 3, 4, 5, 6 and 7) has been published in peer reviewed scientific journals.

Chapter 2 reports on the outcomes of a technology transfer facilitation meeting that was carried out in response to the concern that information generated as part of numerous CST R & D projects was not getting traction at the production level. A two day technology transfer meeting held on the 22-23 March 2011 with CST management and production staff, participating students and R & D project participants was designed with the specific aims of: 1) making CST staff members aware of the project objectives and specific aims; 2) allow R & D providers to present preliminary project R & D findings for fish meal and fish oil substitution in diets for Yellowtail Kingfish; 3) in consolation with CST staff and R & D providers, identify areas for improvement for sea cage stock-taking; and 4) discuss ways to develop a draft framework for the identification, uptake, preliminary testing and implementation into production systems of technology developed during the project. Results from preliminary laboratory based nutrition and health research investigating fish meal and fish oil substitution for Yellowtail Kingfish were implemented into CST farm based production.

The main aims of Chapters 3 to 7 in this project were to improve our understanding of the somatic and physiological responses of Yellowtail Kingfish, at suboptimal water temperatures, and to dietary fish meal and fish oil substitution using alternative dietary protein and lipid ingredients. Information arising from the research presented in these chapters is currently being used by Australian feed companies to aid in the development of more cost-effective and sustainable diets for the culture of healthy Yellowtail Kingfish during the early stages of the grow-out production cycle.

In Chapters 3 and 4, the interactive effects of water temperature and dietary fish oil substitution with poultry oil or canola oil on the performance of Yellowtail Kingfish were examined. Under the conditions tested, poultry oil is an acceptable ingredient for Yellowtail Kingfish, provided the dietary essential omega-3 long chain polyunsaturated fatty acids (2.0-2.4% of the dry diet), dietary cholesterol and taurine levels are met. However, canola oil is not recommended. The substitution of fish oil with canola oil resulted in poor growth and the occurrence of the green liver syndrome, with the effect being significantly more pronounced at 18 °C.

In Chapters 5 and 6, the substitution of fish meal with increasing levels of terrestrial plant protein ingredients, i.e., solvent extracted soybean meal (SESBM) and soy protein concentrate (SPC), were examined in diets for Yellowtail Kingfish at optimal and suboptimal water temperatures. It is recommended that caution be used when substituting fish meal with soy products. The substitution of fish meal with SPC is acceptable at 10-20% inclusion, assuming the dietary amino acids are balanced and taurine is supplement. Higher dietary inclusions of SPC may be attained if palatability issues are addressed with the addition of supplemental attractants and feeding stimulants, and again assuming amino acids are balanced and taurine is supplement. While SPC is an excellent ingredient to replace fish meal in diets for Yellowtail Kingfish, the use of this product will be dictated by price and sustainable production issues. In contrast, it is not recommended to use SESBM in Yellowtail Kingfish production diets due to poor growth and potential digestive tract health issues.

Taurine, a sulphonated acid, has been reported to be conditionally required to maintain or enhance growth for *Seriola* spp. and some other marine species, particularly when fish meal is replaced using plant proteins (Gaylord *et al.* 2006; 2007; Lunger *et al.* 2007; NRC 2011). In this project, taurine defiiciencies appeared evident in Yellowtail Kingfish when fish oil was substituted with poultry oil and more so with canola oil. The closely related Japanese Yellowtail cannot synthesise taurine endogenously, therefore, the dietary provision of this sulphonated organic acid is conditionally essential. Levels of 5 to 8 g kg^{-1} diet have been recommended for Yellowtail Kingfish when dietary fish meal levels of at least 30% are used. More, taurine is required when fish meal taurine levels are low, or when dietary fish meal substitution reduced dietary fish meal levels below 30% to avoid the occurrence of the green liver syndrome and other health related problems. Further research into the specific requirements of taurine, with respect to fish meal replacement, and the interactive effects of taurine and cholesterol on the lipid metabolism of Yellowtail Kingfish during fish oil substitution are warranted.

Chapter 7 investigated growth and digestive enzyme activity of Yellowtail Kingfish at a range of water temperatures under normal (normoxic, >69% oxygen saturation) or low oxygen levels (hypoxic, <50% oxygen saturation). The optimal water temperature and dissolved oxygen level for maximum growth and digestive enzyme activities in juvenile Yellowtail Kingfish were investigated. Yellowtail Kingfish were best grown within 3 °C either side of the optimal temperature of 24 °C, under normoxic conditions, as growth was significantly reduced under hypoxic conditions. Digestive enzyme activities were not affected by dissolved oxygen, but water temperature had a significant effect, with large reductions in trypsin activity at <24 °C. This may impact dietary protein digestion and utilisation.

In Chapter 8, a literature review investigating “the current status of knowledge of the nutritional requirements of Yellowtail Kingfish” reported that very little is known about the requirements of this species and we are dependent on information from related and unrelated species to formulate production diets. Apart from the temperature and size dependent protein and energy requirement information for Yellowtail Kingfish provided by Booth et al. (2010), most of the other nutrient requirements specific to Yellowtail Kingfish

have not been determined. Information for the closely related *Seriola* spp. and other marine species may be used to formulate diets for Yellowtail Kingfish in the interim. However, this must be done with caution. There have been many instances of large unexpected differences in the nutrient requirements on inter- and intra-specific levels with fish. To add to this, many of the nutrient requirement studies examined and discussed throughout the review were carried out under near optimal conditions. Suggested dietary requirements may differ considerably when fish are exposed to stressors in the challenging environment of the sea cage (Lygren et al., 2001).

Chapter 9 describes the project benefits and adoption, and outcomes. A list of future research topics and recommendations based on the findings arising from this project is provided in Chapter 10. The extension of results arising from this project directly to industry was rapid. Results described in each of these Chapters were communicated directly to Michael Thomson, and Dr Trent D'Antignana (CST R & D Managers) and Dr Richard Smullen (Ridley Aquafeed) as the work was undertaken, and in many instances also provided to other relevant CST employees (e.g. Fish production and health managers) as soon as they became available and subsequently as draft reports and scientific publications. CST is currently using the information from the literature review to ensure that nutrient specifications of current Yellowtail Kingfish production diets are adequate, and also to formulate, and test, new diet formulations prior to their incorporation into production.

In conclusion, in response to this research and the directives of CST, the feed companies in Australia have altered their production diets for Yellowtail Kingfish to no longer contain canola oil or SESBM. However, further research is necessary to validate these outcomes. Furthermore, as the experiments were short in duration (~5 weeks), it is recommended that long-term studies are carried out. The longer period may cover a full production cycle to validate the outcomes of this research. Based on the findings of the literature review it is evident that additional nutritional research is required, particularly in the areas of essential amino acid requirements, supplemental taurine, levels, and ratios of DHA to EPA, and vitamins and minerals, to improve our understanding of the nutritional requirements and subsequent commercial diet formulations for this species. There should be a particular focus on ensuring that anti-nutritional factors or dietary deficiencies do not lead to the occurrences of diet-induced enteritis, green liver syndrome or other potential growth limiting factors and health problems in Yellowtail Kingfish.

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Chapter 1. **Preface**

Introduction

Yellowtail Kingfish, *Seriola lalandi*, is a relatively new species to aquaculture that has been cultured in South Australia (SA), Western Australia and New South Wales, with over 90% production from Clean Seas Tuna Ltd (CST, Arno Bay, SA). The production of Yellowtail Kingfish in SA was approximately ~3,000 tonnes in 2010 (Mike Thomson, personal communication) and at this time it was anticipated that its production would increase to 10,000 tonnes within 5 years in the Spencer Gulf region.

Despite the proposed rapid development of this industry the nutritional requirement and feeding methods of sea cage reared Yellowtail Kingfish are not well studied. Additionally, Yellowtail Kingfish cultured in Spencer Gulf are subjected to seasonal variations in water temperatures. For example, water temperatures at Arno Bay range from 13 to 23 °C (Australian Bureau of Meteorology Sea Surface Temperatures, August 2005-August 2007), and greater extremes may be found at other CST sites in Spencer Gulf (10 to 25 °C; Joe Ciura, personal communication). The temperature profile of Spencer Gulf is reasonably consistent between seasons, with summer water temperatures typically ranging from 20 to 25 °C, before falling to 18 °C in autumn, then decreasing rapidly to 10 °C late in winter and increasing to 18 °C by early to mid-spring. Temperature is the single most important factor to influence fish growth by affecting feed intake, digestion, enzyme activity, and metabolism (Jobling, 1994). The optimal temperature for growth reflects the temperature where the difference between the ingested energy and energetic expenditure is largest and energy partitioned into growth is maximal. As optimal growth is found within a narrow temperature range, “thermal tolerance”, deviations from this range often results in decreased feed intake and growth rates. The optimal or preferred growing temperature for Yellowtail Kingfish is not well documented (Pirozzi and Booth, 2009). However, Pirozzi and Booth (2009) investigated the effects of water temperature on the metabolic rate of juvenile Yellowtail Kingfish grown in tanks and reported that the temperature range of 20-25 °C was where changes in metabolic rate (more stable) were least dependent on temperature. CST data suggests that the growth rate of Yellowtail Kingfish in sea cages appears to be minimal below 17 to 18 °C (Joe Ciura, personal communication). Based on this information it would appear that Yellowtail Kingfish cultured at CST operations are exposed to suboptimal water temperatures for more than half of the production cycle. The fluctuating water temperatures throughout the production cycle further complicates the understanding of nutritional requirements, feeding practices, farm management, and ultimately the successful production of Yellowtail Kingfish in these waters.

Current industry practice in SA is to stock 5-10 g Yellowtail Kingfish fingerlings to sea cages in September to coincide with rising seawater temperatures. Fish are then on-grown to a target harvest weight of approximately 3-3.5 kg over a period of 18-24 months. This grow-out period encompasses two winters during which the growth of Yellowtail Kingfish slows considerably. As a consequence feed intake falls and feed conversion ratio (FCR) increases (worsens). Extremely low water temperatures also affect the nutritional and immune status of Yellowtail Kingfish and as a result farms often experience increased rates of mortality during winter months, especially in juvenile stock that have failed to significantly increase their condition during the previous spring/summer. Poor FCR, as well as the additional costs and risks associated with over-wintering stock increase the capital investment required to

support the on-farm standing biomass and reduce profitability. However, winter conditions in SA waters will mean that better survival rather than continued growth is the major production aim during this period.

The AS CRC and CST are investigating new methods and production options that had the potential to attain maximum growth rates and survival of Yellowtail Kingfish grown in sea cages. These new strategies are aimed at truncating the grow-out period of Yellowtail Kingfish (expose fish to one less winter) which would result in more fish per unit of capital investment, ultimately increasing production efficiency. Many of the potential improvements in growth and performance will come through better husbandry and feeding practices, as well as genetic improvement and a better understanding of the impacts that environmental parameters, such as temperature, have on growth. Some of the most influential issues affecting Yellowtail Kingfish growth in sea cages have already been identified and targeted for research. For instance the AS CRC is committed to investigating the effect of stocking size and temperature on growth and performance of small and large Yellowtail Kingfish (AS CRC Project 2008/903). The outcomes of that work will assist CST make production decisions.

CST has also identified four new high priority research topics that will build on information acquired from the AS CRC Project 2008/903 and other related projects. Due to the fact that Yellowtail Kingfish are grown in fluctuating water temperatures CST identified that further research was still required at optimal (22 °C) and suboptimal (18 °C) water temperatures in four key areas to:

1. Improve on-farm feeding strategies: development and validation of a growth-feed intake model for Yellowtail Kingfish cultured in sea cages;
2. Implement new, less labour intensive, more accurate methods for the determination of sea cage biomass. Development of this component is critical for successful on farm feed management;
3. Determine the protein to energy ratios of 3 to 5 kg Yellowtail Kingfish; and
4. Improve the sustainability of Yellowtail Kingfish farming by investigating the maximum dietary inclusion levels of alternative protein and lipid sources to replace fish meal and fish oil, at optimal (22 °C) and suboptimal (18 °C) water temperatures.

Originally, the proposed research outlined in this project investigated aspects of the aforementioned four high priority research topics. However, due to changing research and production priorities of CST, an AS CRC approved project variation and alteration resulted in the planned project work aligned with several of these research priorities being removed from the project. The project variations and alterations are listed in the following text and also in the Objectives section presented on pages 1-8 and 1-9.

Research Priority 1: Improve on-farm feeding strategies: development and validation of a growth-feed intake model for Yellowtail Kingfish cultured in sea cages (Removed from project).

Relatively little was known about the optimal feeding strategy for Yellowtail Kingfish under SA sea cage conditions and production managers were seeking information on feed regimes they can employ to target either maximal weight gain or highest economic return (i.e. establishment of growth – ration curves). Early feeding practices used at CST for the production of Yellowtail Kingfish in sea cages involved feeding fish pellets twice daily to apparent satiation seven days per week. However, this may have unintentionally resulted in under- or over-feeding, leading to high feed conversion efficiencies and reduced dietary nutrient digestibilities (Jobling, 1994).

Japanese producers of *Seriola* spp. often feed their larger fish on alternate days or only twice to three times a week to maximise economic return and or deal with seasonal changes in water temperature or unfavourable weather conditions (Nakada, 2002). CST was concerned about the efficiency, in terms of production costs and the environment, of their early feeding practices and considered improvements in this area as a high priority.

CST has been developing growth-ration models for Yellowtail Kingfish with the aim of improving FCR and production efficiency. To date, the majority of these models have been developed 'in-house' by external consultants (Dr Brett Glencross and Dr Clive Talbot) using farm records for Yellowtail Kingfish that were grown in sea cages at various locations such as Port Lincoln or Arno Bay. The reliability of the farm data used to construct the growth-ration models was questioned, especially with respect to accurate measures of feed intake as opposed to feed input, measures of weight gain, survival and the impact of top-cropping. Feed input has a great bearing on the potential growth rate of fish at any temperature, thus if data collected on feed inputs are unreliable then the resultant growth-ration curves will also be unreliable. Likewise, if those charged with accurately delivering the quantum of feed prescribed by the model being used do not follow the model carefully, then the model will have limited value.

NSW Department of Primary Industries (DPI NSW) Port Stephens Fisheries Institute (PSFI) has also developed a growth-feed intake model for Yellowtail Kingfish using a factorial approach. This model, and the one for mulloway, were outputs of the Aquafin CRC Project 2004/220. These models required the determination of digestibility coefficients, determination of requirements for maintenance and measurement of carcass composition for different size fish. Integration of these data for each species into a single model allows estimation of dietary requirements for digestible energy and digestible protein and allows growth rate and feed intake of Yellowtail Kingfish to be predicted for diets with different protein or energy levels. However, the Yellowtail Kingfish model developed by NSW DPI was based on data for fish up to approximately 2 kg and reared at water temperatures between 20 and 25 °C. As no farm data were available, the NSW DPI model has been constructed exclusively using data for Yellowtail Kingfish reared under experimental conditions. To ensure the model was applicable to farm situations, we needed to consider historic data from CST Yellowtail Kingfish operations in sea cages in SA and test the effectiveness of the model in a controlled and well replicated study in sea cages.

Research Priority 2: Implement new, less labour intensive, more accurate methods for the determination of sea cage biomass (Removed from project).

Estimating fish biomass within individual sea cages is important for efficient and effective feeding and farm management practices. The knowledge of the sea cage population in combination with the average fish weight enables an accurate determination of the sea cage biomass, making it possible to monitor fish growth rates and control feeding rates. Additionally, the knowledge of cage biomass makes it possible to apply correct medication dosing and plan harvesting and sales more accurately.

At the commencement of this project, CST estimated fish numbers in their sea cages at two control points. Control point 1 was when the ~5 g fingerlings were transferred from the hatchery nursery to the sea cages; and Control point 2 was at the end of the first autumn (May/June) in the sea cages. This approach was prone to two major problems. The first problem was that the small size of the fingerlings at stocking, compounded by the high level of abnormalities from the hatchery, left the fish prone

to high mortality in the early stages. In the sea cage environment it was not possible to reliably keep track of mortalities of fish in the first phase of production due to their small size. Small weak, sick and dead fish were eaten by larger fish in the sea cage and this resulted in no apparent visual evidence of mortality. The second problem was the timing of the first sea cage grading/thinning procedure. At the commencement of the project CST's management practice of grading/thinning occurred at the end of autumn. The timing of this procedure was designed to reduce the possibility of a pronounced growth-check on rapidly growing fish during the warmer production phase. On first glance, this production strategy appeared sound; however grading/thinning the sea cages and counting stock during late autumn/early winter months exposed the fish to low water temperature handling stress, which in turn may have led to additional losses in growth, morbidity and mortality. The stress would be further compounded when the fish were confronted with additional drops in water temperatures soon after handling.

The inability to track the mortality rate of young fish coupled with the 7 to 9 month period before stock-taking during the grading/thinning procedure made biomass estimation difficult and over-feeding/under-feeding was highly likely. This phenomenon occurred at CST Yellowtail Kingfish operations on a regular basis (Joe Ciura, personal communication). Over-feeding will contribute to a poorer FCR, increased feed costs and a reduced profit for the company, not to mention additional environmental and potential fish health problems associated with the introduction of uneaten feed into the immediate vicinity of the sea cages. Under-feeding will result in a loss of productivity and impact on fish health.

The problem of high fingerling abnormalities and low fingerling survival rate were addressed by CST in other AS CRC larval rearing research projects as well as in the "Understanding Yellowtail Kingfish" (AS CRC 2008/903) project; however, there was also potential scope to improve on-farm management practices by changing the timing of the grading and cage thinning procedures. Further areas of improvements were also identified such as implementing on-farm fish grading and counting systems (Joe Ciura, personal communication).

At the commencement of this project, CST estimated fish size by manually sub-sampling fish from each cage at each site every month. This invasive process involved crowding, dip-netting, anaesthetising and weighing of fish before they were returned to the cage. This system to estimate fish weight was considered inaccurate (Klontz, 1993), impacted on fish welfare (Ashley, 2007) and labour intensive. CST considered this procedure could be improved by using a non-invasive system. Two commercially available non-invasive systems that CST considered for on-farm implementation were the Vaki biomass counter system (Vaki Aquaculture Systems Limited, Kopagovor, Iceland) and the AM100 stereo camera system (AQ1 Systems Pty. Ltd, Hobart, Tasmania, Australia). Each system has its own specific set of advantages and disadvantages. CST was developing the use of a Vaki biomass counter system within their operations. The Vaki system can be transferred from site to site, which requires minimum labour and considered to be "stress free" to the fish. The system electronically gathers data on average fish weight and size distributions as fish voluntarily swim through a submerged sensing frame within the sea-cage. However, when supplied from the manufacturer, the Vaki system is supplied with software that is calibrated for a range of cultured fish species including the Japanese Yellowtail and Atlantic Salmon. Unfortunately the morphometric characteristics of the Yellowtail Kingfish cultured in SA differ considerably with those of the aforementioned species, and therefore, the Vaki software does not provide accurate information regarding Yellowtail Kingfish weights or lengths. The shortcomings of the Vaki

system and associated software may be rectified by calibration and validation based on a substantial amount of field based Yellowtail Kingfish morphometric information.

The AM100 stereo camera system uses a two dimensional image of a three dimensional object to estimate weight. Stereo camera systems have been used for the determination of fish weight for a range of species (Shortis et al., 2007). For the stereo camera system to work correctly it needs to identify morphometric features on the fish that are easily visible and that can also be used to accurately estimate fish weight (Beddow and Ross, 1996; Shortis et al., 2007). The successful application of this technology requires species specific information, which was not currently available for Yellowtail Kingfish. CST has conducted initial work on developing this method for Yellowtail Kingfish, including the development of a tentative equation using data collected from harvested individual fish weights, lengths and heights. In this study morphometric information for the calibration, validation and implementation of both systems to CST Yellowtail Kingfish sea cages was planned to be developed.

Research Priority 3: Determine the protein to energy ratios of 1 to 4 kg Yellowtail Kingfish (Removed from project).

When culturing fish to achieve maximum growth it is important to feed a diet that balances the dietary protein requirement with the dietary energy content (Halver and Hardy, 2002). Protein is recognised as the most expensive macro-nutrient in formulated fish diets (Halver and Hardy, 2002). The amount of dietary protein in aquafeeds may be optimised ("reduced") by adding non-protein energy sources, such as lipid and carbohydrate. The addition of non-protein sources for energy may create a 'protein sparing' effect (Shiau and Lan, 1996; Halver and Hardy, 2002). This reduces the catabolism of protein for energy and improves protein retention efficiencies (Lupatsch et al., 2001, Halver and Hardy, 2002). However, not all fish species are tolerant of high levels of lipid or carbohydrate in their diet and an imbalance of either class of nutrient may affect growth efficiency and carcass composition.

When a new species is cultured the diet is often formulated based on the nutritional requirements of a well-studied, closely related species, or a species with a similar diet in the wild (Talbot et al., 2000). At the commencement of this project, commercial diets in Australia for Yellowtail Kingfish were based on salmonid/barramundi diets (Dr Richard Smullen, personal communication). These grow out feeds typically contained a minimum of 45% protein and 20% fat with an energy content of 23 MJ/kg and were used throughout the entire sea cage production phase. Since Yellowtail Kingfish are cultured and harvested at different sizes and water temperatures in the Spencer Gulf CST were interested to understand the relationship between protein:energy ratios (i.e. protein:lipid ratios), and carcass composition at optimal and suboptimal water temperatures at different harvest sizes. Slight reductions in dietary protein may equate to large savings in diet costs for CST.

Research Priority 4: Improve the sustainability of Yellowtail Kingfish farming by investigating the maximum dietary inclusion levels of alternative protein and lipid sources to replace fish meal and fish oil, at optimal (22 °C) and suboptimal (18 °C) water temperatures

Fish meal has been the main protein contributor to marine fish production diets due to its balanced amino acid profile and good digestibility (Alexis and Nengas, 2001; Halver and Hardy, 2002); and fish oil has been the main lipid contributor due to its high content of n-3 long chain-highly unsaturated fatty acid (HUFA) and digestibility (Halver and Hardy, 2002; Izquierdo et al., 2003). However, both fish meal and fish oil

are also the most expensive macro-ingredients in fish diets and their global supplies and availability are limited. Consequently, partial replacement of fish meal and fish oil with cheaper alternative protein and lipid sources from plants and terrestrial animals is gaining interest as a more economical way of feeding fish (Tacon, 1995, Allan et al., 2000, Valdimarsson and James, 2001). The use of alternative ingredients also addresses the critical issues of sustainability, such as “fish in fish out” ratio espoused by the World Wildlife Fund and other non-government organisations as measure of the sustainable use of natural resources for fish production. A demonstration of addressing sustainability issues, such as this ratio, by fish producers is becoming highly relevant for market access globally. The sustainable use of fish meal and fish oil are also included as important components of the Best Aquaculture Practices (BAP) codes which have either been developed and implemented, or are currently being developed, for a range of commercially important aquaculture species world-wide.

Meals or protein concentrates derived from a range of plant ingredients including soybean, canola and lupins have shown great potential to replace fish meal protein in fish feeds for a range of marine fish species (Tacon, 1995; Alexis and Nengas, 2001; Valdimarsson and James, 2001). However, the problem with the inclusion of plant-derived protein sources is that they have an amino acid imbalance (typically low in lysine), contain high levels of relatively indigestible carbohydrate and may contain anti-nutritional substances. Anti-nutrients are substances which interfere with food utilisation and affect the health and production of animals by inhibiting growth, decreasing feed efficiency, altering gut histology, reducing digestive capabilities and inducing other pathological conditions (Francis et al., 2001).

Our feed industry AS CRC project participant, Ridley Aquafeeds, expressed a desire to test solvent extracted soybean meal (SESBM) and soy protein concentrates (SPC) with Yellowtail Kingfish. These products were chosen as they are readily available and proven to be cost effective dietary ingredients for the culture of a range of fish species. However, soybean meal is known to contain a range of anti-nutrients that have been demonstrated to impede growth and impact on health for several species of freshwater and marine fish (Francis et al., 2001). In order to determine the maximum acceptable dietary inclusion of SESBM and SPC in diets for Yellowtail Kingfish we investigated the growth performance, digestive tract physiology and health of Yellowtail Kingfish fed increasing levels of these ingredients.

There have already been many studies on various fish species investigating the partial or complete replacement of fish oil with cheaper, alternative sustainable lipid sources from plants and land animals. Full replacement of fish oil with vegetable or animal oils in marine fish is not common in the commercial situation and is dependent on the fatty acid requirements of the given species, and may not be possible (Thompson et al., 1996; Bell et al., 2002; Grisdale-Helland et al., 2002; Bell and Dick, 2004). Another major problem with the inclusion of plant-derived oils is that they are poor sources of omega 3 fatty acids in comparison to fish oil, and contain no HUFA which are essential for the growth of healthy marine fish. The same problem occurs for the inclusion of animal oils, whereby the omega 3 HUFA content is very limited. The use of alternative oils in diets can affect the fatty acid composition, particularly the ratio of omega 3 to omega 6 fatty acids. This will have an impact on organoleptic and health characteristics of the product. Often when alternative lipid sources are incorporated into diets in the early stages of production, a finishing diet containing fish oil can be fed to improve the fatty acid composition of the flesh prior to harvest and sale. However, the evaluation of alternative oil sources for growth and product quality has not been conducted for Yellowtail Kingfish and was considered a high priority research topic by CST.

Additionally, a literature review addressing the current status of knowledge of the nutritional requirements of Yellowtail Kingfish was incorporated into the project. While there is a vast amount of literature addressing the nutritional requirements of a range of marine carnivorous species, relatively little is currently known of the nutritional requirements of Yellowtail Kingfish. Information arising from the literature review will enable CST to make informed management decisions, in collaboration with feed companies, on dietary specifications and formulations in an attempt to improve the health and productivity of Yellowtail Kingfish in their operations.

The approaches taken, and the information gained in this study, will directly address the industry R & D priorities and lead to an improvement in diets and feed management and in the economical and sustainable production of Yellowtail Kingfish by CST.

Need

Yellowtail Kingfish is the main closed cycle cultured finfish in SA and this industry has great potential to expand in other states of Australia. One of the major inefficiencies identified was feeds and feed management in CST's Yellowtail Kingfish production. Henceforth, the newly formed CST Research and Management Group (RMAG) identified improving FCRs in Yellowtail Kingfish operations as an urgent priority. The production efficiency of Yellowtail Kingfish by CST has been hindered by the poor understanding of feeding strategies, sea cage biomass determination, growth performance and the nutrient requirements of Yellowtail Kingfish at different life stages and water temperatures. Research in this project was designed to specifically address each of these topics. This information is essential as Yellowtail Kingfish are exposed to suboptimal water temperatures at most times of the year in SA. All-year round management strategies based on sound information needs to be developed and implemented to maximise the production efficiency.

Despite the development of growth-ration curves for Yellowtail Kingfish by external consultants, CST was still experiencing problems establishing profitable feeding regimes that aimed to reduce end of cycle FCR from above 2.0 to less than 1.7. This level may or may not be achievable and, apart from accurate feeding, depends on many factors including diet composition, seasonal water temperature fluctuations, genetic background, etc. It has also become apparent that in order for CST to gain market acceptance for their Yellowtail Kingfish products on a global basis the issue of sustainable production needed to be addressed. In order to address the issue of sustainability, detailed changes to current dietary formulations for Yellowtail Kingfish, which contain high levels of fish meal and fish oil, had to be made. Species specific information regarding nutrient availability, growth performance, fish health and maximum dietary inclusion levels of alternative sustainable ingredients for Yellowtail Kingfish cultured at fluctuating water temperatures were required in order to make these changes. An understanding of the current status of knowledge pertaining to the nutrient requirements of Yellowtail Kingfish was also required.

Objectives

The four objectives for this project, corresponding to the four research priorities identified by Clean Seas Tuna Ltd, are provided in the following list (Note: some objectives were subsequently removed from the project prior to its early termination, on the request of CST and are noted below):

Objective 1: Improve on-farm feeding strategies: development and validation of a growth-feed intake model for Yellowtail Kingfish cultured in sea cages (removed from project).

- Subproject 1: Run a technology transfer facilitation framework meeting with CST staff, and project R & D participants (remained in the project).

- Subproject 2: Develop a growth-feed intake model specific to Yellowtail Kingfish. Due to an AS CRC approved variation this subproject was removed from the project.

- Subproject 3: Test and validate the growth-feed intake model developed in Subproject 2 with Yellowtail Kingfish cultured in replicated sea cages on the CST R & D farm at summer water temperatures. Due to an AS CRC approved variation this subproject was removed from the project.

Objective 2: Implement new, less labour intensive, more accurate methods for the determination of sea cage biomass (removed from the project).

- Subproject 4: Review current fish counting methods used by CST to establish a more accurate on-farm fish counting system. Due to an AS CRC approved variation this subproject was removed from the project.

- Subproject 5: Calibrate, validate and implement two electronic fish biomass counter systems to CST Yellowtail Kingfish sea cages for the determination of Yellowtail Kingfish biomass. Due to an AS CRC approved variation this subproject was removed from the project.

Objective 3: Determine the protein to energy ratios of 1 to 4 kg Yellowtail Kingfish (removed from the project).

- Subproject 6: Test the effects of varying protein to energy ratios on the growth performance, and feed efficiency of ~1 to 4 kg Yellowtail Kingfish cultured in sea cages in the CST research farm. Due to an AS CRC approved variation this subproject was removed from the project.

Objective 4: Improve the sustainability of Yellowtail Kingfish farming by investigating the maximum dietary inclusion levels of alternative protein and lipid sources to replace fish meal and fish oil, at optimal (22 °C) and suboptimal (18 °C) water temperatures (remained in the project).

- Subproject 7: Investigate the suitability of canola oil, and poultry oil as alternative sources to fish oil for Yellowtail Kingfish when cultured at optimal and suboptimal temperatures (remained in the project).

- Subproject 8A and 8B: Explore the suitability of soybean products as alternative protein sources (8A, solvent extracted soybean meal (soy 48); and 8B, soy

protein concentrate) in juvenile diets at optimal and suboptimal temperatures (remained in the project).

- Subproject 9: Test the effects of alterative protein sources on the growth performance of ~4 kg Yellowtail Kingfish cultured in the CST research farm. Due to an AS CRC approved project variation this subproject was removed from the project.

- Subproject 10: Evaluate the enzyme activity of Yellowtail Kingfish under a range of temperatures (8-28 °C) (remained in the project).

- Subproject 11: A literature review: Current status of knowledge of the nutritional requirements of Yellowtail Kingfish (*Seriola lalandi*). Due to an AS CRC approved project variation the literature review was an AS CRC approved addition to the original project that was requested by CST in September 2012.

Chapter 2. **Technology transfer meeting**

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Abstract

Concerns had been raised at all levels regarding the uptake and implementation of technology developed throughout R & D projects with CST. A two day technology transfer meeting was designed with the specific aims of:
1) Making CST staff members aware of the project objectives and specific aims;
2) Allow R & D providers to present preliminary project R & D findings for fish meal and fish oil substitution in diets for Yellowtail Kingfish;
3) In consolation with CST staff and R & D providers, identify areas for improvement for sea cage stock-taking; and
4) Discuss ways to develop a draft framework for the identification, uptake, preliminary testing and implementation into production systems of technology developed during the project.

The meeting was well received by CST staff and actions were taken to address the aims of the meeting. Results from preliminary laboratory based nutrition and health research investigating fish meal and fish oil substitution for Yellowtail Kingfish were immediately implemented into CST farm based production.

The information presented in this Chapter addresses part of Objective 1 (Subproject 1): Improve on-farm feeding strategies: development and validation of a growth-feed intake model for Yellowtail Kingfish cultured in sea cages.

Introduction

Concerns had been raised at all levels regarding the uptake and implementation of technology developed throughout R & D projects with CST. This subproject was designed to address these concerns. A two day technology transfer meeting was designed to allow time for CST staff and R & D providers to be made aware of the aims and objectives of the AS CRC "Sustainable feeds and feed management for Yellowtail Kingfish" project, and also communicate their views on specific strategies that would facilitate rapid technology transfer from the R & D experimental phase of the project through to incorporation into on-farm routine production practices.

Aim

The aims of the meeting were to:

- Make CST staff members aware of the project objectives and understand the specific project aims;
- Present preliminary project R & D findings for fish meal and fish oil substitution in diets for Yellowtail Kingfish;
- Identify areas for improvement for sea cage stock-taking; and
- Discuss ways to develop a draft framework for the identification, uptake, preliminary testing and implementation into production systems of technology developed during the project.

Methods

The meeting with CST management and production staff and R & D project participants and students was held on 22-23 March 2011 at the Port Lincoln Marine Science Centre, Port Lincoln, SA. Refer to Appendix 2 for the list of meeting participants and meeting Agenda.

Results and Discussion

The two day meeting was well attended and preliminary information from all of the preceding work in the project was presented to CST management and production staff. Briefly, the meeting defined the specific aims of the project and passed on relevant preliminary R & D findings from laboratory based nutrition research with regards to fish meal and fish oil substitution with soy products and canola and poultry oils, respectively, for Yellowtail Kingfish (refer to information in Chapters 3-6; Bowyer et al., 2012a, b, c; 2013a, c). Areas for the improvement of sea cage stock-taking were identified, and meeting participants discussed the development of a framework for the identification, uptake, preliminary testing and implementation into production systems of technology developed during the project. Actions were taken to address issues of feeding and FCR reductions across CST operations. Refer to Appendix 2 for the list of meeting outcomes and actions.

Technology transfer from project R & D to CST production systems occurred during the meeting. CST management acted on the information provided on fish meal and fish oil substitution for Yellowtail Kingfish, presented by Bowyer et al. (2012 a, b, c; 2013a, c), Bansemer (2011) and Bansemer et al. (In press; Appendix 4) (Chapters 3-6), and instructed the commercial feed suppliers to exclude canola oil and soy product from their diets until further research was done to ascertain their potential deleterious effect on Yellowtail Kingfish health.

It was decided that technology transfer would be best achieved by R & D providers maintaining regular contact with the CST R & D manager, and by regular meetings with CST farm management staff. Also the establishment of an R & D farm, within the sustainable feeds and feed management project, was planned at Port Lincoln. The facility would allow CST to test relevant R & D findings on a pilot scale, prior to application across CST operations.

The meeting was used as a forum to examine current fish stock-taking methods used in the CST Yellowtail Kingfish production sea cages (Objective 2, Subproject 4). During the meeting, CST farm management staff, led by Joe Ciura and Mike Thomson, identified critical key points in the production cycle where improvements were to be made. This information was gathered over the 12 months preceding the meeting, and in response to issues identified with previously used CST on-farm fish counting and weighting systems during the development of this project. Mike Thomson reported that as a result of this process CST have developed and implemented a range of standard operating procedures to improve on-farm fish counting and stock-taking systems.

Conclusion and Recommendations

The meeting gave CST management and production staff an opportunity to become aware of the project aims, meet the R & D providers and have direct input into the development of the project. Technology transfer occurred at this meeting. R & D findings regarding Yellowtail Kingfish growth and health in relation to fish meal and fish oil substitutions were transferred from the R & D providers directly to CST management, who implemented changes to production diets. Round table discussions were held, and in consultation with CST management staff and farm mangers, areas for improvements in sea cage stock-taking were identified and actions were planned to address them. CST management and R & D providers also discussed ways to development of a draft framework for the identification, uptake, preliminary testing and implementation into production systems of technology developed during the project. The establishment of a pilot scale R & D farm was decided upon to help to facilitate the successful uptake of R & D into CST farm operations. Actions were planned for future R & D transfer meetings. Overall, the meeting was considered a success by CST management.

Chapter 3. **Investigate the impacts of canola oil, and poultry oil as alternative sources to fish oil on the growth performance of Yellowtail Kingfish when cultured at optimal and suboptimal water temperatures**

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Abstract

Fish oil has been replaced by alternative oils to reduce the cost of aquaculture diets, but fish growth may be compromised by these oils at suboptimal temperatures. A 5-week trial was run to examine the interactive effects of optimal (22 °C) and suboptimal (18 °C) water temperature and the partial or total replacement of fish oil with poultry oil or canola oil on the performance of juvenile Yellowtail Kingfish. Practical diets were identical in composition, except the dietary lipid component was supplemented with 100% lipid as either poultry oil, canola oil , a blend of fish oil and poultry oil (50:50) or a blend of fish oil and canola oil (50:50). The dietary lipid component of the control diet contained 100% fish oil. Fish fed the canola oil diet at 18 °C had inferior growth, feed efficiency and nutrient retention, and showed higher incidences of green liver and lower plasma cholesterol levels. Whole body proximate composition was influenced by temperature, but not diet, except moisture content which was highest in fish fed canola oil. The fatty acid composition of fillet lipid correlated with the poultry oil and canola oil inclusion, in that the proportions of 18:1n-9, 18:2n-6 and 18:3n-6 all increased with increasing dietary poultry oil and canola oil. The concentrations of 20:5n-3 (EPA), 22:6n-3 DHA) and 20:4n-6 (ARA) in the fillet lipid was reduced with increasing dietary poultry oil and canola oil levels. Results confirmed that 100% poultry oil and 50% canola oil can replace fish oil in diets without reducing growth, but 100% canola oil results in poor fish growth compared with the fish oil control, regardless of temperature. However, we recommend only partial fish oil substitution using poultry oil. Diets must be formulated to contain sufficient levels of essential EPA, DHA and ARA. The level of these essential fatty acids in the fish oil and dietary ingredients will determine the actual level of fish meal substitution possible. The results are useful for dietary formulation to reduce feed costs and improve sustainability without compromising fish health and growth.

The information in this Chapter addresses part of Objective 4 (Subproject 7): Determine the maximum inclusion levels of alternative lipid sources to replace fish oil at optimal and suboptimal temperatures. The contents of this Chapter have been published in Aquaculture: 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 (22 °C) and suboptimal (18 °C) water temperatures. Aquaculture, 356-357, 211-222.

Introduction

Water temperature is an important physical factor influencing fish growth (Brett, 1979; Jobling, 1997; Tucker, 1998) because it directly affects feed intake, digestion, enzyme activity and metabolism. At optimal water temperatures, most food energy can be partitioned into fish growth (Brett, 1979). So when a marine carnivorous species is cultured in an environment where water temperature falls outside the fish's optimal range, fish growth will be reduced. Yellowtail Kingfish (*Seriola lalandi*, Valenciennes, 1833) is a temperate, carnivorous, marine fish species that is widely distributed throughout the Pacific and Atlantic oceans (Nugroho et al., 2001). Yellowtail Kingfish are well suited to sea cage culture conditions and are a fast-growing, economically valuable species (Fernandes and Tanner, 2008). However, one of the key issues with farming Yellowtail Kingfish during the grow-out period (5 g fingerling to 3-4 kg market weight) is managing their nutrition during seasonal fluctuations in water temperatures. In southern Australia for example, where Yellowtail Kingfish are grown, the average annual sea surface temperatures range from 10 °C in winter to 25 °C in summer (Miegel et al., 2010), but the optimal water temperature for good growth is reported to be 22.8 °C (Pirozzi and Booth, 2009). When water temperatures drop below 18 °C, growth conditions are suboptimal, leading to reductions in growth performance, feed efficiency and resistance to disease. Therefore, there is a need to investigate nutrition-environment interactions to sustain Yellowtail Kingfish production and quality year round in this region.

Fast-growing marine carnivorous fish, from temperate and cold water environments, require high energy diets with dietary lipid as the favoured source of non-protein energy (Shimeno et al., 1996; Sargent et al., 2002). Fish oil contains long chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), and arachidonic acid (20:4n-6, AA). The LC-PUFA are essential r marine carnivorous fish to maintain optimum growth, health and development (Tocher, 2010). It is well known that the n-3 LC-PUFA (EPA and DHA) present in fish have important health benefits for human nutrition, such as prevention of cardiovascular disease and Alzheimer's disease (Mozaffarian and Rimm, 2006; Schaefer et al., 2006). Fish oil is a commodity used not only by the aquaculture feed industry, but there is also competition for its use in the terrestrial animal feed industry and human nutritional use. The global supply of fish oil production has reached a maximum of about 1.3 million tonnes (FAO, 2010; Jackson, 2010) and competition between industries, as well as natural environmental impacts such as El Nino events, affects the price of fish oil (Turchini et al., 2009).

The future expansion and long-term sustainability of the aquaculture industry is reliant upon identifying suitable alternative lipid ingredients to fish oil. In recent years, efforts have been made in replacing dietary fish oil with lipids from terrestrial plant and animal sources. Canola oil and poultry oil are two lipid ingredients available to replace fish oil in aquaculture feeds for marine fish. Canola oil is the trademark name given to rapeseed cultivars that contain a low concentration of erucic acid (<2%) (Canola Council of Canada, 2004; Higgs et al., 2006). Canola oil is a fully refined, bleached and deodorised edible oil obtained from certain varieties of *Brassica napus* or *Brassica campestris*, whereas poultry oil is obtained from processing chicken by-products. The prices of canola oil and poultry oil are generally cheaper than fish oil (Turchini et al., 2009) and both ingredients are high in supply and are good sources of digestible energy (Turchini et al., 2009). Canola oil and poultry oil are rich in monounsaturated fatty acids (MUFA) (particularly oleic acid, 18:1n-9) and total n-6 polyunsaturated fatty acids (PUFA), but devoid of the essential n-3 LC-PUFA, EPA and DHA (Higgs et al., 2006). Marine fish have a poor ability to convert linoleic acid (18:2n-6, LA) to AA, and α-linolenic acid (18:3n-3, ALA) to EPA and DHA (Sargent et

al., 2002). Therefore, vegetable oils have successfully replaced up to 60% dietary fish oil in European seabass and gilthead seabream without negative effects on growth, survival or health (Izquierdo et al., 2005; Mourente et al., 2005), but replacement at higher levels such as 80% led to decreased performance (Montero et al., 2003; Menoyo et al., 2004). Replacement of fish oil with alternative oils also influences the muscle fatty acid (FA) profile (Glencross et al., 2003; Higgs et al., 2006; Huang et al., 2007). Prior to this project there was limited published literature on the inclusion of canola oil or poultry oil in diets for Yellowtail Kingfish (Booth et al., 2010a) and the associated impacts on growth performance, FA profile or health parameters have not been reported.

The replacement of fish oil with alternative oils can lead to significant changes to the metabolism of lipids (Glencross, 2009). The liver plays a major role in the process of lipid metabolism (Peng et al., 2008), such as synthesising bile salts that are formed by the conjugation of bile acids and taurine for the emulsification of lipids (Goto et al., 2001). In Japanese Yellowtail (*Seriola quinqueradiata*) and red seabream (*Pagrus major*) if the excretion of bile pigments from the liver into bile is impaired it can lead to a symptom called 'green liver' (Takagi et al., 2005; Takagi et al., 2010). Green liver has been linked to the replacement of fish meal and is thought to be caused by a deficiency in the non-essential amino acid taurine (Goto et al., 2001; Takagi et al., 2005).

Since Yellowtail Kingfish farming in Australia is conducted in sea cages with fluctuating water temperatures, it is necessary to identify the interactive effect of water temperature on fish oil substitution with alternative lipid ingredients. Fish are able to alter the structural diversity of lipids within their membranes to adapt to changes in ambient water temperature (Miller et al., 2006). At cold water temperatures the chemical structure of n-3 LC-PUFA permits a greater degree of unsaturation compared to more saturated fatty acids, which allows for more fluidity and flexibility within the membrane structure. Fish oil contains high levels of EPA and DHA, which function as structural and physiological components of the cell membrane in most fish tissues (Glencross, 2009). In contrast, canola oil and poultry oil are devoid of EPA and DHA. The interactive effects of water temperature and fish oil replacement on muscle FA composition has been studied in species such as Atlantic Salmon (Jobling and Bendiksen, 2003; Ng et al., 2007), but not Yellowtail Kingfish. Understanding changes to growth performance and FA profile when fish oil is replaced at different water temperatures is essential to maximise growth potential using cost-effective ingredients, while maintaining the health aspects for both fish and humans.

Aims

The aim of this Chapter was to investigate the effect of partial (50%) or total (100%) replacement of dietary fish oil with poultry oil and canola oil on the growth performance, feeding efficiency, nutrient retention, fillet fatty acid profile and plasma cholesterol and triacylglyceride levels in Yellowtail Kingfish during the early grow-out stage at optimal (22 °C) and suboptimal (18 °C) water temperatures.

Materials and Methods

Experimental diets

The five experimental diets were made from the same basal formulation and provided the equivalent of 45% crude protein and ~25% crude lipid on a dry weight basis. In all diets, ~5% lipid was provided from the residual oil in the fish meal and other dietary ingredients, while the other 20% lipid was achieved through separate

additions of fish oil, canola oil or poultry oil to the diets. As a result, the five test diets were Diet 1: 100% fish oil (FO), Diet 2: 100% poultry oil (PO), Diet 3: 100% canola oil (CO), Diet 4: 50% fish oil + 50% poultry oil (FO/PO), and Diet 5: 50% fish oil + 50% canola oil (FO/CO, Table 3.1). The diets were formulated to satisfy the nutritional requirements of a carnivorous marine fish and supply sufficient n-3 LC-PUFA for normal growth and development (NRC, 2011). The diets were produced at the Australasian Experimental Stockfeed Extrusion Centre (Roseworthy, Adelaide, Australia) as cooked, extruded, slow sinking 3 mm pellets. After extrusion, feed pellets were coated with the oils. Enough feed for approximately 1 week was maintained at 4 °C, otherwise feeds were kept frozen (-20 °C) until used. The fatty acid profiles of the experimental oils and diets are shown in Table 3.1.

Experimental design and system

All experiments were conducted at the SARDI, Aquatic Science Centre, West Beach, Australia. Experimental protocols followed the guidelines approved by the Animal Welfare Committee of Flinders University (E286). Two independent trials were run, with each trial using separate cohorts of juvenile Yellowtail Kingfish, but fertilised eggs came from the same broodstock. Experiment 1 was run at 22.1 ± 0.3 °C (mean ± SD) in March-April 2010, and Experiment 2 was run at 17.6 ± 0.9 °C in July-August 2010.

Yellowtail Kingfish eggs were obtained from Clean Seas Tuna Ltd (Arno Bay, SA, Australia) and juveniles were on-grown in 5000-L fibreglass tanks at SARDI Aquatic Science Centre at ambient temperature until the beginning of both trials. Fish were fed a commercial diet (Skretting, NOVA, 3 mm sinking pellet; 45% protein, 20% lipid, Cambridge, Tasmania) prior to the commencement of both experiments. Yellowtail Kingfish were anaesthetised using AQUI-S® (AQUI-S® New Zealand Ltd.) at a concentration of 25 mg L^{-1} in seawater before weighing each individual. A total of 210 fish (mean weight ± SE, 95.6 ± 0.1 g in Experiment 1 and 101.1 ± 0.1 g in Experiment 2) were randomly assigned to fifteen 700-L fibreglass experimental tanks with 14 fish per tank, and the five experimental diets were randomly assigned to three replicate tanks. Fish were fed to apparent satiation twice a day for 34 days in Experiment 1 and 33 days in Experiment 2. The following water quality parameters were monitored daily for both experiments: water temperature (optimal, 22.1 ± 0.3 °C; suboptimal, 17.6 ± 0.9 °C), dissolved oxygen (>7.3 mg L^{-1}), pH (7.6-7.9), ammonia nitrogen (<0.17 mg L^{-1}) and salinity (38-39 ppt). The re-circulating systems (RAS) were housed in a temperature and photoperiod (14 h light:10 h dark) controlled room. There were two identical RAS units each consisting of a 780-L sump, an electric pump, a sand filter, and 780-L moving bed bio-filter (0.3 m^3), UV light, and eight 700-L cylindrical tanks. The water temperature of each RAS system was controlled to meet the experimental requirement in both experiments, i.e. 18 and 22 °C.

Sample collection

At the end of Experiments 1 and 2, feeding was stopped at 24 h and 48 h, respectively, to ensure digestive tracts were empty before harvesting. All fish were euthanised and measured for weight (nearest 0.1 g) and fork length (nearest 0.1 mm). Blood samples were taken from the caudal vasculature from three randomly selected fish from each tank and the right side fillet (de-boned and skinned) was collected for fatty acid profiling. The livers were weighed and used to determine hepatosomatic index (HSI). Three more fish from each tank were pooled to analyse whole body proximate composition. All dissected fish were checked for the presence of green liver, a positive case was identified by the presence of a green colour, irrespective of the area and the intensity. The fillets were snap frozen in liquid

nitrogen and stored at -80 °C until analysed. Blood samples were centrifuged immediately at 3000 rpm (804 × *g*) for 5 min (Houston, 1990). Plasma was separated and stored frozen at -80 °C until analysed. Whole fish for proximate composition were stored frozen at -20 °C until analysed.

Table 3.1. Ingredient formulation and fatty acid profile of the test oils, and the proximate composition and fatty acid composition of the five experimental diets.

Variable	Ingredient				Diet (g kg^{-1} dry basis)				
	Fish meal	Fish oil	Poultry oil	Canola oil	FO	PO	CO	FO/PO	FO/CO
Ingredients[1]									
Herring meal					500.0	500.0	500.0	500.0	500.0
Wheat gluten meal					95.3	95.3	95.3	95.3	95.3
Fish oil					203.6			101.8	101.8
Poultry oil						203.6		101.8	
Canola oil[2]							203.6		101.8
Tapioca starch					96.1	96.1	96.1	96.1	96.1
Mill run					97.0	97.0	97.0	97.0	97.0
Choline chloride					3.0	3.0	3.0	3.0	3.0
Vitamin/ mineral premix[3]					2.0	2.0	2.0	2.0	2.0
Vitamin C (Stay C)[4]					3.0	3.0	3.0	3.0	3.0
Total					*1000.0*	*1000.0*	*1000.0*	*1000.0*	*1000.0*
Analysed composition (% DM)									
Dry matter					95.8	96.3	96.5	96.6	96.7
Crude protein					42.4	42.8	44.2	43.8	44.7
Crude lipid					24.2	24.0	24.2	24.4	25.8
Ash					9.2	9.5	9.9	8.9	9.4
Gross energy (MJ kg^{-1})					23.1	23.4	23.3	23.4	23.4
Fatty acids (% total fatty acid dry basis)[5]									
14:0	4.3	8.8	1.3	0.1	9.7	1.5	0.8	4.9	4.2
16:0	24.3	20.7	27.9	4.7	26.8	23.5	8.6	23.5	15.1
18:0	7.4	3.9	7.4	2.2	5.5	6.4	3.3	5.7	3.9
Total SFA[6]	39.8	35.3	37.6	8.1	44.7	32.7	14.4	35.9	25.2
16:1n-7	4.2	8.7	7.4	0.2	9.0	5.6	0.9	6.9	4.2
18:1n-9	10.7	11.6	28.3	61.0	13.3	39.3	55.6	26.4	36.6
18:1n-7	2.8	3.1	3.0	3.2	3.6	2.6	3.2	2.9	3.2
20:1n-11	1.0	2.7	0.2	0.01	1.9	0.3	0.2	1.3	1.1
20:1n-9	0.9	0.6	0.5	1.0	0.8	0.6	1.1	0.6	0.9
22:1n-11	0.7	1.2	0.04	0.0	1.0	0.1	0.1	0.6	0.5
Total MUFA[7]	22.2	29.2	41.4	65.8	31.3	50.1	61.7	40.3	47.6
18:2n-6	1.6	1.0	16.3	18.3	3.7	13.2	17.2	8.3	11.1
20:4n-6	2.1	1.0	0.3	0.0	0.9	0.4	0.3	0.7	0.6
Total n-6 PUFA[8]	5.5	2.9	17.0	18.4	5.5	14.2	17.8	9.7	12.2
18:3n-3	1.0	0.6	2.5	7.5	0.7	1.9	5.9	1.3	3.7
20:5n-3	7.1	18.2	0.1	0.0	11.2	1.0	0.9	7.7	7.0
22:5n-3	1.9	2.2	0.1	0.0	1.5	0.3	0.2	1.1	1.0
22:6n-3	20.9	10.5	0.2	0.0	8.1	2.7	2.3	6.5	5.9
Total n-3 PUFA[9]	31.1	31.7	3.0	7.5	21.9	6.0	9.5	16.8	17.8
Total PUFA	36.7	34.6	20.0	25.9	27.3	20.2	27.2	26.5	30.0
EPA+DHA	28.0	28.7	0.3	0.0	19.4	3.7	3.2	14.2	13.0
n-3:n-6	5.6	10.8	0.2	0.4	4.2	0.4	0.6	1.8	1.5

Diet abbreviations: FO, 100% fish oil; PO, 100% poultry oil; CO, 100% canola oil; FO/PO, 1:1 blend of fish oil and poultry oil; FO/CO, 1:1 blend of fish oil and canola oil.

[1] Supplied by Ridley Aquafeeds, QLD, Australia.

[2] Crisco canola oil, Goodman Fielder Food Services, SA, Australia.

[3] A proprietary product supplied by Lienert Australia Pty Ltd, Australia.

[4] Rovimix® Stay-C® 35 DSM Nutritional Products, Basel, Switzerland.

FA, fatty acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

[5] Values are the mean for test oils and experimental diets of triplicate analyses (n = 3).

[6] Includes 15:0, 17:0, 20:0, 22:0, 24:0.

[7] Includes 16:1n-9, 17:1, 18:1n-9, 19:1, 22:1n-9, 24:1n-9.

[8] Includes 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6, 22:5n-6.

[9] Includes 16:2n-3, 20:3n-3.

Chemical analyses

All proximate composition analyses of ingredients, diets, whole body and muscle fillet tissue were conducted according to methods in the British Pharmocopoeia Commission (2004) or DIN 51900-1 (2000). Fish collected (per tank) for whole body proximate analyses were pooled, half thawed, cut into pieces and then blended in a food processor. Fillet tissue samples were individually blended. Subsamples of tissue were freeze-dried to a constant weight at -50 °C to determine moisture. Diet samples were oven dried to a constant weight at 105 °C for 16 h to determine moisture. Crude protein (N × 6.25) was determined by the Kjeldahl method (BP A219 H Determination of Nitrogen). Crude lipid was extracted with chloroform-methanol (2:1 v/v) solvent (Bligh and Dyer, 1959). Fatty acid methyl esters (FAME) were extracted from the fillet tissue, separated and quantified using a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA), following method in Bowyer et al. (2012b). Ash was determined by a muffle furnace at 550 °C for 16 h. Gross energy content was determined using a bomb calorimeter (DIN Standards, 2000), calibrated with benzoic acid). Total plasma triacylglycerols (TAG) were measured using a triacylglyceride kit (Sigma, TR0100) and quantified spectrophotometrically at 540 nm. Total plasma cholesterol was measured using a cholesterol/cholesteryl ester kit (BioVision, K603-100) and quantified spectrophotometrically at 570 nm.

Calculation of performance indices

Performance indices were calculated using the following formulae (Hardy and Barrows, 2002; Stone et al., 2011), where the weight (wt) unit was in grams. All calculations using fish weight and body composition were based on wet values and feed consumption and compositions were based on dry values.

- Weight gain (g / fish) = final wt – initial wt
- Specific growth rate (SGR, % BW day^{-1}) = (ln final wt – ln initial wt) × 100 / days
- Apparent feed conversion ratio (Apparent FCR) = dry wt feed consumed / wet wt fish gain
- Hepatosomatic index (HSI, %) = (liver wt / fish wt) × 100
- Protein efficiency ratio (PER) = wt gain / protein consumed
- Energy efficiency ratio (EER) = wt gain / energy consumed
- Apparent protein retention (PR, %) = (final body protein – initial body protein) × 100 / protein intake
- Apparent energy retention (ER, %) = 100 × (final body energy – initial body energy) / energy intake

Statistical analyses

All statistical analyses were carried out using SPSS (version 18, Chicago, Illinois, USA). The effects of dietary oil type (5 levels) and water temperature (2 levels) on growth performance, feed efficiency, somatic parameters, compositional data, nutrient retention, and plasma constituents were tested with two-factor ANOVA. Initial body weight was included as a covariate in the two-factor ANOVA model, but no significance was found and was therefore excluded from the final analyses of the measured performance parameters. If a significant interaction was detected between the main effects, then the variable was analysed using a one-factor ANOVA. Assumptions of homogeneity of variances were checked using Levene's equal variance test. Where necessary, data were transformed to satisfy the assumptions of ANOVA. All percentage data was arcsine transformed before analyses. Tukey's Honestly Significantly Different (HSD) test with P=0.05 was applied when a significant main effect was detected. Data analyses and graphical relationships between selected dietary fatty acids and their respective fillet FA concentrations were examined according to Bell et al. (2001).

Results

Growth, survival and feed efficiency

All diets were readily accepted by the fish and there were no signs of disease during the experiment. Over the course of the feeding trial only one fish died in Experiment 1 when fed the fish oil/poultry oil diet. Weight gain was significantly affected by water temperature (P<0.001) and diet (P<0.001), and there was no significant interaction (P=0.058, Table 3.2). Fish gained significantly less weight at 18 °C than at 22 °C (P<0.001). Fish fed the fish oil/poultry oil, poultry oil and fish oil/canola oil diets had similar weight gain to fish fed the fish oil control diet (P≥0.056). In contrast, fish fed the canola oil diet gained significantly less weight than fish fed the other four diets (P<0.001). The effect of diet on final weight and SGR was dependent on water temperature (P≤0.005, Table 3.2). The reason for the interaction was due to the more pronounced decrease in final weight and SGR in fish fed the canola oil diet at 18 °C compared to all other treatments. Despite this, the final weight and SGR was clearly higher at 22 °C than at 18 °C, and fish fed the canola oil diet had the lower final weight and SGR than fish fed the other four diets (P<0.001). At 22 °C, the best performing diet was the fish oil/poultry oil diet, but this was similar to fish fed the fish oil control diet (P≥0.081).

Feed intake was significantly affected by water temperature (P<0.001) and diet (P=0.002) and there was no significant interaction between these two factors (P=0.693, Table 3.2). Feed intake at 18 °C was 1.5-fold lower than at 22 °C (P<0.001), regardless of diet. Fish fed the canola oil diet consumed less feed than fish fed any of the other diets (P≤0.003). The effect of diet on apparent FCR was dependent on water temperature (P=0.001, Table 3.2). The reason for the interaction was due to the more pronounced increase (worse) in apparent FCR in fish fed the canola oil diet at 18 °C than fish fed in any other treatments (P=0.001). At 22 °C the apparent FCR was significantly lower (better) for fish fed fish oil/poultry oil diet, but was similar to all other diets (P≥0.309), except the fish fed the canola oil diet (P=0.047).

Somatic parameters and nutrient retentions

The effect of water temperature on the hepatosomatic index (HSI) was dependent on the diet (P=0.048, Table 3.2). The reason for the interaction was due to the slight increase of HSI in fish fed the canola oil diet and held at 22 °C compared to all the other treatments. At both 18 and 22 °C fish fed the poultry oil and canola oil diets had significantly higher HSI values than fish fed the fish oil control diet (P≤0.001). The green liver symptom was affected by water temperature (P=0.003) and diet (P=0.001) and there was no significant interaction (P=0.750). The frequency of the green liver symptom was greater at 18 °C (36.2 ± 4.8%) than at 22 °C (14.3 ± 4.8%, P=0.003). The presence of green liver was higher in fish fed the canola oil diet (64.3 ± 7.5%) compared with all other diets (<19.0 ± 5.5%, P≤0.001), but there was no difference between fish fed the fish oil, poultry oil, fish oil/poultry oil and fish oil/canola oil diets (P=0.672).

The effects of water temperature on PER, EER, and apparent PR were all independent of diet (P≥0.081, Table 3.2). PER, EER and apparent PR were lower at 18 °C than at 22 °C (P≤0.039). Fish fed the canola oil diet had significantly lower PER and EER compared to fish fed all the other diets (P≤0.001). Apparent PR was significantly lower in fish fed the canola oil diet compared with fish fed the fish oil/poultry oil and poultry oil diets (P≤0.034), but it was not significantly different to fish fed the fish oil and fish oil/canola oil diets (P≥0.072). The effect of diet on apparent ER was dependent on water temperature (P=0.029, Table 3.2). At 18 °C fish fed the canola oil diet had a lower apparent ER than fish fed the fish oil, poultry

oil or fish oil/canola oil diets ($P \leq 0.016$), but this parameter was similar for fish fed the fish oil/poultry oil diet ($P=0.126$). At 22 °C fish fed the canola oil diet had a significantly lower apparent ER than fish fed the fish oil/poultry oil diet ($P=0.017$), but there was no significant difference found in fish fed the fish oil, poultry oil or fish oil/canola oil diets ($P \geq 0.269$, Table 3.2).

Whole body proximate composition

Whole body moisture composition was affected by both water temperature ($P<0.001$) and diet ($P=0.026$) and there was no significant interaction ($P=0.196$, Table 3.3). Body moisture content was higher at 18 °C than at 22 °C ($P=0.004$). Fish fed the canola oil diet had the highest body moisture content, but this was similar to all diets ($P \geq 0.056$) except that fish fed the canola oil diet contained more moisture than fish fed the fish oil/canola oil diet ($P=0.026$). Whole body fat, protein and gross energy values were all lower at 18 °C than at 22 °C ($P \leq 0.020$), but diet had no effect ($P \geq 0.058$), and there was no interaction ($P \geq 0.054$). The whole body ash content was affected by neither water temperature ($P=0.106$) nor diet ($P=0.527$), and there was no interaction ($P=0.176$).

Fillet fatty acid composition

The percent fillet FA composition was significantly affected by water temperature and diet (Table 3.4). An interaction between diet and water temperature was identified for all FA, except 20:4n-6, 22:5n-3, 22:6n-3 and total PUFA. The relationships between the concentrations of selected FA in fillet lipids (% total FA, Table 3.4) were explored in relation to the relevant concentrations of the respective FA in the dietary lipids (% total FA, Table 3.1). The correlation coefficients (R^2) and slopes of the lines for selected individual FA for the poultry oil containing diets (fish oil, fish oil/poultry oil and poultry oil, Table 3.5) and the canola oil containing diets (fish oil, fish oil/canola oil and canola oil, Table 3.6), demonstrate that the relationship between the concentration of FA in the fillet lipids compared to the concentrations in the dietary lipids varied for individual FA. This can also be seen in the difference (Δ values) between the concentration of individual FA in dietary lipids and fillet lipids for fish fed the diets containing either 0% poultry oil (100% fish oil) and 100% poultry oil or 0% canola oil (100% fish oil) and 100% canola oil. In fish fed the poultry oil and canola oil diets, the negative Δ values for 18:1n-9 and 18:3n-3 indicates that these FA decreased in the fillet lipids when their concentrations increased in the dietary lipids (Table 3.5 and 3.6). Fish fed both poultry oil and fish oil maintained the level of 18:2n-6 in the fillet lipids as dietary inclusion levels increased, but for fish fed the canola oil diet, the negative Δ value indicates that this FA was utilised.

The FA concentrations of 20:4n-6, 20:5n-3 and 22:6n-3 decreased in the dietary lipids with increasing poultry and canola oil inclusion, but positive Δ values indicated that regardless of dietary oil type (i.e. poultry oil or canola oil) these FA were being preferentially retained in the fillet lipids.

Water temperature influenced the relationship between the FA concentration in the fillet lipid and the FA concentration in the dietary lipid (Table 3.5 and 3.6). Fish fed the poultry oil and canola oil diets utilised higher amounts of 18:1n-9 at 22 °C than at 18 °C. Under the same conditions, fish fed the canola oil diet utilised 18:1n-9 in the fillet lipid 8.1 and 1.8-fold more than in fish fed the poultry oil diet. The level of 22:6n-3 was retained at a higher level in the fillet lipid at 18 °C than at 22 °C, regardless of dietary oil type. At 18 °C, fish fed the poultry oil and canola oil diets had 22:6n-3 levels 1.7 and 2.2-fold higher than at 22 °C, signifying the higher retention and importance of this FA at cooler water temperatures.

Table 3.2. Growth performance, feed efficiency, somatic parameters and nutrient retentions of Yellowtail Kingfish acclimated to two different water temperatures and fed diets differing in oil source[1].

Temperature	18 °C					22 °C					ANOVA[2]						
Diet	FO	PO	CO	FO/PO	FO/CO	FO	PO	CO	FO/PO	FO/CO	Temp °C	Diet					Interaction
											18 vs. 22	FO	PO	CO	FO/PO	FO/CO	
Growth performance																	
Initial weight (g)	101.0 ± 0.3	100.9 ± 0.2	101.2 ± 0.1	100.9 ± 0.4	101.5 ± 0.3	95.8 ± 0.4	95.5 ± 0.2	95.6 ± 0.1	95.5 ± 0.2	95.8 ± 0.1							
Final weight (g)	184.6 ± 2.0^{a}	188.2 ± 3.3^{a}	147.1 ± 3.5^{b}	187.6 ± 0.9^{a}	186.1 ± 1.4^{a}	266.9 ± 6.0^{ab}	262.2 ± 4.4^{b}	236.9 ± 4.1^{c}	281.2 ± 1.2^{a}	259.2 ± 2.3^{b}	*	*					*
Weight gain (g)	83.7 ± 2.3^{a}	87.3 ± 3.3^{a}	46.0 ± 3.6^{b}	86.7 ± 1.3^{a}	84.6 ± 1.4^{a}	170.2 ± 4.7	166.7 ± 4.3	141.3 ± 4.2	180.6 ± 3.9	163.4 ± 2.4	<	*yz*	*yz*	*x*	*z*	*y*	NS
SGR (% BWday-^{1})	1.83 ± 0.04^{a}	1.89 ± 0.05^{a}	1.13 ± 0.08^{b}	1.88 ± 0.03^{a}	1.84 ± 0.02^{a}	3.01 ± 0.06^{ab}	2.97 ± 0.05^{b}	2.67 ± 0.05^{c}	3.17 ± 0.01^{a}	2.93 ± 0.03^{b}	*	*					*
Feed efficiency																	
Feed intake (g fish^{-1}day^{-1})	3.7 ± 0.0	3.8 ± 9.2	3.1 ± 0.1	3.9 ± 0.2	3.8 ± 0.1	5.9 ± 0.2	6.0 ± 0.1	5.5 ± 0.1	5.9 ± 0.2	5.8 ± 0.1	<	*z*	*z*	*y*	*z*	*z*	NS
Apparent FCR	1.47 ± 0.05^{b}	1.49 ± 0.04^{b}	2.13 ± 0.14^{a}	1.47 ± 0.06^{b}	1.48 ± 0.05^{b}	1.14 ± 0.05^{bc}	1.25 ± 0.07^{bc}	1.32 ± 0.04^{a}	1.12 ± 0.01^{c}	1.20 ± 0.01^{bc}	*	*					*
Somatic parameters																	
HSI (%)	0.91 ± 0.03^{c}	1.29 ± 0.08^{a}	1.18 ± 0.07^{ab}	0.94 ± 0.04^{bc}	1.02 ± 0.06^{abc}	1.08 ± 0.05^{c}	1.45 ± 0.02^{b}	1.68 ± 0.04^{a}	1.24 ± 0.04^{c}	1.24 ± 0.04^{c}	*	*					*
Green liver (%)	29.0 ± 0.0	23.7 ± 17.2	76.0 ± 5.0	19.0 ± 5.0	33.3 ± 20.6	0	9.3 ± 4.7	52.7 ± 17.2	9.3 ± 4.7	4.7 ± 4.7	>	*y*	*y*	*z*	*y*	*y*	NS
Nutrient retention																	
PER	1.6 ± 0.1	1.6 ± 0.0	1.1 ± 0.1	1.6 ± 0.1	1.5 ± 0.0	2.1 ± 0.1	1.9 ± 0.1	1.7 ± 0.1	2.0 ± 0.0	1.9 ± 0.0	<	*z*	*z*	*y*	*z*	*z*	NS
ERE	2.9 ± 0.1	2.9 ± 0.1	2.0 ± 0.1	2.9 ± 0.1	2.9 ± 0.1	3.8 ± 0.2	3.8 ± 0.2	3.2 ± 0.1	3.8 ± 0.0	3.6 ± 0.0	<	*z*	*z*	*y*	*z*	*z*	NS
Apparent PR (%)	29.6 ± 0.6	29.6 ± 1.3	19.4 ± 2.1	28.5 ± 1.1	28.6 ± 0.8	31.8 ± 1.7	33.8 ± 3.7	28.9 ± 2.7	37.1 ± 5.2	31.4 ± 2.1	<	*yz*	*z*	*y*	*z*	*yz*	NS
Apparent ER (%)	26.1 ± 2.3^{a}	26.3 ± 2.2^{a}	15.6 ± 1.1^{b}	22.5 ± 2.5^{ab}	26.3 ± 0.7^{a}	32.3 ± 1.1^{ab}	30.3 ± 1.4^{ab}	27.7 ± 0.5^{b}	36.3 ± 2.1^{a}	32.3 ± 0.3^{ab}	*	*					*

Diet abbreviations: FO, 100% fish oil; PO, 100% poultry oil; CO, 100% canola oil; FO/PO, 1:1 blend of fish oil and poultry oil; FO/CO, 1:1 blend of fish oil and canola oil.

$^{x, y, z}$ For variables with a significant effect of diet and no interaction, values without a common letter are different (*z* indicated the highest value; $P<0.05$).

$^{a, b, c, d}$ For variables with a significant interaction, differences in diets are compared within each temperature (one-factor ANOVA, Tukey's HSD test), values without a common superscript are different ($P<0.05$).

[1] Mean ± SE; n = 3, SE less than 0.1 are reported as "0.0".

[2] NS, non-significant; *, $P<0.05$. For variables with a significant effect of temperature ($P<0.05$) and no interaction, < or > indicates whether the values measured at 18 °C was less than or greater than that measured at 22 °C.

Table 3.3. Whole body proximate composition of Yellowtail Kingfish acclimated to two different water temperatures and fed diets differing in oil source[1].

Temperature	18 °C					22 °C					ANOVA[2]					
Diet	FO	PO	CO	FO/PO	FO/CO	FO	PO	CO	FO/PO	FO/CO	Temp °C	Diet				
											18 vs. 22	FO	PO	CO	FO/PO	FO/CO
(% wet basis)																
Moisture	71.8 ± 0.9	71.6 ± 0.3	73.9 ± 0.2	72.8 ± 0.7	71.5 ± 0.1	69.0 ± 0.5	69.0 ± 0.1	69.7 ± 0.4	68.3 ± 0.3	68.7 ± 0.8	>	*yz*	*yz*	*z*	*yz*	*y*
Crude protein	17.5 ± 0.6	17.2 ± 0.4	17.1 ± 0.4	17.9 ± 0.2	16.6 ± 0.5	17.5 ± 0.6	18.7 ± 0.9	18.4 ± 0.6	18.6 ± 1.2	18.3 ± 0.7	<	NS				
Crude lipid	7.0 ± 0.4	7.7 ± 0.3	5.9 ± 0.4	6.3 ± 0.5	8.0 ± 0.5	9.4 ± 0.7	8.7 ± 0.1	9.0 ± 0.3	10.9 ± 1.0	9.8 ± 0.5	<	NS				
Ash	2.8 ± 0.1	2.6 ± 0.1	2.7 ± 0.3	2.5 ± 0.1	2.5 ± 0.0	2.6 ± 0.1	2.7 ± 0.1	2.9 ± 0.1	2.9 ± 0.1	2.7 ± 0.2	<	NS				
GE (MJ kg^{-1})	6.9 ± 0.3	7.0 ± 0.2	6.2 ± 0.1	6.4 ± 0.3	7.0 ± 0.1	7.8 ± 0.1	7.8 ± 0.1	7.6 ± 0.1	8.2 ± 0.1	8.0 ± 0.4	<	NS				

Diet abbreviations: FO, 100% fish oil; PO, 100% poultry oil; CO, 100% canola oil; FO/PO, 1:1 blend of fish oil and poultry oil; FO/CO, 1:1 blend of fish oil and canola oil.

Interaction non-significant for all variables.

[y, z] For variables with a significant effect of diet and no interaction, values without a common letter are different (*z* indicated the highest value; $P<0.05$).

[1] Values are presented as mean ± SE (n = 3), SE less than 0.1 are reported as "0.0".

[2] NS, non-significant; *, $P<0.05$. For variables with a significant effect of temperature ($P<0.05$) and no interaction, < or > indicates whether the values measured at 18 °C was less than or greater than that measured at 22 °C.

Table 3.4. Muscle total fat and fatty acid levels (% dry basis) of Yellowtail Kingfish grown at two different water temperatures and fed diets differing in oil source[1].

Temperature	18 °C					22 °C					ANOVA[2]						
Diet	FO	PO	CO	FO/PO	FO/CO	FO	PO	CO	FO/PO	FO/CO	Temp °C	Diet					Interaction
											18 vs. 22	FO	PO	CO	FO/PO	FO/CO	
% total fat	11.0 ± 0.9	11.9 ± 1.4	9.8 ± 2.3	11.0 ± 1.4	10.1 ± 1.6	14.1 ± 1.0	14.6 ± 2.3	13.8 ± 1.0	15.0 ± 1.9	15.2 ± 0.5	>	NS					NS
14:0	3.7 ± 0.2^{a}	1.2 ± 0.1^{c}	1.2 ± 0.1^{c}	2.5 ± 0.1^{b}	2.3 ± 0.1^{b}	4.9 ± 0.0^{a}	1.2 ± 0.0^{d}	0.9 ± 0.0^{e}	3.1 ± 0.0^{b}	2.7 ± 0.1^{c}	<	*					*
16:0	16.4 ± 0.3^{a}	15.6 ± 0.0^{a}	11.7 ± 0.3^{c}	16.1 ± 0.1^{a}	13.6 ± 0.2^{b}	17.7 ± 0.1^{a}	16.5 ± 0.1^{b}	9.9 ± 0.2^{d}	17.4 ± 0.0^{a}	13.4 ± 0.1^{c}	<	*					*
18:0	6.8 ± 0.1^{ab}	7.1 ± 0.1^{a}	6.3 ± 0.3^{b}	7.1 ± 0.1^{a}	6.4 ± 0.2^{ab}	6.3 ± 0.0^{a}	6.6 ± 0.0^{a}	4.6 ± 0.2^{c}	6.6 ± 0.1^{a}	5.4 ± 0.0^{b}	>	*					*
Total SFA	29.2 ± 0.4^{a}	25.2 ± 0.1^{b}	20.8 ± 0.6^{c}	27.6 ± 0.1^{a}	24.1 ± 0.4^{b}	30.9 ± 0.2^{a}	25.6 ± 0.2^{c}	16.9 ± 0.4^{e}	28.7 ± 0.1^{b}	23.2 ± 0.1^{d}	>	*					*
16:1n-7	6.1 ± 0.2^{a}	4.9 ± 0.2^{b}	2.1 ± 0.1^{d}	5.4 ± 0.1^{ab}	3.6 ± 0.2^{c}	7.0 ± 0.1^{a}	5.1 ± 0.1^{c}	1.5 ± 0.1^{e}	6.1 ± 0.1^{b}	3.7 ± 0.1^{d}	NS	*					*
18:1n-9	13.9 ± 0.2^{d}	33.2 ± 0.7^{a}	35.3 ± 1.4^{a}	22.6 ± 0.4^{c}	27.6 ± 0.9^{b}	13.6 ± 0.3^{e}	36.8 ± 0.5^{b}	44.7 ± 0.9^{a}	24.8 ± 0.3^{d}	31.4 ± 0.4^{c}	<	*					*
18:1n-7	3.4 ± 0.0^{a}	2.9 ± 0.0^{c}	3.1 ± 0.0^{b}	3.2 ± 0.0^{b}	3.2 ± 0.0^{b}	3.3 ± 0.0^{a}	2.8 ± 0.0^{c}	3.1 ± 0.0^{b}	3.1 ± 0.0^{b}	3.2 ± 0.0^{a}	>	*					*
20:1n-11	1.6 ± 0.1^{a}	0.3 ± 0.0^{c}	0.3 ± 0.0^{c}	1.0 ± 0.0^{b}	0.8 ± 0.1^{b}	2.0 ± 0.1^{a}	0.3 ± 0.0^{d}	0.2 ± 0.0^{d}	1.1 ± 0.0^{b}	1.0 ± 0.0^{c}	<	*					*
Total MUFA	27.9 ± 0.4^{c}	43.5 ± 1.0^{a}	42.9 ± 1.4^{a}	34.7 ± 0.6^{b}	37.6 ± 1.1^{b}	28.8 ± 0.4^{e}	47.0 ± 0.6^{b}	51.4 ± 0.8^{a}	37.6 ± 0.4^{d}	41.6 ± 0.5^{c}	<	*					*
18:2n-6	4.6 ± 0.0^{e}	13.6 ± 0.1^{b}	14.5 ± 0.3^{a}	8.2 ± 0.1^{d}	9.7 ± 0.2^{c}	4.3 ± 0.1^{e}	13.9 ± 0.2^{b}	16.3 ± 0.2^{a}	8.4 ± 0.1^{d}	10.3 ± 0.1^{c}	<	*					*
20:4n-6	1.5 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.0	0.8 ± 0.0	0.6 ± 0.0	1.0 ± 0.0	0.9 ± 0.0	>	*z*	*wx*	*w*	*y*	*x*	NS
Total n-6	7.3 ± 0.1^{e}	15.6 ± 0.1^{b}	16.4 ± 0.1^{a}	10.5 ± 0.0^{d}	11.8 ± 0.1^{c}	6.7 ± 0.2^{e}	15.4 ± 0.2^{b}	17.5 ± 0.2^{a}	10.3 ± 0.0^{d}	12.0 ± 0.0^{c}	NS	*					*
18:3n-3	0.7 ± 0.0^{e}	1.6 ± 0.0^{c}	3.2 ± 0.2^{a}	1.1 ± 0.0^{d}	2.5 ± 0.1^{b}	0.8 ± 0.0^{e}	1.7 ± 0.0^{c}	4.4 ± 0.1^{a}	1.2 ± 0.0^{d}	2.9 ± 0.0^{b}	<	*					*
20:5n-3	13.1 ± 0.1^{a}	2.9 ± 0.1^{e}	3.9 ± 0.2^{d}	8.5 ± 0.1^{b}	7.8 ± 0.0^{c}	14.6 ± 0.2^{a}	2.1 ± 0.1^{d}	2.3 ± 0.1^{d}	8.4 ± 0.1^{b}	7.4 ± 0.1^{c}	>	*					*
22:5n-3	3.6 ± 0.2	1.4 ± 0.1	1.7 ± 0.1	2.7 ± 0.1	2.5 ± 0.1	2.8 ± 0.0	1.0 ± 0.1	0.9 ± 0.1	2.0 ± 0.0	1.9 ± 0.0	>	*z*	*x*	*x*	*y*	*y*	NS
22:6n-3	17.7 ± 0.5	8.8 ± 0.8	10.9 ± 1.0	14.2 ± 0.6	13.3 ± 1.0	14.5 ± 0.5	6.2 ± 0.4	6.2 ± 0.4	10.9 ± 0.4	10.4 ± 0.5	>	*z*	*x*	*x*	*y*	*y*	NS
Total n-3	35.2 ± 0.7^{a}	14.9 ± 0.0^{d}	19.8 ± 1.0^{c}	26.6 ± 0.5^{b}	26.2 ± 1.0^{b}	32.9 ± 0.3^{a}	11.0 ± 0.5^{d}	13.9 ± 0.5^{c}	22.6 ± 0.3^{b}	22.7 ± 0.4^{b}	>	*					*
Total PUFA	42.5 ± 0.8	30.5 ± 1.0	36.2 ± 0.9	37.2 ± 0.5	38.0 ± 0.9	39.6 ± 0.3	26.5 ± 0.5	31.3 ± 0.4	32.9 ± 0.3	34.6 ± 0.4	>	*z*	*w*	*x*	*xy*	*y*	NS
EPA+DHA	30.8 ± 0.5^{a}	11.8 ± 0.9^{c}	14.7 ± 1.1^{c}	22.7 ± 0.6^{b}	21.1 ± 1.0^{b}	29.1 ± 0.3^{a}	8.3 ± 0.5^{d}	8.4 ± 0.6^{d}	19.2 ± 0.3^{b}	17.7 ± 0.4^{c}	>	*					*
n-3:n-6	4.8 ± 0.0^{a}	1.0 ± 0.1^{c}	1.2 ± 0.1^{c}	2.5 ± 0.1^{b}	2.2 ± 0.1^{b}	5.0 ± 0.1^{a}	0.7 ± 0.0^{c}	0.8 ± 0.0^{c}	2.2 ± 0.0^{b}	1.9 ± 0.0^{b}	>	*					*

FO, 100% fish oil; PO, 100% poultry oil; CO, 100% canola oil; FO/PO, 1:1 blend of fish oil and poultry oil; FO/CO, 1:1 blend of fish oil and canola oil.

$^{w, x, y, z}$ For variables with a significant effect of diet and no interaction, values without a common letter are different (z indicated the highest value; $P<0.05$).

$^{a, b, c, d}$ For variables with a significant interaction, differences in diets are compared within each temperature (one-factor ANOVA, Tukey's HSD test), values without a common superscript are different ($P<0.05$).

[1] Values are presented as mean ± SE, n = 3. SE less than 0.1 are reported as "0.0".

[2] NS, non-significant; *, $P<0.05$. For variables with a significant effect of temperature ($P<0.05$) and no interaction, < or > indicates whether the values measured at 18 °C was less than or greater than that measured at 22 °C.

Table 3.5. Correlation coefficients (R^2) and slope values from linear plots of FA concentrations in the fillet total lipid (%) of Yellowtail Kingfish fed fish oil (FO), fish oil/poultry oil and poultry oil (PO) against FA concentrations in the dietary total lipid (%), including the difference (Δ) between fillet FA and dietary FA values for fish oil and poultry oil (100% poultry oil) treatments.

Temperature	18 °C					22 °C				
Fatty acid	R^2	Slope	*P*	ΔFO[1]	ΔPO[1]	R^2	Slope	*P*	ΔFO[1]	ΔPO[1]
18:1n-9	0.993	0.745	0.019	0.3	-6.1	0.999	0.892	0.008	0.6	-2.5
22:1n-11	0.971	0.762	0.039	-0.3	0.0	0.960	1.023	0.045	0.1	0.0
18:2n-6	0.980	0.949	0.032	0.9	0.4	0.991	1.009	0.022	0.6	0.7
20:4n-6	0.998	0.966	0.011	0.6	0.6	0.954	1.073	0.049	0.4	0.4
18:3n-3	0.979	0.723	0.032	0.0	-0.3	0.992	0.733	0.020	0.1	-0.2
20:5n-3	0.967	0.977	0.041	1.9	1.9	0.939	1.187	0.056	3.4	1.1
22:6n-3	0.978	1.607	0.033	9.6	6.1	0.951	1.480	0.050	6.4	3.5

[1] Negative Δ values indicate lower values in fillet compared with diet, whereas positive values indicate accumulation in fillet relative to diet.

Table 3.6. Correlation coefficients (R^2) and slope values from linear plots of FA concentrations in the fillet total lipid (%) of Yellowtail Kingfish fed fish oil (FO), fish oil/canola oil and canola oil (CO) against FA concentrations in the dietary total lipid (%), including the difference (Δ) between fillet FA and dietary FA values for fish oil and canola oil (100% canola oil) treatments.

Temperature	18 °C					22 °C				
Fatty acid	R^2	Slope	*P*	ΔFO[1]	ΔCO[1]	R^2	Slope	*P*	ΔFO[1]	ΔCO[1]
18:1n-9	0.977	0.509	0.034	0.6	-20.3	0.999	0.737	0.008	0.3	-10.9
22:1n-11	0.961	0.735	0.045	-0.3	0.1	0.984	0.435	0.029	0.1	0.0
18:2n-6	0.997	0.732	0.012	0.9	-2.7	0.994	0.883	0.017	0.6	-0.9
20:4n-6	0.866	0.822	0.083	0.6	0.7	0.969	1.238	0.040	0.4	0.3
18:3n-3	0.958	0.483	0.046	0.0	-2.7	1.000	0.697	0.003	0.1	-1.5
20:5n-3	0.923	0.883	0.063	1.9	3.0	0.917	1.172	0.065	3.4	1.4
22:6n-3	0.825	1.125	0.096	9.6	8.6	0.967	1.410	0.041	6.4	3.9

[1] Negative Δ values indicate lower values in fillet compared with diet, whereas positive values indicate accumulation in fillet relative to diet.

Plasma triacylglycerol and cholesterol contents

The effect of water temperature on plasma total cholesterol (TC) level was independent of diet (*P*=0.062). Plasma TC level was higher at 18 °C than at 22 °C (*P*<0.001, Fig 3.1). Fish fed the canola oil and poultry oil diets had significantly lower TC levels, compared to all other diets (*P*<0.001). The effect of water temperature on plasma total triacylglyceride (TAG) level was independent of diet (*P*=0.365). In contrast to the TC levels, the plasma TAG levels were lower at 22 °C than at 18 °C for all diets except for canola oil (*P*=0.01, Fig 3.2). Fish fed the canola oil diet had higher TAG levels than fish fed the poultry oil and fish oil/canola oil diets, but this value was similar for fish fed fish oil and fish oil/poultry oil diets (*P*=0.013).

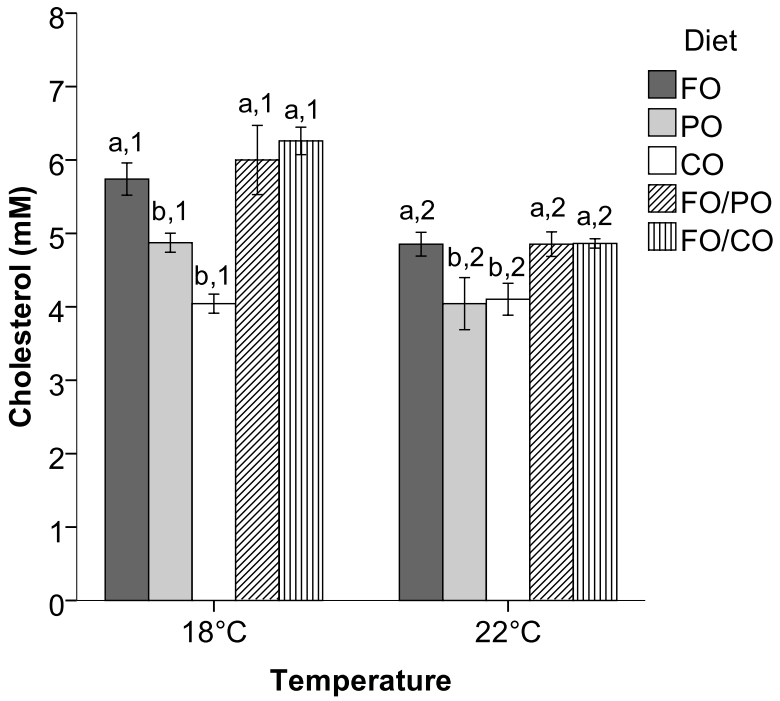

Figure 3.1. Plasma cholesterol level (mM) of Yellowtail Kingfish acclimated to two different water temperatures and fed diets differing in oil source. Values are means (n = 3 per diet treatment based on 3 fish per replicate). Different alphabetical letters indicate significant differences (P<0.05) between diets within each water temperatures. Different numbers indicate significant differences (P<0.05) between fish held at different water temperatures. Diet abbreviations refer to Table 3.1.

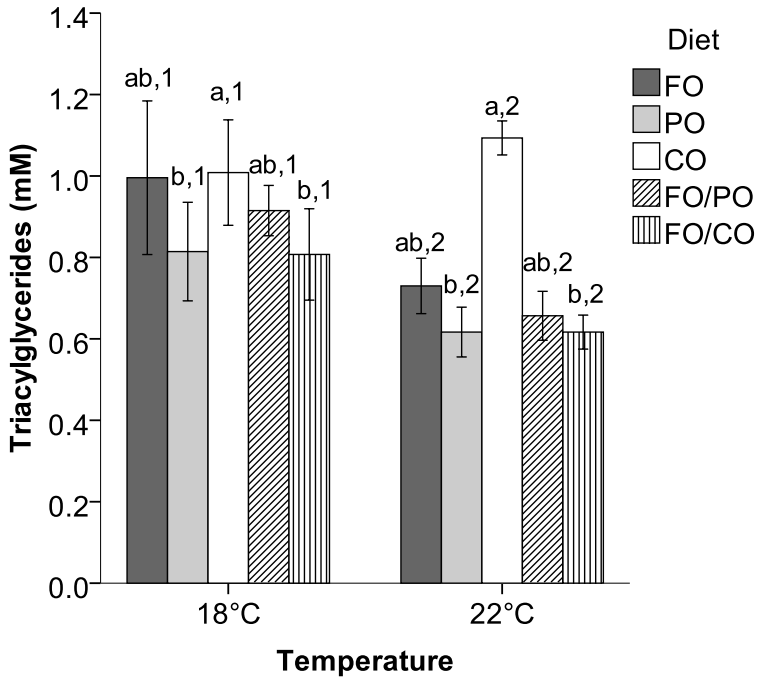

Figure 3.2. Plasma triacylglyceride level (mM) in Yellowtail Kingfish acclimated to two different water temperatures and fed diets differing in oil source. Values are means (n = 3 per diet treatment based on 3 fish per replicate). Different alphabetical letters indicate significant differences (P<0.05) between diets within each water temperatures. Different numbers indicate significant differences (P<0.05) between fish held at different water temperatures. Diet abbreviations refer to Table 3.1.

Discussion

Poultry oil and canola oil have considerable potential as replacements for fish oil in diets for Yellowtail Kingfish. However, the effect of these ingredients on growth performance, feeding efficiency, somatic parameters, and plasma constituents varied according to lipid ingredient, inclusion level and water temperature. The proximate composition, nutrient retention and muscle tissue fatty acid profile of Yellowtail Kingfish were also significantly influenced by these same parameters.

Growth and feed utilisation

The weight gain of Yellowtail Kingfish in this study demonstrated that up to 100% replacement of fish oil with poultry oil did not compromise growth performance or feed efficiency compared to the fish oil control diet. However, while canola oil may be appropriate as an alternative to fish oil at 50% replacement, the 100% total replacement caused a significant reduction in most measured parameters. The effect of water temperature on fish held at 18 °C significantly reduced growth performance and feed efficiency regardless of diet compared to fish held at 22 °C. The reduced feed efficiency reflects the decreased metabolic rate at the cooler water temperature, a common occurrence in Yellowtail Kingfish, as well as other marine carnivorous fish species (Pirozzi and Booth, 2009). In species such as Asian seabass (*Lates calcarifer*) and European seabass (*Dicentrarchus labrax*) growth rate declined in response to low water temperatures, which was largely concomitant to the decline in feed intake (Person-Le Ruyet et al., 2004; Bermudes et al., 2010). The replacement of 50% poultry oil in diets for Yellowtail Kingfish resulted in the highest weight gain (133.7 g) at both water temperatures and this was significantly higher than the weight gain for the fish fed the fish oil control diet (127.0 g). The feed intake and apparent FCR of fish fed fish oil/poultry oil and poultry oil diets were similar to fish fed the fish oil control diet. The successful inclusion of high levels of poultry oil in diets for Yellowtail Kingfish has also been found for other species such as Atlantic Salmon (*Salmo salar*) with up to 60% fish oil replacement having no significant effect on growth performance or feeding efficiency (Rosenlund et al., 2001; Higgs et al., 2006).

The replacement of 50% of the dietary fish oil with canola oil resulted in similar weight gain, feed intake and apparent FCR results as for fish fed both the poultry oil based diets and the fish oil control diet. In contrast, there was an increase in apparent FCR of fish fed the 100% canola oil diet and this was significantly pronounced at 18 °C. Other studies using canola/rapeseed oil as a lipid source in diets for Atlantic Salmon and red seabream (*Pagrus major*) at dietary inclusion levels of up to 50% and 70%, respectively, showed no reduction in growth rate and feed efficiency with dietary lipid contents between 10-19% (Rosenlund et al., 2001; Huang et al., 2007). In the present study, the acceptance of the diet containing 100% canola oil may be a contributing factor since all diets were formulated to have the same amount of dietary lipid (234 g kg^{-1}) and gross energy level (22.5 MJ kg^{-1}). Glencross et al. (2003) found decreased growth and increased apparent FCR in red seabream (*Pagrus auratus*) when fed low lipid diets (4% crude lipid) replacing 100% of dietary fish oil with crude canola oil, but not when using refined canola oil. These authors proposed that there was some type of anti-nutritional factor present in the crude canola oil. In the present study the canola oil was refined and of human-grade quality, but at high inclusion levels the palatability of the canola oil diet may have reduced the feed intake in Yellowtail Kingfish, influencing weight gain, particularly at 18 °C.

Somatic parameters

A major finding of this study was a physiological abnormality where the liver had a green discolouration, which was found at different percentages in fish fed all the diets at both water temperatures, except in fish fed the fish oil diet at 22 °C. Several causes of ‘green liver syndrome’ in fish have been reported, such as adverse reactions to inferior feed quality, bile duct occlusion by parasites, dietary taurine deficiency (Takagi et al., 2008; Takagi et al., 2010; Takagi et al., 2011), as well as frequently occurring during periods of low water temperature (Sakaguchi and Hamaguchi,

1979). In the closely related Japanese Yellowtail (*Seriola quinqueradiata*), green liver symptom has been ascribed to the replacement of fish meal, rich in taurine, with soybean products which are deficient in taurine (Takagi et al., 2008; Takagi et al., 2010). All experimental diets in the present study were formulated to contain 50% fish meal as the major protein ingredient, therefore, it is assumed that the dietary taurine requirement for Yellowtail Kingfish was met. In the present study, the occurrence of green liver was significantly higher at 18 °C compared to 22 °C, which is consistent with the previous findings for Japanese Yellowtail (Sakaguchi and Hamaguchi, 1979), as well as practical observations from the Yellowtail Kingfish growers in SA (Mike Thomson, personal communication). Sakaguchi and Hamaguchi (1979) found that a drop in water temperature lead to a decrease in the physiological function of the hepatopancreas impacting on the secretion of bile. The functioning of the liver and pancreas was not measured in the present study, but further research in this area would be beneficial towards fully understanding these results.

Interestingly, the occurrence of green liver was profoundly increased in fish fed the diet containing 100% canola oil, compared with fish fed all of the other diets at both optimal and suboptimal water temperatures. However, there has been no published literature on the occurrence of green liver in response to replacing fish oil with any alternative lipid ingredients in any fish species. Bile made from cholesterol in the liver is released into the intestine and plays an important role in the digestion and absorption of fat (Goto et al., 2001). Red seabream fed non-fish meal diets displaying the effects of green liver syndrome had a reduction in the digestion and absorption of lipids (Goto et al., 2001). In the current study the functionality of the bile process from the liver to the intestine may have been compromised in fish fed the 100% canola oil diet at 18 °C. This reasoning is supported by the numerically lower whole body total fat level (P=0.059) and apparent energy retention values of fish fed this diet at 18 °C.

Not only did the livers show signs of green liver, but fish fed 100% poultry oil and canola oil had enlarged livers, which were very pale in colour compared to fish fed the 100% fish oil control diet. Pale liver colour has been associated with fat deposits in other marine fish species such as red drum (*Sciaenops ocellatus*) fed high lipid diets (10-20% crude lipid, Craig et al., 1999). In fish, the accumulation of fat in the liver can lead to a fatty liver syndrome, which may be associated with increased lipid peroxidation and impaired liver function such as inefficient nutrient utilisation and necrosis (Tucker et al., 1997; Craig et al., 1999). Regrettably, liver total fat levels were not measured as part of this study, but the high HSI values of the poultry oil and canola oil fed fish indicated that the fish were receiving enough energy from the diets and storing the excess energy in the liver.

Proximate composition and nutrient retentions

Yellowtail Kingfish utilised the 50% and 100% poultry oil diets as efficiently as the fish fed 100% fish oil, but the ability of Yellowtail Kingfish to convert dietary protein and energy into body protein and energy was influenced by water temperature. In response to the increase in whole body lipid and gross energy level, the protein and energy efficiency ratio and apparent protein and energy retention were higher at 22 °C than 18 °C. The present study agrees with research on Asian seabass in which protein retention values for two different fish sizes (20 g and 140 g) decrease at suboptimal water temperatures (23.1% and 10%) compared to optimal temperatures (>41%) for this species (Bermudes et al., 2010). The decreased nutrient retentions of fish fed experimental diets may simply be a result of the decreased feed intake and weight gain of fish held at 18 °C compared to 22 °C.

The inclusion of 50% canola oil resulted in similar whole body proximate composition and nutrient retention results as fish fed up to 100% poultry oil, but 100% canola oil significantly influenced these parameters. The whole body moisture content was significantly increased in fish fed canola oil compared to all other diets, while whole body fat levels were lower at 18 °C, there was no difference between dietary lipid treatments at 22 °C. The influences of 100% canola oil on the whole body proximate composition was reflected in the decreased protein and energy efficiency ratio and apparent protein and energy retention values. The retention values indicate that the amount of

protein and energy the fish gained from the canola oil diet intake was not fully utilised by the fish. In contrast, Glencross et al. (2003) found no effect of dietary canola oil inclusion on protein retention (32%) in red seabream, but the total level of lipid replacement was much lower (4% fish oil) in the study by Glencross et al (2003). In the current study all diets contained the same level of dietary protein, but the type of oil varied.

Fatty acid composition

The production performance of fish is not typically affected by the replacement of fish oil with alternative lipid sources, as long as the minimum essential fatty acid (EFA) requirements, specifically for EPA and DHA, are met. The n-3 LC-PUFA requirement for most marine fish such as Japanese Yellowtail, red seabream, and gilthead seabream (*Sparus aurata*) is 0.5-2.0% of total dietary lipid (Deshimaru and Kuroki, 1983; Takeuchi et al., 1990; Ibeas et al., 1994). Where the EFA requirements have not been met, reductions in growth and feed intake have commonly been reported in species such as gilthead seabream, black seabream (*Acanthopagrus schlegeli*) and cobia (*Rachycentron canadum*) when fed 100% fish oil replacement diets (Peng et al., 2008; Benedito-Palos et al., 2009; Trushenski et al., 2010). In the present study, the poultry oil and canola oil diets contained 3.6 and 3.1% of n-3 LC-PUFA, respectively, which is much higher than the requirement for most marine fish species (Glencross, 2009; NRC, 2011). Therefore, the reduction in weight gain and feed intake of fish fed the canola oil diet is not likely due to an essential fatty acid deficiency. The EFA requirements in these two diets were met from the inclusion of fish meal in the diet, but it must be noted that if fish meal is also replaced then the residual fish oil level will be reduced as well.

It is well known in other marine species that diet is the primary determinant of tissue FA composition (Bell et al., 2001). As expected, the fillet lipid FA composition of Yellowtail Kingfish showed considerable differences between diets and water temperature. The most notable were the differences between the fish fed the canola oil and poultry oil diets and the fish fed the fish oil diet. Partial fish oil replacement (50%) showed graded changes relative to the level of substitution between the 0% and 100% replacement levels as indicated by the linear correlations between the percentage of individual FA in dietary lipids and fillet lipids. The PUFA fatty acids 18:2n-6 and 18:3n-3 are precursors used for elongation and desaturation into LC-PUFA in freshwater fish, but marine fish have lost this enzymatic capacity (Sargent et al., 1995). Therefore, it is most likely that the higher dietary lipid concentrations of 18:1n-9, 18:2n-6 and 18:3n-3 in the poultry oil and canola oil diets than in the fillet lipids suggests that these FA were being catabolised for energy. The high utilisation of 18:1n-9 is consistent with previous research on fish where it is known that both saturated fatty acids and MUFA are more readily catabolised by mitochondrial β-oxidation (Sargent et al., 1989; Bell et al., 2001). In fish fed all diets, the concentrations of 20:4n-6, 20:5n-3 and 22:6n-3 in the fillet lipids was higher than in the dietary lipids, regardless of water temperature. This indicates that fish fed diets containing small amounts of 20:5n-3, 20:4n-6 and 22:6n-3, i.e. 100% canola oil and poultry oil, the fish were selectively retaining and accumulating these fatty acids in the fillet lipids, particularly 22:6n-3. Similar to other studies in marine fish, the selective deposition of 22:6n-3 into the fillet lipids may also be due to the relative resistance of 22:6n-3 to β-oxidation due to its complex catabolic pathway (Bell et al., 2001; Tocher, 2003). However, it must be noted that even though there was retention of the important FA, the values were still significantly reduced compared to the fish oil diet. This has important implications in the final product and must be addressed closer to when fish reach market weight.

Fish can exploit the structural diversity of lipids within their membranes to adapt to changes in water temperature (Miller et al., 2006). At cold water temperatures, n-3 LC-PUFA permit a greater degree of unsaturation compared to the more saturated animal fatty acids (Hazel, 1984). In the present study, the LC-PUFA were present in the fillet lipids in higher concentrations than in the dietary lipids. Interestingly, at 18 °C the level of 20:5n-3, present at low concentrations in the canola oil and poultry oil diets, was retained in the fillet lipids at higher levels than at 22 °C. However, under the same conditions the level of 20:5n-3 in fish fed the fish oil diet was found to be lower at 18 °C than at 22 °C. On the other hand, the level of 20:4n-6 and 22:6n-3 was retained at higher levels at 18 °C

compared to 22 °C, regardless of diet. Similar to the present studies results, previous studies in Atlantic Salmon and cobia found that 22:6n-3 was preferentially retained over 20:5n-3 (Bell et al., 2001; Trushenski et al., 2010). In respect to the tissue lipid FA composition, these results suggest that 100% dietary fish oil can be replaced with both poultry oil and canola oil in diets for Yellowtail Kingfish. These diets did contain residual fish oil from the dietary fish meal satisfying the EFA requirements, but Yellowtail Kingfish did demonstrate the ability to adapt the FA profile to retain the required levels of essential long-chain PUFA fatty acids, 20:5n-3, 20:4n-6 and 22:5n-3.

Plasma constituents

Differences in growth performance may also be due to nutritional differences between fish oil and terrestrial plant oils and animal fats, such as dietary cholesterol content. Fish oil and animal fats are rich in cholesterol, but plant ingredients are deficient (Cheng and Hardy, 2004). Cholesterol is a known essential dietary nutrient for crustaceans and an important precursor for many physiologically active compounds (Hernández et al., 2004). However, since vertebrates have the ability to synthesise cholesterol from sterol precursors, limited research has been focused on the cholesterol requirements for fish (NRC, 2011). Plasma cholesterol level is a function of dietary cholesterol (exogenous cholesterol) and *de novo* cholesterol synthesis in the liver (endogenous cholesterol). In the current study, the complete replacement of dietary fish oil with either canola or poultry oil led to a hypocholesterolemic effect. Similar findings have been made for marine species such as European seabass and black seabream when fish oil was replaced with plant oils (Richard et al., 2006; Peng et al., 2008) due to the fact that diets containing plant oils rich in 18:1n-9, 18:2n-6 and 18:3n-3 can reduce cholesterol levels (Fernandez and West, 2005). Plant oils also contain phytosterols (Phillips et al., 2002), which are known to reduce the levels of total cholesterol in some fish species by decreasing the intestinal cholesterol absorption (Gilman et al., 2003). The supplementation of dietary cholesterol may be necessary when replacing high levels of fish oil with oil ingredients containing low cholesterol to prevent hypocholesterolemia.

The level of plasma triacylglyceride can be used as an indicator of liver function and nutritional status of fish (Lee et al., 2009). The TAG level in fish fed the 100% canola oil diet was not significantly higher than fish fed the 100% fish oil diet, but it was numerically more pronounced at 22 °C. In contrast, other studies have found decreasing plasma triacylglyceride levels with increasing inclusion levels of plant oils (Peng et al., 2008). The tendency towards hypertriglyceridemia in fish fed the 100% canola oil supports the conclusions that the functioning of the liver is compromised when high levels of canola oil are included in the diets for Yellowtail Kingfish. However, to understand the complexities of the change of liver function requires further research.

Conclusion and Recommendations

In conclusion, the complete replacement of fish oil with 100% poultry oil, but only 50% replacement with canola oil, had no detrimental effects on the growth of Yellowtail Kingfish under our test conditions. However, complete replacement of fish oil with 100% canola oil resulted in poor fish growth compared with the 100% fish oil diet, regardless of temperature, and lead to a pronounced occurrence of green liver. These results are certainly useful in dietary formulation to reduce feed cost in Yellowtail Kingfish farming. However, a longer term study using 100% poultry oil and 50% canola oil over the entire production cycle of Yellowtail Kingfish is recommended to confirm the application of our finding to commercial production. In particular, changes in the FA composition of the muscle tissue, specifically n-3 LC-PUFA and the n-3:n-6 ratio, induced by the replacement of 100% fish oil with alternative lipid ingredients may influence the market value of these fish. Therefore, the flesh lipid composition should be evaluated once fish are nearer to market size. If necessary, a feeding strategy including the use of ‘finisher diets’ should be considered when fish are closer to market size range. In the interim, for juvenile Yellowtail Kingfish diets, we recommend a maximum level of fish oil replacement with poultry oil in the range of 50-75%, to provide 10-15% of total dietary lipid.

Chapter 4. **Investigation of digestive enzyme activity and gut histology of Yellowtail Kingfish fed canola and poultry oils as alternatives to fish oil at optimal and suboptimal temperatures.**

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Abstract

Yellowtail Kingfish were fed five diets in which the dietary lipid component was replaced with 100% lipid as either poultry oil, canola oil, a blend of fish oil and poultry oil (50:50) or a blend of fish oil and canola oil (50:50) and held at optimal (22 °C) and suboptimal (18 °C) water temperature. After five weeks, the changes in gastrointestinal histology and digestive enzyme activity of fish were examined. Digestive trypsin, lipase and α-amylase enzyme activities were down regulated in fish held at 18 °C. The α-amylase activity was not influenced by diet, but the trypsin activities were significantly lower in fish fed the canola oil diet than fish fed the fish oil and fish oil/poultry oil diets. Although the lipase activities were significantly lower in fish fed the canola oil diet than fish fed the fish oil/poultry oil diet at 18 °C and 22 °C, there were no significant differences between fish fed other diets at both temperatures. The reduction in trypsin activity in fish fed the canola oil diet may be attributed to anti-nutritional factors present in the canola oil. The histology of the foregut and hindgut showed no signs of diet-induced enteritis. However, there was a high influx of goblet cells and severe reduction in supranuclear vacuolisation across all dietary treatments, including the fish oil control diet at both water temperatures and in initial fish samples. As no information exists on the histology of juvenile Yellowtail Kingfish this study provides baseline information for further study. However, reference tissue from wild fish is recommended to determine the validity of these findings and for the future histological assessment of this economically important fish species to dietary or environmental temperature changes.

The information presented in this Chapter addresses part of Objective 4 (Subproject 7): Determine the maximum inclusion levels of alternative lipid sources to replace fish oil at optimal (22 °C) and suboptimal (18 °C) water temperatures. The contents of this Chapter have been published in Aquaculture: Bowyer J.N., Qin J.G., Adams L.R., Thomson, M.J.S., Stone, D.A.J., 2012c. 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.

Introduction

The increasing growth of the global aquaculture industry has led to the need for replacing protein and lipid ingredients from the traditional fish meal and fish oil from wild fish stocks with terrestrial-based plant and animal ingredients (De Silva and Anderson, 1995; Gatlin et al., 2007; Turchini et al., 2009). In conjunction with the development of more energy-dense diets, a significant proportion of the total global production of the world's fish oil has primarily been used as the main dietary lipid component in aquafeeds (Sargent et al., 2002; Bowyer et al., 2012d). Marine fish have a requirement for the long chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA), and to some extent arachidonic acid (20:4n-6, AA). EPA and DHA are found in high levels in fish oil, but are devoid in terrestrial plant oils and animal fats. A number of studies on marine fish have showed that the level of maximum fish oil substitution with terrestrial plant and animal oils without affecting growth performance is 60-75% (Glencross, 2009; Turchini et al., 2009; NRC, 2011). The growth performance of fish is not typically affected by the substitution of fish oil with alternative lipid sources, as long as the minimum essential fatty acid (EFA) requirements, specifically EPA and DHA, are met. The n-3 LC-PUFA requirement for most marine fish such as Yellowtail Kingfish (*Seriola lalandi*), Japanese Yellowtail (*Seriola quinqueradiata*), red seabream (*Pagrus major*) and gilthead seabream (*Sparus aurata*) is 0.5-2.0% of dry diet (Deshimaru and Kuroki, 1983; Takeuchi et al., 1990; Ibeas et al., 1994; NRC, 2011). Although the histological response of the digestive tract and the liver of carnivorous fish to alternative terrestrial-based lipid ingredients has been documented (Caballero et al., 2002; Fountoulaki et al., 2009), the response of digestive enzyme mechanisms to dietary fish oil substitution is not well understood (Morais et al., 2004).

The process of digestion is the first step in the metabolism of lipids. Lipids are digested in the gut, releasing their fatty acids, which are then absorbed and resynthesised back into lipid and circulated within the bloodstream (De Silva and Anderson, 1995). The digestion of lipids occurs mainly in the pyloric caeca and is facilitated by emulsifiers (bile salts), such as cholate or taurocholate, which are produced in the liver and stored in the gallbladder (Denton et al., 1974). The majority of lipid absorption occurs in the pyloric caeca as well as in the foregut for most carnivorous fish species (Denstadli et al., 2004) and is facilitated by the activation of bile salt dependent lipases (Iijima et al., 1998). It is well known that the ability of fish to digest and absorb lipids from an ingredient is based on the composition, degree of unsaturation and chain length of the constituent fatty acid (Morais et al., 2004; Turchini et al., 2009). The apparent lipid digestibility coefficients of polyunsaturated fatty acids (PUFA) are higher than most saturated fatty acids (SFA) (Koven et al., 1994; Caballero et al., 2002). In rainbow trout (*Oncorhynchus mykiss*) fed diets containing different oil ingredients, Caballero et al. (2002) found a higher reduction in the fatty acid apparent digestibility coefficient (ADC) when diets contained higher levels of SFA and when the level of unsaturation decreased. This also corresponds to the order of preference for bile-salt activated lipase (Iijima et al., 1998). Parallels have been found between the digestive and absorptive processes of fish with pigs and rats, and it is assumed that these processes are similar in fish (Ricketts and Brannon, 1994; Hedemann et al., 2001). There have been studies on the effect of dietary fish oil substitution on digestive enzyme activities in larval fish (Morais et al., 2004; Morais et al., 2006), but the effect has been less studied in juvenile fish at the grow-out stage (Carter et al., 2003; Santigosa et al., 2011). Despite this, Santigosa et al. (2011) found that the total substitution of fish oil with plant oils, in diets where 75% of the protein was plant based, caused an accumulation of lipid droplets in the enterocytes of the proximal intestine of gilthead seabream, which resulted in an impaired digestion rate from the intestinal mucosa into the blood (Salhi et al., 1999).

The knowledge of how dietary adaptation affects the digestive enzyme profile is necessary to understand the nutritional physiology of fish and to choose dietary ingredients suitable to satisfy the nutritional requirements for fish during the grow-out period. Data comparisons on digestive enzyme activities between studies can be difficult due to the variability in collection and analytical methods as well as other factors such as fish size, environment and nutritional status (Hidalgo et al., 1999). Fish are poikilothermic animals so in a sea cage grow-out environment, changes to the ambient

surrounding water temperatures can influence physiological functions such as gut transit rate and nutrient absorption. The contact time of digestive enzymes to nutrients and the time spent at absorption sites are dependent on the nutrients rate of passage through the digestive tract (Fauconneau et al., 1983). At cooler water temperatures, gut transit rates decrease and can lead to an accumulation of digestive enzymes in the chyme and a higher level of nutrient digestion in the anterior intestine in Yellowtail Kingfish (Miegel et al., 2010). While at warmer temperatures, as the rate of metabolism increases, this subsequently leads to an increase in feed intake and enzyme activities (Hardewig and van Dijk, 2003).

The substitution of fish oil with plant oils and animal fats into aquafeeds can induce histological changes in fish tissues which can affect digestive and absorptive processes. For example, the dietary fat level and the degree of unsaturation of the lipid ingredient have been described to modify the composition of enterocyte and hepatocyte membranes in fish tissues (Caballero et al., 2003; Fountoulaki et al., 2009). For example, Arctic charr (*Salvelinus alpinus*) fed linseed oil containing high levels of α-linolenic acid (18:3n-3) exhibited an accumulation of lipid droplets in the enterocytes of the pyloric caeca and midgut (Olsen et al., 1999; Olsen et al., 2000). Similarly, Caballero et al. (2002) fed rainbow trout with different vegetable oils (soy, rapeseed, palm or olive oil) and an animal fat (lard), and observed supranuclear accumulation of lipid droplets in the intestinal cells of fish fed vegetable oils. These digestive tract alterations can compromise the transport and/or metabolism of fat and can reduce lipid and protein digestion (Francis et al., 2007).

Yellowtail Kingfish is a commercially important marine carnivorous finfish species in Australia. However, it is susceptible to a disease called diet-induced enteritis, which leads to low morbidity but high mortality (Sheppard, 2004). The onset of this disease has been linked to an antagonistic combination of factors including a) cool temperature (i.e. slowing the movement of digesta through the tract), b) high-fat feeds, c) presence of high levels of plant ingredients and proteins, d) low feed intake resulting in low vitamin/mineral intake, and e) opportunistic bacteria. These factors have the potential to cause an inflamed and irritated bowel (enteritis), necrosis, ulceration, electrolyte imbalance, reduced nutrient uptake and ultimately death (Gallardo et al., 2003; Sala-Rabanal et al., 2003; Sheppard, 2004). Some of the symptoms of this disease such as an inflamed and irritated bowel appear similar to that of soybean-meal induced enteritis in Atlantic Salmon (*Salmo salar*) fed diets containing high levels of soybean products (van den Ingh et al., 1991; Krogdahl et al., 2003). In addition, another symptom common to Japanese Yellowtail, which was also identified in Yellowtail Kingfish in a previous study by Bowyer et al. (2012a; Chapter 3), was the tentative identification of the green liver syndrome. In Chapter 3, we also suggested that the functioning of the liver in Yellowtail Kingfish fed high levels of canola oil in particular, but also poultry oil, was compromised (Bowyer et al.,2012a). The reduced dietary cholesterol level, particularly in the canola oil diet at 18 °C, may have led to the reduced synthesis of bile salts required for conjugating with taurine, leading to the accumulation of bile pigments in the liver and the manifestation of the green liver syndrome.

Aim

Chapter 3 (Bowyer et al., 2012a) reported on the effects of partial and total substitution of fish oil by plant and terrestrial animal oils on the growth performance, feed efficiency, nutrient retention, fillet fatty acid profile and blood plasma constituents of Yellowtail Kingfish, while this present Chapter focuses on the response of digestive enzyme activity and intestinal histology to the dietary lipid composition at different temperatures. In this Chapter, we aimed to:

1. Understand the role of water temperatures in regulating the activity of digestive enzymes (e.g. trypsin, lipase and α-amylase) in juvenile Yellowtail Kingfish; and
2. Determine whether the partial (50%) or total (100%) substitution of fish oil with poultry oil or canola oil affected the digestive enzyme activities and digestive tract histology.

The results of this Chapter are drawn from the animals used in Chapter 3 and provide evidence to demonstrate that alternative lipid ingredients and their inclusion level have an effect on the morphology and physiological functioning of the digestive tract of juvenile Yellowtail Kingfish.

Materials and Methods

Fish and diets

The fish, diets (Table 3.1) and experimental design are given in Chapter 3. In brief, two independent experiments were run, with each trial using separate cohorts of juvenile Yellowtail Kingfish, but fertilised eggs came from the same broodstock. Experiment 1 was run at 22.1 ± 0.3 °C (mean ± SD) in March-April 2010, and Experiment 2 was run at 17.6 ± 0.9 °C in July-August 2010. A total of 210 Yellowtail Kingfish averaging 95.6 ± 0.3 g (mean weight ± SD) in Experiment 1 and 101.1 ± 0.5 g in Experiment 2 were evenly distributed into fifteen 700-L fibreglass tanks at the SARDI Aquatic Science Centre, West Beach, Australia. Fish were fed five experimental diets where the added oil component composed of either: Diet 1: 100% fish oil (FO), Diet 2: 100% poultry oil (PO), Diet 3: 100% canola oil (CO), Diet 4: 50% fish oil + 50% poultry oil (FO/PO), and Diet 5: 50% fish oil + 50% canola oil (FO/CO). For further details of dietary composition please refer to Table 3.1 in Chapter 3. At stocking, nine fish (three groups of three fish) were randomly selected and digestive tract samples were collected and analysed for initial fish histology.

Sample collection

At the end of Experiments 1 and 2, feeding was stopped at 24 h and 48 h, respectively, to ensure digestive tracts were void of food residuals before harvesting. All fish were euthanised using an overdose of AQUI-S® (AQUI-S® New Zealand Ltd., Lower Hutt, New Zealand) and measured for weight (nearest 0.1 g) and fork length (nearest 0.1 mm). The abdomens from three fish per tank were opened ventrally and the whole gastrointestinal tract was dissected out and weighed. A 1-cm section at the anterior of the midgut and hindgut, immediately posterior to the ileal valves was taken, immediately flushed with saline, opened longitudinally and fixed in 10% neutral buffered formalin for 24 h, before being transferred to 70% ethanol for storage before analyses. The pyloric caeca, foregut/midgut and hindgut sections (combined tissue and mucus) were dissected out from three other fish per tank, individually weighed (wet basis), and then snap-frozen in liquid nitrogen and stored at -80 °C until analysed for the determination of digestive enzyme activity.

Preparation of gut histology and gut extracts and enzymatic assays

Gastrointestinal sections for histological determination were processed for paraffin histology. These samples were analysed by Dr Louise Adams of the National Centre for Marine Conservation and Resource Sustainability (NCMCRS), Australian Maritime College, University of Tasmania, Launceston, Tasmania. Refer to Appendix 3, “The assessment of soybean enteritis like condition in juvenile Yellowtail Kingfish *Seriola lalandi* held under different feed and temperature regimes” for a more detailed description of methods. Briefly, the foregut and hindgut samples were sectioned at 5 µm and stained with haematoxylin and eosin (H & E) and the structure examined under light microscopy. Goblet cell abundance was confirmed by differentiation with H & E with alcian blue (pH 2.5), which stains acidic mucopolysaccharides blue. The morphology of the foregut and hindgut sections was assessed according to the criteria used by Baeverfjord and Krogdahl (1996): (1) widening and shortening of the intestinal folds, (2) loss of supranuclear vacuolisation in the absorptive cells (enterocytes), (3) widening of the central lamina propria within the intestinal folds, with increased amounts of connective tissue, (4) infiltration of a mixed leucocyte population in the lamina propria and submucosa, and (5) goblet cell proliferation. Sections were randomised and blind read, and allocated semi quantitative scores based on a scale of 1-5 according to Urán (2008), which is a classification scale to identify the condition of soybean meal-induced enteritis in Atlantic Salmon. For each enzyme assay, the pyloric caeca, foregut/midgut and hindgut from three fish per replicate tank (n = 3) were partially thawed, each tissue type was pooled together and rinsed with distilled water before being blotted dry, weighed, and homogenised in four volumes of ice-cold phosphate buffered saline (PBS, pH 7.4) (*W/V*) using a hand-held homogeniser (CAT, X 120, Staufen, Germany). The suspensions were centrifuged at an acceleration of 3893 *g* for 30 min at 4 °C. Supernatants were kept in aliquots and stored at -80 °C until analysed for specific digestive enzyme activity.

The specific enzyme activity of each pooled gut section sample was analysed in triplicate at the temperature at which the respective tissue sample had been collected (i.e. either 18 or 22 °C) and analysed using spectrophotometric techniques and commercial enzyme test kits. Each specific enzyme kit included internal standard solutions and had set pH levels. Trypsin (E.C 3.4.21.4) activity (Biovision, Catalogue No. K771-100) was determined after reading the absorbance of samples at a wavelength of 405 nm for 1 h. Lipase (E.C 3.1.1.) activity was determined according to the method described by Furukawa et al. (1982) with absorbance of samples read at a wavelength of 412 nm after 10 and 20 min (QuantiChrom^{TM}) (BioAssay Systems, Catalogue No. DLPS-100). α-Amylase (E.C 3.2.1.1) activity was determined after reading the absorbance of samples at a wavelength of 412 nm at 0 and 20 min (BioAssay Systems, Catalogue No. K711-100). Total protein was determined using a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich), with bovine serum albumin solution as the standard. The specific enzyme activities were defined as the amount of enzyme that catalysed the conversion of one micro mole of substrate per minute per mg of protein (i.e. U mg soluble protein^{-1}) at the respective temperature.

Statistical analyses

All statistical analyses were carried out using SPSS (version 18, Chicago, Illinois, USA). The digestive enzyme activity was determined for each digestive tract section. The difference in enzyme activity between each section for each temperature and dietary oil treatment (i.e. fish oil at 18 °C, or poultry oil at 22 °C) was analysed using a one-factor ANOVA. In addition, the enzyme activity measured in each gut section was also pooled for gut section to give a representation of the specific trypsin, lipase and α-amylase activities in the whole gut. The effect of water temperature (two levels: 18 or 22 °C) and diet (five levels: fish oil, poultry oil, canola oil, fish oil/poultry oil or fish oil/canola oil) on the response of the pooled digestive enzyme activity was tested with two-factor ANOVA. Visceral weight was initially included as a covariate in the two-factor model for measuring digestive enzyme activity, but no significance was found and was therefore excluded from the final analyses. Assumptions of homogeneity of variances were checked using Levene's equal variance test. Where necessary, data were log transformed to satisfy the assumptions of ANOVA. Multiple comparisons were made using Tukey's Honestly Significantly Different (HSD) test and P=0.05 was applied when a significant main effect was detected. Correlation analyses were performed on the activity of the pooled digestive enzymes with weight gain, specific growth rate (SGR), feed intake and feed conversion efficiency. The enzyme activity was also pooled for water temperature and diet.

Results

Growth performance and fatty acid composition

Fish were fed the experimental diets for five weeks. Growth performance, feed efficiency, proximate composition and nutrient retention data were given in Chapter 3 (Bowyer et al., 2012a). In brief, the results identified that the complete substitution of fish oil with canola oil had detrimental effects on the growth of Yellowtail Kingfish under the test conditions. However, no negative effects were found with either the complete substitution of fish oil with poultry oil or the partial substitution of fish oil with canola oil by 50%. In the present study, the fish oil diet contained 19.4% of n-3 LC-PUFA, while the poultry oil and canola oil diets contained 3.7 and 3.2% of n-3 LC-PUFA, respectively, which is higher than the essential fatty acid requirement for most marine fish species (Glencross, 2009; NRC, 2011). The fatty acid (FA) composition of the muscle tissue generally reflected the dietary FA profile. Two-factor ANOVA identified that the visceral weight was significantly higher at 22 than at 18 °C (P<0.001), but was not affected by diet (P=0.131).

Histology

Refer to Appendix 3 for the full histological examination report. Foregut and hindgut tissues had normal histological appearance and consisted of a sub-mucosal layer, sub-epithelial connective tissues and epithelial mucosa with abundant villi (mucosal folds, both simple and complex). Mucosal folds (villi) were composed of epithelial mucosal cells, with a central distinct lamina propria

containing a mixed leucocyte population. There were no observed differences in gut morphology due to dietary treatment or temperature. The foregut and hindgut from initial fish and fish fed the control diet and the poultry oil or canola oil diets did not show any signs of acute enteritis. Overall, the scores for mucosal fold height, lamina propria width, cellular infiltration of the sub-epithelial mucosa, goblet cell abundance and supranuclear vacuolisation were similar for initial fish and fish fed the different dietary treatments. Scores for mucosal fold height, lamina propria width and cellular infiltration of the sub-epithelial mucosa were normal and ranged from 1 to 2 in initial fish and in fish across all dietary treatments. However, goblet cell abundance was prolific with scores of 4-5 in initial fish and fish fed all dietary treatments (including control diet), at both 18 and 22 °C (Fig. 4.1a, b). In addition, there was a severe reduction in supranuclear vacuolisation in initial fish and in fish fed all dietary treatments (including control diet), scoring 3.5-5, at both 18 and 22 °C (Fig. 4.1c, d).

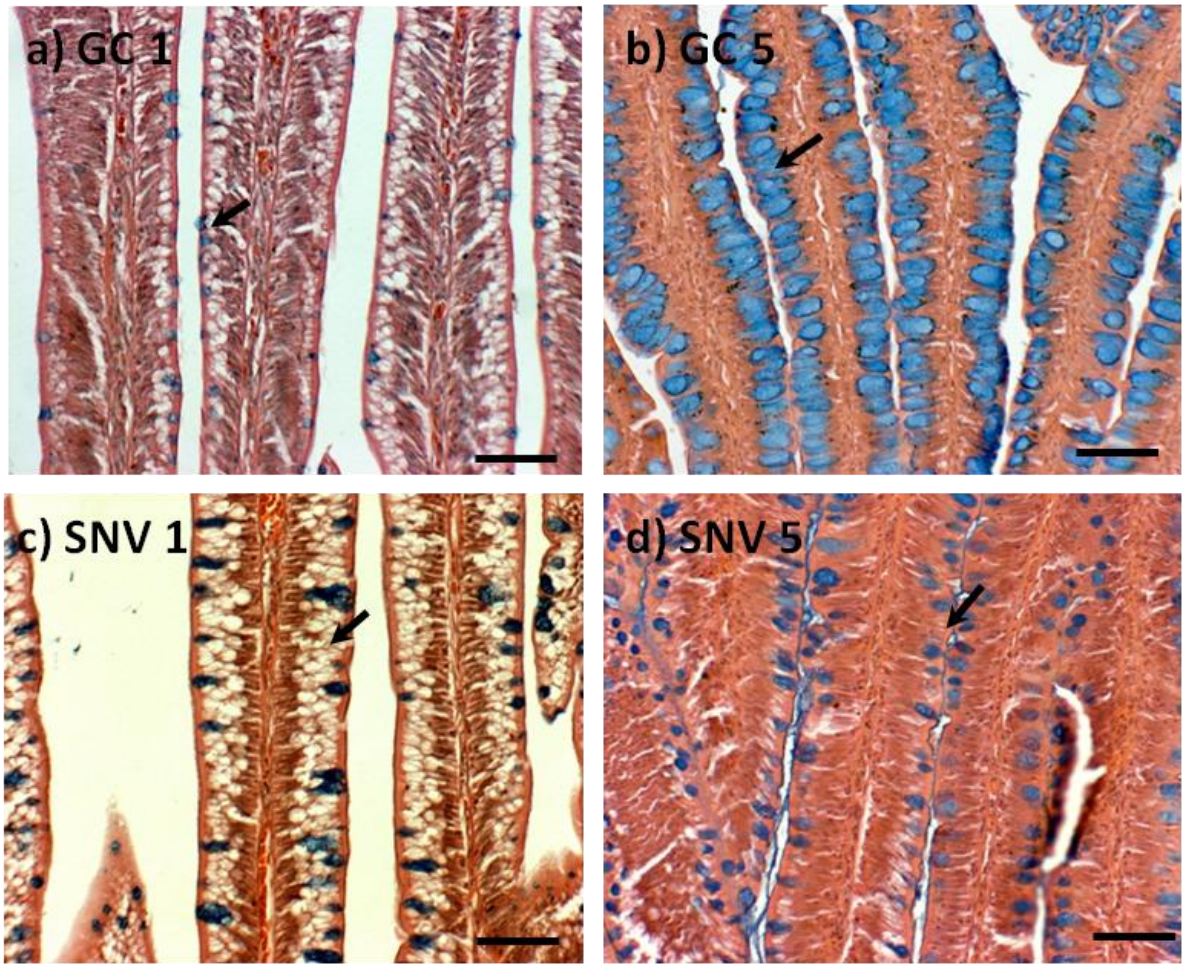

Figure 4.1. Intestine histology from juvenile Yellowtail Kingfish fed different oil sources. The morphological changes seen in a) is the occasional goblet cells (GC) distributed at the proximal edge of the intestinal epithelium, b) prolific abundance of goblet cells along the entire margin of the intestinal epithelium, c) wide margin of supranuclear vacuoles (SNV) within enterocytes of the epithelium and uniformly arranged nuclei adjacent to the lamina propria, and d) extinction of all SNV along the epithelial margin and disorganization of nuclei (H & E alcian blue, pH 2.5, scale bar = 100 µm).

Digestive enzymes

The specific trypsin, lipase and α-amylase activities varied between the pyloric caeca, foregut/midgut and hindgut sections (Table 4.1). The difference in enzyme activity between the pyloric caeca, foregut/midgut and hindgut sections were examined for each water temperature and diet combination separately. The trypsin, lipase and α-amylase activities were significantly higher in the pyloric caeca than in the foregut/midgut and hindgut sections, while the difference in activities between the foregut/midgut and hindgut depended on the diet type and temperature.

Table 4.1. Specific trypsin, lipase and α-amylase activity in the pyloric caeca, foregut/midgut and hindgut of Yellowtail Kingfish fed one of five experimental diets and acclimated to either 18 or 22 °C [1,2].

Temperature	18 °C					22 °C					ANOVA[3]						
Diet	FO	PO	CO	FO/PO	FO/CO	FO	PO	CO	FO/PO	FO/CO	Temp °C	Diet					Interaction
											18 vs. 22	FO	PO	CO	FO/PO	FO/CO	
Trypsin activity (U mg protein^{-1})																	
Pyloric caeca	4.772 ± 0.486^{A}	3.827 ± 0.638^{A}	1.925 ± 0.532^{A}	4.348 ± 1.462^{A}	4.070 ± 0.041^{A}	4.873 ± 0.622^{A}	4.638 ± 1.099^{A}	4.163 ± 0.486^{A}	5.790 ± 0.626^{A}	4.547 ± 0.590^{A}	<	*z*	*yz*	*y*	*z*	*yz*	NS
Foregut/midgut	0.014 ± 0.003^{B}	0.013 ± 0.006^{B}	0.005 ± 0.002^{B}	0.014 ± 0.007^{B}	0.016 ± 0.004^{B}	0.015 ± 0.004^{B}	0.010 ± 0.001^{B}	0.009 ± 0.001^{B}	0.018 ± 0.004^{B}	0.013 ± 0.005^{B}	NS	*z*	*yz*	*y*	*z*	*yz*	NS
Hindgut	0.008 ± 0.001^{C}	0.007 ± 0.000^{B}	0.006 ± 0.001^{B}	0.009 ± 0.003^{B}	0.008 ± 0.001^{B}	0.008 ± 0.001^{C}	0.008 ± 0.001^{B}	0.009 ± 0001^{B}	0.009 ± 0.002^{C}	0.008 ± 0.002^{B}	NS	NS					NS
Lipase activity (U mg protein^{-1})																	
Pyloric caeca	3.944 ± 0.900^{A}	3.439 ± 0.638^{A}	2.503 ± 0.648^{A}	4.372 ± 0.490^{A}	3.967 ± 1.051^{A}	5.289 ± 0.582^{A}	4.897 ± 0.160^{A}	4.932 ± 0.449^{A}	5.177 ± 0.281^{A}	4.879 ± 0.572^{A}	<	NS					NS
Foregut/midgut	1.331 ± 0.079^{B}	0.886 ± 0.053^{B}	0.830 ± 0.094^{B}	1.220 ± 0.118^{B}	1.014 ± 0.092^{B}	1.326 ± 0.181^{B}	1.287 ± 0.104^{B}	1.113 ± 0.127^{B}	1.550 ± 0.227^{B}	1.244 ± 0.158^{B}	<	*yz*	*x*	*x*	*z*	*xy*	NS
Hindgut	0.152 ± 0.046^{C}	0.171 ± 0.036$^{\C}$	0.170 ± 0.065^{C}	0.192 ± 0.061^{C}	0.247 ± 0.034^{C}	0.391 ± 0.114^{C}	0.534 ± 0.119^{C}	0.508 ± 0.076^{B}	0.515 ± 0.074^{C}	0.414 ± 0.050^{B}	<	NS					NS
α-Amylase activity (U mg protein^{-1})																	
Pyloric caeca	0.066 ± 0.004^{A}	0.049 ± 0.003^{A}	0.035 ± 0.003^{A}	0.064 ± 0.013^{A}	0.062 ± 0.001^{A}	0.066 ± 0.008^{A}	0.077 ± 0.022^{A}	0.062 ± 0.002^{A}	0.069 ± 0.005^{A}	0.073 ± 0.015^{A}	<	*z*	*yz*	*y*	*z*	*z*	NS
Foregut/midgut	0.010 ± 0.003^{B}	0.015 ± 0.000^{B}	0.018 ± 0.005^{B}	0.011 ± 0.002^{B}	0.012 ± 0.003^{B}	0.016 ± 0.002^{B}	0.010 ± 0.004^{B}	0.012 ± 0.003^{B}	0.017 ± 0.001^{B}	0.015 ± 0.005^{B}	NS	NS					*
Hindgut	0.005 ± 0.002^{B}	0.004 ± 0.001^{C}	0.010 ± 0.001^{B}	0.005 ± 0.001^{C}	0.007 ± 0.002^{B}	0.007 ± 0.001^{C}	0.007 ± 0.001 ^{B}	0.007 ± 0.002^{C}	0.007 ± 0.002^{C}	0.005 ± 0.001^{B}	NS	NS					*

[x, y, z] For variables with a significant effect of diet and no interaction, values without a common letter are different (*z* indicated the highest value; $P<0.05$).
[A, B, C] For comparing between digestive tract section for each temperature and diet combination, values without a common superscript letter within each column are different ($P<0.05$).
[1] Diet abbreviation: FO, 100% fish oil; PO, 100% poultry oil; CO, 100% canola oil; FO/PO, 50% fish oil + 50% poultry oil; FO/CO, 50% fish oil + 50% canola oil.
[2] Mean ± SD, n = 3, three pooled tissue sections per replicate. SD less than 0.001 are reported as "0.000".
[3] NS, non-significant; *, $P<0.05$. For variables with a significant effects of temperature ($P<0.05$) and no interaction, < indicates the value measured at 18 °C was less than that measured at 22 °C. Comparing between digestive tract section in each temperature and diet combination, values without a common uppercase superscript letter within each column are different ($P<0.05$).

The trypsin activity in the pyloric caeca was significantly lower at 18 °C (P=0.001), but the activity was not affected by temperature in the foregut/midgut or hindgut (P≥0.181). The pyloric caeca and foregut/midgut were significantly affected by diet with the activity being higher in fish fed the fish oil and fish oil/poultry oil diets than the canola oil diet, but similar to fish fed the poultry oil and fish oil/canola oil diets (P≤0.012). The trypsin activity in the hindgut was not affected by diet (P=0.443) (Table 4.1). The lipase activities in the pyloric caeca, foregut/midgut and hindgut were all significantly lower at 18 °C (P<0.001). The lipase activity in the foregut/midgut was affected by diet (P<0.001), but the activities in the pyloric caeca and hindgut were not affected (P≥0.066). In the foregut/midgut the lipase activity in fish fed the fish oil and fish oil/poultry oil diets was significantly higher than fish fed the poultry oil and canola oil diets, while the activity in fish fed the fish oil/canola oil diet was similar to the fish oil diet (Table 4.1).

The α-amylase activity in the pyloric caeca was significantly lower at 18 °C (P=0.001), but there was no effect of temperature on the activity in the foregut/midgut or hindgut sections (P≥0.412). The effect of diet on the α-amylase activity in the pyloric caeca was significant (P=0.019), but there was no effect on the foregut/midgut or hindgut sections (P≥0.074). In the pyloric caeca the α-amylase activity in fish fed the fish oil, fish oil/poultry oil and fish oil/canola oil diets was similar to fish fed the poultry oil diet, but significantly higher than in fish fed the canola oil diet (Table 4.1).

A two-factor ANOVA analysed the specific trypsin, lipase and α-amylase activity of the pooled gut sections (pyloric caeca, foregut/midgut and hindgut) to determine if the activities, representative of the whole gut, were significantly affected by temperature or diet (Fig. 4.2). Trypsin, lipase and α-amylase activities were all significantly lower at 18 °C (P≤0.001). Specific trypsin and lipase activities were significantly affected by diet (P≤0.005) with the activity lower in fish fed the canola oil diet compared to fish fed the fish oil and fish oil/poultry oil diets, while the α-amylase activity was not affected by diet (P=0.052).

Table 4.2. Non-linear correlation coefficient (R) between the pooled digestive enzyme activities and weight gain, specific growth rate, feed intake and feed conversion efficiency in Yellowtail Kingfish, using pooled enzyme activity data that has been pooled for both water temperature and dietary lipid.

Variable	Weight gain		SGR		Feed intake		FCE	
	R	*P*	*R*	*P*	*R*	*P*	*R*	*P*
Trypsin	-0.656	<0.001	-0.678	<0.001	*0.723	*<0.001	*0.721	*<0.001
Lipase	-0.909	<0.001	-0.911	<0.001	0.847	<0.001	*0.863	*<0.001
α-Amylase	-0.695	<0.001	-0.695	<0.001	0.660	<0.001	-0.697	<0.001

*Pearson correlation coefficient analyses data presented as the relationship was linear (n = 30).
Enzyme activities (U mg protein^{-1}); Specific growth rate (% BW day^{-1}); feed intake (% BW day^{-1}); feed conversion efficiency (FCE).

Correlation analyses were performed on the activity of the pooled digestive enzymes with weight gain, specific growth rate (SGR), feed intake and feed conversion efficiency. The enzyme activity was also pooled for water temperature and diet (Table 4.2). Out of the three digestive enzymes the strongest correlations were identified for lipase activity, in particular a strong negative non-linear correlation with both weight gain (r=0.909, P<0.001) and SGR (r=0.911, P<0.001, Table 4.2).

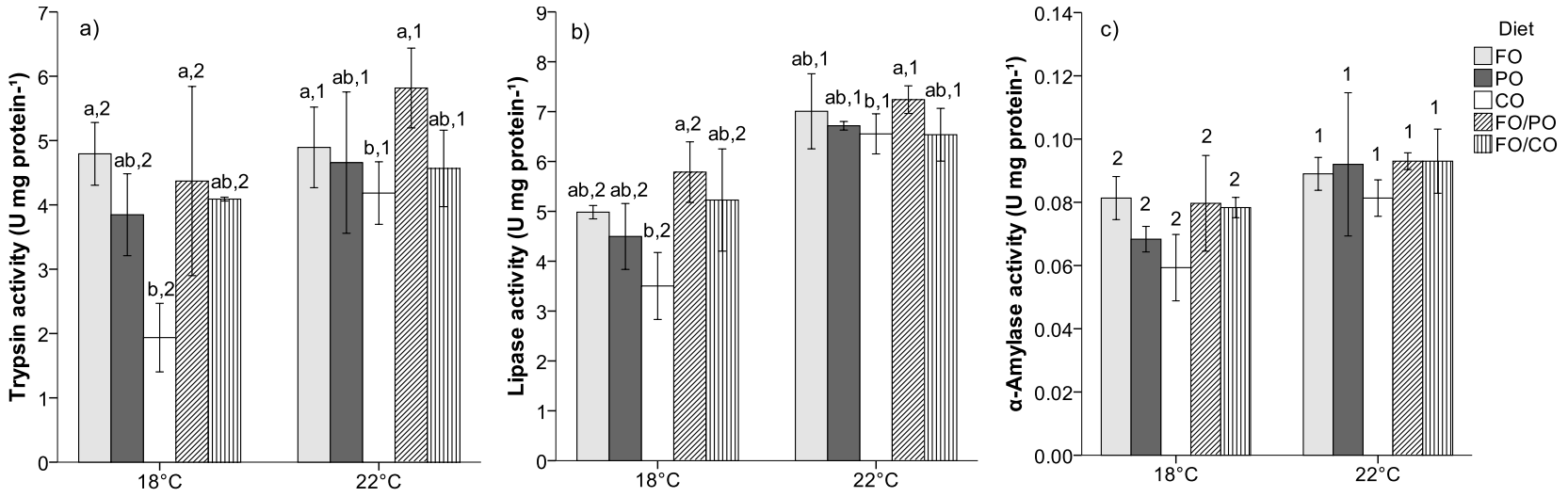

Figure 4.2. Specific trypsin, lipase and α-amylase activities in the pooled whole gut sample in Yellowtail Kingfish fed one of the five experimental diets. Values are means ± SD (n = 3). Values without a common number between temperatures are different (P<0.05). Values without a common alphabetical letter between diets within each temperature group indicate a significant difference (Two-factor ANOVA, Tukey's HSD test, P<0.05).

Discussion

Digestive enzyme activity

In general, the response of the digestive enzyme activity closely followed that of the growth performance results reported in Chapter 3 (Bowyer et al., 2012a). The level of trypsin, lipase and α-amylase activities differed between gut sections, with all activities found to be significantly higher in the pyloric caeca compared to the foregut/midgut and hindgut sections. This study has demonstrated that the partial and total substitution of fish oil with poultry oil and canola oil in diets for Yellowtail Kingfish affected the digestive enzyme activities of trypsin, lipase and α-amylase differently. The enzyme activities were also significantly influenced by water temperature, with lower activities at the suboptimal temperature of 18 °C.

The trypsin activity was significantly influenced by fish oil substitution. The most notable reduction in trypsin activity occurred in the fish fed the canola oil diet at 18 °C which was down-regulated by almost 60% compared to the fish oil diet at the same temperature. Additionally, the trypsin activity at 22 °C in fish fed the canola oil diet was down regulated by almost 15% compared to the fish oil diet at the same temperature. In Chapter 3, the growth performance of Yellowtail Kingfish suggested that an increase in dietary canola oil inclusion reduced the palatability of the canola oil diet. In turn, this significantly reduced the feed intake and weight gain of fish fed the canola oil diet at 18 °C more than at 22 °C (Bowyer et al., 2012a). Anti-nutritional factors (ANF) causing reductions in diet palatability are usually associated with the inclusion of plant protein ingredients into fish diets (Kissil et al., 2000; Deng et al., 2010). The presence of ANF in various protein sources has caused changes to the digestive enzyme activity in fish species such as gilthead seabream (Robaina et al., 1995; Alarcón et al., 1999), Japanese flounder (*Paralichthys olivaceus*) (Deng et al., 2010) and Japanese Yellowtail (Nguyen et al., 2011). However, the presence of an ANF in crude canola oil was suggested as the possible cause of decreased growth and worsening FCR in red seabream (*Pagrus auratus*) (Glencross et al., 2003), and in Yellowtail Kingfish fed a high inclusion level of refined canola oil (Bowyer et al., 2012a). These results are consistent with a recent study by Santigosa et al. (2011), who suggested that changes to the protease activity in gilthead seabream were related to an inhibition of luminal proteases due to the presence of ANF in vegetable oils. Morais et al. (2006) also found that a high inclusion of soybean oil had a depressing effect on trypsin activity in Senegalese sole (*Solea senegalensis*) larvae. Our data showed a reduction in trypsin activity in fish fed the 100% canola oil diet compared to the fish oil control diet. This suggests that there may have been an inhibition or depression of luminal proteases by the presence of ANF in high levels of canola oil regardless of water temperature; but in fact the effect was more pronounced at suboptimal water temperatures.

In addition, the digestive enzyme activity in fish has been directly related to feed intake (Eusebio and Coloso, 2002; Hardewig and van Dijk, 2003). In the current study, when determining if there were any correlations between trypsin activity and the growth performance and feed efficiency parameters, it was found that trypsin activity had a moderately positive linear relationship with feed intake and feed conversion efficiency. Trypsin activity has been significantly correlated with feed conversion efficiency in Atlantic cod (*Gadus morhua*) and was suspected to potentially limit the growth rate (Lemieux et al., 1999). In Asian seabass (*Lates calcarifer*), a higher level of protein intake led to an increase in protease activity caused by stimulating the secretion of enteropeptidase in the intestinal mucosa (Eusebio and Coloso, 2002). In contrast, the current study found a significant reduction in trypsin activity of fish fed the 100% canola oil diet at 18 °C. This corresponds to the poor growth and feed intake of fish fed the 100% canola oil diet,

particularly at 18 °C. This is reflective of the presence of ANFs in the canola oil used in this study (Bowyer et al., 2012a; Chapter 3).

Canola oil is the trademark name given to rapeseed cultivars that contain a low concentration of erucic acid (<2%) and are low in glucosinolates (Canola Council of Canada, 2004; Higgs et al., 2006). The erucic acid (22:1n-9) content of the canola oil used in the current study was only 0.04% of the component fatty acids. This suggests that some other type of anti-nutrient was present. Plants typically contain trypsin inhibitors, which are a defence mechanism that plants have evolved to inhibit the digestion of seeds (Dong et al., 2000). There is evidence that trypsin inhibitors may be fat soluble in soy oil and have been isolated and identified as some free fatty acids, with trypsin found to be extremely sensitive to oleic and linoleic acids (Wang et al., 1975). A rapeseed trypsin inhibitor has previously been isolated from the rapeseed (*Brassica napu*s) seed (Ceciliani et al., 1994), but these inhibitors are water soluble. Therefore, this area of research is complex and requires a more in-depth examination to identify which ANF were responsible for the large reduction in trypsin activity in fish fed the 100% canola oil diet.

In Chapter 3 we reported that when Yellowtail Kingfish were fed the 100% canola and poultry oil diets at both 18 and 22 °C, the fish displayed an increased occurrence of green liver syndrome and exhibited liver dysfunction (Bowyer et al., 2012a). These effects were significantly more pronounced in fish fed the 100% canola oil diet at 18 °C. From the results of the current study, it can be hypothesised that in fish fed the 100% canola oil diet the reduced level of protein available for digestion due to the reduction in protein intake and trypsin activity may have contributed to a reduced level of dietary available taurine. It is well known that *Seriola* spp. cannot sufficiently synthesise taurine endogenously (Takagi et al., 2005). Therefore, the reduction in available taurine in combination with reduced cholesterol levels may have been responsible for the accumulation of bile pigments in the liver. This may then have caused the higher occurrence of the green colouration in fish fed the alternative lipid diets, but significantly more so in fish fed the 100% canola diet at 18 °C (Bowyer et al., 2012a; Chapter 3). This suggests that caution needs to be taken when alternative oils, particularly canola oil, are used to substitute fish oil in aquafeed formulations. This becomes particularly important when fish are experiencing stressful winter temperatures, since cold water temperatures are an additional cause of green liver syndrome in *Seriola* spp. (Sakaguchi and Hamaguchi, 1979).

In the current study, the lipase activity in the fish fed the canola oil or poultry oil diet was not significantly different from that in the fish fed the fish oil diet at both water temperatures. However, the lipase reduction was more pronounced in fish fed the canola oil diet at 18 °C. The lipase activity of fish fed the canola oil and poultry oil diets at 18 °C were down regulated by almost 47% and 37%, respectively, compared to fish fed the same diets and held at 22 °C. This temperature related reduction in enzyme activity also occurred for trypsin and α-amylase activity, regardless of diet. The digestive capacity of a fish is dependent on both the digestive enzyme level as well as gut transit time (Fountoulaki et al., 2005). It has previously been reported that a reduction in water temperature can lead to an increase in enzyme activities in the intestinal chyme of both Japanese Yellowtail (Kofuji et al., 2005) and Yellowtail Kingfish (Miegel et al., 2010). The increase in activity was reported to be in response to the increase in gut transit time at the colder water temperature (Kofuji et al., 2005; Miegel et al., 2010). This allowed for a longer retention time of the enzymes to be in contact with the digesta. However, in the study by Kofuji et al. (2005) the trypsin activity was also measured in the pyloric caeca tissue of starved (48 h) Japanese Yellowtail. The authors found that the trypsin activity was reduced at the lower water temperatures (i.e., 16-18 °C vs. 22-25 °C). This was reflective of the longer re-

synthesis time of trypsin after secretion and subsequent depletion of storage levels in the tissue. This is in agreement with the current study where the digestive enzyme activities in the gut tissue and mucus of starved fish were significantly reduced in fish held at 18 °C. Similar to Kofuji et al. (2005), the reduced activity in Yellowtial Kingfish at 18 °C may be a result of reduced enzyme synthesis and storage levels. In the previous Chapter we reported that the fish size, specific growth rate, feed intake and whole body proximate composition parameters, except for moisture, were all significantly reduced at 18 °C (Bowyer et al., 2012a; Chapter 3). The culmination of all these results are in support of a previous notion found in other marine species where digestive enzymatic activity is reduced at low water temperatures (Brett, 1979; Jobling, 1982; Kaushik, 1986; Einarsson et al., 1996; Jobling, 1997; Hardewig and van Dijk, 2003). This may require the addition of feeding stimulants into diets for Yellowtail Kingfish cultured at low water temperatures to improve digestive enzyme capabilities, as has been found in other species (Carter et al., 1994; Kofuji et al., 2006; Kader et al., 2010; Sarker et al., 2012).

Fatty acid composition

The substitution of fish oil in diets with alternative oils results in significant reductions in the level of LC-PUFA, particularly EPA and DHA available for fish (Turchini et al., 2009). The fatty acid specificity of lipolytic activity towards different fatty acids has been well documented in fish (Tocher, 2003), and that the lipase activity is related to the acyl chain length and degree of saturation (Brannon, 1990). The apparent digestibility of individual fatty acids is also dependent on its composition and has been shown to decrease with increasing chain length and to increase with unsaturation (Olsen et al., 1998). In the current study, the lipase activity was higher in fish fed diets containing fish oil compared to the 100% poultry oil or canola oil diets. This is indicative of the lipase activity having a higher affinity towards fish oil compared to alternative oils. This is also in support of the higher retentions of EPA+DHA found in the muscle tissue of fish fed the fish oil diet compared to that of the canola oil and poultry oil diets in our previous study in Chapter 3 (Bowyer et al., 2012a). The reduction in lipase activity with alternative oil inclusion is also supported by other dietary studies on mammals where the lipase activity decreased when fed alternative oils compared to fish oil (Ricketts and Brannon, 1994; Hedemann et al., 2001). Although the response of lipase activity to alternative oils in grow-out stage fish has been little investigated, one study by Morais et al. (2004) on larval seabass (*Dicentrarchus labrax*) found a contrasting result, where the lipase activity was higher in fish fed coconut oil compared to a fish oil diet. It was suggested that the higher lipolytic activity was stimulated by the medium chain fatty acids and/or SFA present in the coconut oil. In the current study, the lipase activity in Yellowtail Kingfish did have a higher preference towards the diets containing fish oil compared to that of either canola or poultry oil diets at high inclusion levels. However, in our previous study reported in Chapter 3, the inclusion of canola oil and poultry oil into the diets did not have a significant effect on the whole body total fat compared to the fish oil diet, although the apparent nutrient retention data in fish fed the canola oil diet was significantly reduced compared to all other diets (Bowyer et al., 2012a). This is reflective of the digestive enzyme activity results presented in the current study.

Gut histology

The histological analyses were used to assess if any signs of acute enteritis in the digestive tract were associated with effects of temperature and dietary oil type. Refer to Appendix 3 for the full histological report. The mucosal fold height, lamina propria width, and sub-epithelial mucosal infiltration were not changed by either temperature or the type and quantity of dietary lipids. However, the significant abundance of goblet cells and severe reduction in supranuclear vacuolisation were unexpected

because, based on the scoring system by Urán (2008), scores between 4-5 suggest that the goblet cells are densely grouped to highly abundant and tightly-packed and that the supranuclear vacuoles are at the stage of extinction or no vacuoles present. The lack of differential responses of fish in histological changes to temperature, dietary oil type and quantity in this study was at odds with the distinct response in fish growth to different temperatures and dietary oil manipulations reported in Chapter 3 (Bowyer et al., 2012a). The incompatible indications between histological evidence and fish growth performance to environmental and dietary changes suggest the need to establish baseline histological data firstly from wild Yellowtail Kingfish as reference tissue to detect any abnormities of histological tissues in farmed fish. In addition, the scoring system used in this study was based on the criteria developed for Atlantic Salmon, which may not be suitable to evaluate the histological response of Yellowtail Kingfish gastrointestinal tracts to dietary changes. This needs to be further assessed.

Conclusions and Recommendations

In conclusion, under the conditions tested in this study, this research has provided insights into the digestive functioning of Yellowtail Kingfish fed diets substituting fish oil with poultry oil and canola oil. The substitution of fish oil with 100% canola oil led to a reduction in trypsin and lipase activity, particularly at the cooler water temperatures, which corroborates with our previous findings that fish growth is negatively related to the inclusion of canola oil. The lipase activity in Yellowtail Kingfish was found to be higher in diets containing fish oil compared to the 100% poultry oil and canola oil diets. It is important to note that the diets were formulated to ensure the requirements for essential fatty acids were met, despite the high substitution of fish oil with poultry and canola oil. Although the histological study did not reveal any signs of dietary-induced enteritis in the digestive tract, a high number of goblet cells and low supranuclear vacuolisation were identified in fish fed all diets and in the initial fish prior to the experiment. This suggests the need to validate the histology scoring system specifically for Yellowtail Kingfish. In addition, a longer period of exposure to inappropriate nutrition may result in different or more pronounced structural and functional changes to the digestive system. Therefore, we would recommend longer term studies to investigate the effects of fish oil substitution and suboptimal water temperatures on the digestive enzyme activities and digestive tract health and function in Yellowtail Kingfish.

Chapter 5. **Investigate the maximum inclusion level of solvent extracted soybean meal as a fish meal substitute in juvenile Yellowtail Kingfish diets at optimal and suboptimal water temperatures**

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Abstract

Juvenile Yellowtail Kingfish were fed four iso-nitrogenous and iso-calorific (digestible basis) diets containing 0, 10, 20 or 30% solvent extracted soybean meal (SESBM) for 34 days at optimal (22 °C) and suboptimal (18 °C) water temperatures to determine the effects of diet and temperature on growth, feed efficiency, nutrient retention, apparent nutrient digestibility and digestive functions. The substitution of fish meal with up to 20% SESBM did not significantly affect growth. No differences were detected in any of the other parameters measured between 0 and 10% inclusion. However, second-order polynomial regression demonstrated that increasing SESBM had a negative effect on growth, feed efficiency, nutrient retention and the digestibility of diets. Whole body moisture, crude lipid, ash and gross energy were affected by SESBM inclusion. Digestibility coefficients decreased linearly with increasing SESBM. Foregut and hindgut digestive enzyme activities varied with SESBM inclusion. Fish held at 18 °C had significantly reduced growth, feed efficiency and nutrient retention. The whole body moisture increased at 18 °C, while the nutrient and energy digestibilities and whole body protein and gross energy content were lower at 18 °C. The impact of temperature on digestive enzyme activities depended on the section of the digestive tract. No gross signs of digestive tract enteritis were observed. However, increasing levels of SESBM significantly eroded the mucus layer of the digestive tract. The use of SESBM in diets for Yellowtail Kingfish is not recommended until the health problems associated with the erosion of the mucus layer of the digestive tract are clarified.

The information presented in this Chapter addresses part of Objective 4 (Subproject 8A): Determine the maximum inclusion levels of alternative protein and lipid sources to replace fish meal at optimal (22 °C) and suboptimal (18 °C) temperatures. The contents of this Chapter are published in Aquaculture: 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.

Introduction

Soybean ingredients are widely used in diets for many cultured fish species as a cost-efficient alternative protein source to fish meal (FM) due to their constant availability, consistent protein content and relatively well-balanced amino acid profile (Gatlin et al., 2007; Lim et al., 2008). The substitution of fish meal with increasing levels of solvent extracted soybean meal (SESBM) has been tested in many cultured fish species with varying success (Tomas et al., 2005; Gatlin et al., 2007). Differences in the effects of SESBM inclusion on growth performances and health parameters reflect the sensitivity of the individual species to the variety of problems associated with SESBM inclusion. Atlantic Salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) are more sensitive to the inclusion of SESBM, causing reductions in growth and distinct changes to the distal intestinal epithelium (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003). Whereas other species such as Japanese flounder (*Paralichthys olivaceus*) and red drum (*Sciaenops ocellatus*) can tolerate equal amounts of SESBM and FM without adverse effects on growth performance and feed efficiencies (Reigh and Ellis, 1992; Kikuchi, 1999). Juvenile Japanese Yellowtail (*Seriola quinqueradiata*) (14 g) have been successfully fed diets with 20% SESBM inclusion (Shimeno et al., 1992) and longer-term trials with larger Japanese Yellowtail (230 g) fed diets with 30% SESBM produced no significant reduction on growth performance (Shimeno et al., 1993), whereas in another study, 40% soybean meal inclusion gave significantly poorer growth rates (Lee et al., 1991). There is limited information of the inclusion of SESBM in diets for Yellowtail Kingfish (*Seriola lalandi*), but changes to the mucus layer thickness and goblet cell abundance have been reported to occur when fed diets containing 10, 20 and 30% SESBM inclusion (Bansemer, 2011; Bansemer et al., In press; Appendix 4).

Some of the major problems with soybean meals are their low level of the essential amino acids, methionine and lysine, as well as high levels of indigestible carbohydrates, and the presence of anti-nutritional factors (Francis et al., 2001). Even though SESBM is subjected to various processes such as heat-treatment to reduce the level of anti-nutrients, there can still be residual levels of anti-nutritional factors present, such as protease inhibitors, phytates and soya saponins (Francis et al., 2001; Francis et al., 2002). These factors can affect feed efficiencies through reduced palatability, digestibility and nutrient utilisation by inhibiting digestive enzyme activities and compromising gut integrity (Mitchell et al., 1993; Alarcón et al., 1999), and ultimately lead to poor fish growth and health (Bureau et al., 1998; Francis et al., 2001; Hansen et al., 2006; Gatlin et al., 2007).

Decreasing water temperatures to a suboptimal range can influence almost every aspect of the grow-out stage of cultured fish species from reductions in basal metabolic rate to feed intake, gut transit time, activities in the whole enzymatic system, and ultimately growth (Kaushik, 1986). Yellowtail Kingfish in southern Australia are cultured in sea cages where water temperatures that can fluctuate from 10 °C in winter to 24 °C in summer (Miegel et al., 2010). In previous laboratory-based experiments, the optimal water temperatures for maximum growth in this species have been reported as 22 °C by Pirozzi and Booth (2009), 24 °C by Bowyer et al. (2013b; Chapter 7) and 26 °C by Abbink et al. (2012). However, it has been reported that when water temperatures fall below 17 °C, this species has been found to reduce feed intake and growth, and numerous health problems have been reported (Sheppard, 2004; Miegel et al., 2010).

Cultured Yellowtail Kingfish are subjected to fluctuating water temperatures during their grow-out cycle and are fed commercial diets that generally contain unspecified levels of plant and/or animal protein ingredients. In addition, there is limited published

information on the feasibility of using known quantities of various soybean products as a source of dietary protein in feed formulations for Yellowtail Kingfish as reviewed by Bowyer et al. (2012d). Therefore, it is important to understand the impacts of varying water temperatures and the inclusion of dietary plant protein on fish growth, feed efficiency and digestive functioning to determine maximal inclusion levels.

Aim

The aim of this Chapter was to investigate the suitability of substituting fish meal with SESBM at 0, 10, 20 and 30% inclusion, and the response of growth performance, feed efficiency, nutrient utilisation and digestive functioning of juvenile Yellowtail Kingfish when cultured at suboptimal (18 °C) and optimal water temperature (22 °C).

Materials and Methods

Experimental diets

Solvent extracted soybean meal was included into the basal diet at 0 (control), 10, 20 and 30% inclusion levels, substituting 0, 21.7, 43.5 and 65.2% of the fish meal, respectively. The formulation of the control and three experiment diets are displayed in Table 5.1, and the proximate composition and calculated amino acid values of the diets are shown in Table 5.2.

Table 5.1. Ingredient formulation of the four experimental diets fed to Yellowtail Kingfish.

Ingredients[1]	Diet (g kg^{-1} dry basis)			
	0% SESBM	10% SESBM	20% SESBM	30% SESBM
Herring meal	460.0	360.0	260.0	160.0
Solvent extracted soybean meal	0.0	100.0	200.0	300.0
Wheat 14	90.0	90.0	90.0	90.0
Wheat gluten meal	73.9	77.7	77.7	95.0
Fish oil	93.4	99.5	105.6	111.9
Soy lecithin	5.0	5.0	5.0	5.0
Wheat starch	81.9	46.6	11.8	0.0
Poultry by-product meal	60.6	60.7	60.7	60.7
Blood meal	23.6	45.3	70.8	97.2
Choline chloride	3.0	3.0	3.0	3.0
Corn gluten meal	90.0	90.0	90.0	47.8
Vitamin/ mineral premix[2]	2.0	2.0	2.0	2.0
Vitamin C (Stay C)[3]	3.0	3.0	3.0	3.0
Vitamin E	0.4	0.4	0.4	0.4
Betaine	5.0	5.0	5.0	5.0
Monosodium phosphate	4.6	5.5	6.5	7.6
Taurine	3.6	4.5	5.3	6.1
Lysine	0.0	0.7	1.0	1.6
Methionine	0.0	1.1	2.2	3.7
Total	*1000.0*	*1000.0*	*1000.0*	*1000.0*

Diets formulated on a digestible protein and lipid basis.
Yttrium oxide was added to the diets at a rate of 200 mg kg^{-1}.
[1] Supplied by Ridley Aquafeeds, QLD, Australia.
[2] A proprietary product supplied by Lienert Australia Pty Ltd, Australia.
[3] Rovimix® Stay-C® 35, DSM Nutritional Products, Basel, Switzerland.

Table 5.2. Proximate composition and calculated amino acid composition of the SESBM ingredient and the four experimental diets fed to Yellowtail Kingfish.

Item	SESBM ingredient	Diet			
		0% SESBM	10% SESBM	20 SESBM	30 SESBM
Analysed proximate composition (dry basis)					
Dry matter (g kg^{-1})	881.0	920.2	919.1	918.0	917.2
Crude protein (g kg^{-1})	524.4	496.2	508.0	504.4	498.8
Crude lipid (g kg^{-1})	20.0	208.8	197.0	186.4	178.8
Ash (g kg^{-1})	70.0	82.1	75.7	66.1	61.0
NFE (g kg^{-1})[1]	385.6	212.9	228.8	255.9	270.0
Starch (g kg^{-1})	*27.0	151.7	117.1	83.0	68.1
NSP (g kg^{-1})[2]	358.6	61.2	111.7	172.9	201.9
Phosphorous (g kg^{-1})	n/a	14.8	13.2	12.2	10.8
Gross energy (MJ kg^{-1})	20.0	23.1	23.9	24.9	24.0
Calculated amino acids (g kg^{-1} dry basis)					
Arginine	40.4	29.0	29.7	30.4	30.9
Histidine	13.6	15.8	15.8	15.8	15.5
Isoleucine	23.4	24.3	23.5	22.6	21.4
Leucine	39.3	48.0	49.1	50.5	49.0
Lysine	32.9	33.5	33.4	33.3	33.3
Methionine	6.16	14.0	14.0	13.9	13.8
Phenylalanine	25.9	24.6	25.8	27.1	27.4
Threonine	20.7	21.9	21.9	21.9	21.5
Tryptophan	6.76	5.5	5.7	6.0	6.2
Valine	24.6	29.3	29.9	30.6	30.9
Σ IAA[3]	*233.7*	*246.0*	*248.8*	*252.0*	*250.0*
Taurine	n/a	8.0	8.0	8.0	8.0

Diets formulated on a digestible protein and lipid basis.
NFE, nitrogen-free extract; NSP, non-starch polysaccharides; IAA, indispensable amino acids.
[1] By difference: NFE = (100 – crude protein – total fat – ash).
[2] By difference: NSP = (NFE – starch).
[3] Σ IAA: total indispensable amino acid.
* Reported SESBM ingredient starch value from NRC (2011).

The diets were formulated to contain 41.5% digestible protein (50% crude protein) and 14.5% digestible lipid (20% crude lipid) with a gross energy level of 22 MJ kg^{-1} as described by Booth et al. (2010a). The amino acid composition of the diets were calculated using analysed amino acid ingredient values and the diets were formulated to satisfy the nutritional requirements for a carnivorous marine fish (NRC, 2011). The level of lysine and methionine were balanced in all the diets according to values for Japanese Yellowtail (Ruchimat et al., 1997a; Ruchimat et al., 1997b) and to reflect the content of the fish meal control diet (0% SESBM).

Based on the fact that fish meal often contains taurine in excess of 0.5%, plus the diets contained some animal products which also contain taurine (Gaylord et al., 2006), it was assumed that the dietary levels of taurine were between 0.25-0.5%. Therefore, the taurine level was over supplemented to contain 0.8% across all the diets, which was based on current commercial formulations for Yellowtail Kingfish (Dr Richard Smullen, personal communication). Yttrium oxide was included in all diets (0.02% inclusion) as an inert maker for digestibility determination at the completion of the growth trial. The diets were produced at the SARDI Australasian Experimental Stockfeed Extrusion Centre (Roseworthy, Adelaide, Australia) as cooked, extruded and slow sinking pellets (2.5 mm) using a Wenger X-85 (Sabetha, KS, USA). The production parameters were recorded for each diet. During diet production, the maximum levels reached for the following parameters were: extruder temperature, 85 °C; cone head pressure, 100 psi; feeder screw and extruder rotations, 16 and 300 rpm, respectively, cooler retention time, 11.3 min; and dryer temperature, 75 °C. Enough feed for approximately 1 week was maintained at 4 °C. Otherwise feeds were kept frozen prior to use (-20 °C).

Growth experiment

Experimental work was conducted at the SARDI, Aquatic Science Centre, SA, Australia. Experimental protocols followed the ethical standard approved by the Animal Welfare Committee of Flinders University (E286). Yellowtail Kingfish fingerlings (5-10 g) were obtained from Clean Seas Tuna Ltd (Arno Bay, Australia) and were on-grown in 5000-L fibreglass tanks at SARDI Aquatic Science Centre in ambient seawater temperatures (17 ± 1 °C) until the beginning of the trial. Fish were fed a commercial diet (Skretting, NOVA, 1.8, 2 and 3 mm sinking pellet; 50% protein, 15% lipid, Cambridge, Tasmania, Australia) prior to the commencement of the experiment.

Upon commencement of the trial, Yellowtail Kingfish juveniles were anaesthetised using AQUI-S® (AQUI-S® New Zealand Ltd., Lower Hutt, New Zealand) at a concentration of 20 mg L^{-1} of seawater. A total of 384 fish (mean weight ± SD; 22.61 ± 0.02 g) were randomly assigned to sixteen 700-L fiberglass experimental tanks (24 fish per tank), and the four experimental diets were randomly assigned to two replicate tanks at each water temperature. Fish were fed the experimental diets to apparent satiation twice daily for 34 days. The following water quality parameters were monitored daily during the growth trial for both the 22 °C (optimal, 21.8 ± 0.3 °C) and 18 °C (suboptimal, 17.9 ± 0.2 °C) treatments: dissolved oxygen (6.4-7.2 mg L^{-1}), pH (7.6-7.7), and ammonia levels (0.1-0.3 mg L^{-1}) and weekly measurements for salinity (38 ± 0.5 ppt). At stocking, initial fish were randomly selected and analysed for proximate composition (three groups of six fish) and digestive tract histology (three groups of three fish).

The re-circulating system was housed in a temperature and photoperiod (14 h light: 10 h dark) controlled room. There were two identical re-circulating system units each consisting of a 780-L sump, an electric pump, sand filter, a 780-L moving bed bio-filter, UV light and eight 700-L cylindrical tanks. One re-circulating system unit contained a water chiller system which maintained the suboptimal water temperature. The temperature of the other re-circulating system unit was maintained by heating the ambient air. The ambient incoming seawater was 20 ± 1 °C. At the end of the experiment, feeding was stopped 24 h before sample collection. All fish were euthanised by an overdose of AQUI-S® and measured for weight (nearest 0.1 g) and fork length (nearest 0.1 mm). Liver weight was used to determine hepatosomatic index (HSI). Foregut and hindgut samples were collected from seven fish per tank for histological analyses. One cm sections at the anterior of the midgut and hindgut, immediately posterior to the ileal valves were taken, flushed with saline. Sections

were then opened longitudinally and fixed in 10% neutral buffered formalin for 24 h, before being transferred to 70% ethanol for storage before analyses. The whole gut weights from three other fish per tank were individually weighed (wet basis) and used to determine the viscerosomatic index (VSI). The pyloric caeca, foregut/midgut and hindgut (combined tissue and mucus) were then separated from the whole gut and individually weighed (wet basis) and then snap frozen in liquid nitrogen, then stored at -80 °C until analysed for the determination of digestive enzyme activity. Another three fish from each tank were randomly selected, pooled and stored at -20 °C until analysed for whole body proximate composition.

Preparation of histology, gut extracts and enzymatic assays

Gastrointestinal sections for histological determination were processed for paraffin histology. Foregut and hindgut samples were sectioned at 5 µm and stained with haematoxylin and eosin (H & E) and the structure examined under light microscopy. Goblet cell abundance was quantified by differentiation with H & E with alcian blue (pH 2.5), which stains acidic mucopolysaccharides blue. The morphology of the foregut and hindgut sections were assessed according to the criteria used by Baeverfjord and Krogdahl (1996) for Atlantic Salmon: (1) widening and shortening of the intestinal folds, (2) loss of supranuclear vacuolisation in the absorptive cells (enterocytes), (3) widening of the central lamina propria within the intestinal folds by increased amounts of connective tissue, (4) infiltration of a mixed leucocyte population in the lamina propria and submucosa, and (5) goblet cell proliferation. Sections were randomised and blind read, and allocated semi quantitative scores based on a scale of 1-5 according to Urán (2008), which is a classification scale to identify the condition of soybean meal-induced enteritis in Atlantic Salmon. Refer to Appendix 3 for a detailed description of methods.

For each enzyme assay, each individual gut section, i.e., pyloric caeca, foregut/midgut or hindgut, from three pooled fish per tank (n = 3) were partially thawed, pooled for each gut section, rinsed in distilled water, blotted dry, weighed, and homogenised on ice using a hand-held homogeniser (CAT, X 120, Staufen, Germany) in four volumes of ice-cold phosphate buffered saline (PBS, pH 7.4) (*W/V*). The suspensions were centrifuged at an acceleration of 3893 *g* for 30 min at 4 °C. Supernatants were kept in aliquots and stored at -80 °C until analysed for digestive enzyme activity. All measurements were carried out either in duplicate or triplicate.

The specific enzyme activity of each pooled gut section sample was analysed in triplicate at the temperature at which the respective tissue sample had been collected (i.e., either 18 or 22 °C). Analyses were conducted using spectrophotometry (Multiscan Ex Microplate Reader, Labsystems, Finland) and commercially available enzyme test kits. The loss of substrate over time was used to determine the level of enzyme activity. Each specific enzyme kit included internal standard solutions and had set pH levels. Trypsin (E.C 3.4.21.4) activity (Biovision, Catalogue No. K771-100, California, USA) was determined after reading the absorbance of samples at a wavelength of 405 nm at 0 and 1 h. Lipase (E.C 3.1.1.) activity was determined according to the method described by Furukawa et al. (1982) with absorbance of samples read at a wavelength of 412 nm after 10 and 20 min (QuantiChrom^{TM}, BioAssay Systems, Catalogue No. DLPS-100, California, USA). Amylase (E.C 3.2.1.1) activity was determined after reading the absorbance of samples at a wavelength of 412 nm at 0 and 20 min (BioAssay Systems, Catalogue No. K711-100, California, USA). Total protein was determined using a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich, Missouri, USA), with bovine serum albumin solution as the standard. The specific enzyme activities were defined as the amount of enzyme that catalysed the conversion of one micro mole of substrate per minute per mg of protein (i.e., U mg soluble protein^{-1}) at the respective temperature.

In vivo digestibility procedures

After the end of the growth trial a digestibility trial was carried out to determine the apparent digestibility coefficients (ADC) of the test diets. Individual fish were exposed to four faecal collection events over a period of 19 days. The remaining 11 fish per tank from the growth trial were fed their respective diets to excess twice per day (0900 and 1530 h). Every fourth day, fish were fed their respective diet to excess at 0900 and 1200 h to ensure full guts, and then starting at 1400 h fish were captured by net, rapidly anaesthetised (AQUI-S®, 25 mg L^{-1}, for a maximum duration of 3 min) and faeces were collected from all fish by manual stripping. Fish were recovered in holding bins (100-L aerated seawater) at their respective water temperatures, before being transferred back into their experimental tanks after all fish had undergone the stripping procedure. Care was taken to avoid contamination of faeces with water, mucous or urine. To avoid any influence of sampling technique on faecal collections, the same operator was assigned to the task for each stripping event. Faecal material from each stripping event from all fish within individual tanks was pooled and then analysed as a single unit (two tanks per treatment).

Chemical analyses

Proximate composition analyses of diets, whole body and faeces were conducted according to methods in the British Pharmacopoeia Commission (2004) or DIN 51900-1 (2000). Fish for whole body proximate analyses were half thawed and then blended in a food processor. A sub-sample of tissue and faces were freeze-dried to a constant weight at -50 °C to determine moisture. Diet samples were oven dried at 105 °C for 16 h to determine moisture. Crude protein (N × 6.25) was determined by the Kjeldahl method (BP A219 H Determination of Nitrogen, 2004) (Buchi Speed Digester K439 and FjelFlex K360, Switzerland and Mettler Toledo DL22 Food and Beverage Analyser, Switzerland). Crude lipid was determined using the method of Bligh and Dyer (1959). Ash was determined by muffle furnace at 550 °C for 16 h. Gross energy content was determined using a bomb calorimeter (Parr Instrument Company, Moline, Illinois, USA), and calibrated with benzoic acid (DIN 51900-1, 2000). Faecal samples were not measured for ash content, due to an insufficient sample size. Diet and faecal samples were measured for yttrium oxide using ICPOES analysis (University of Queensland, School of Land, Crop and Food Analytical Services), for the determination of apparent dietary nutrient and energy digestibility.

Calculation of performance indices

Performance indices were calculated using the following formulae (De Silva and Anderson, 1995; Hardy and Barrows, 2002), where the weight (wt) unit was in grams. All calculations using fish weight and body composition were based on wet values and feed consumption and compositions were based on dry values.

- Weight gain (g $fish^{-1}$) = final weight – initial weight
- Specific growth rate (SGR, % day^{-1}) = (ln(final weight) – ln(initial weight))/ days × 100
- Apparent feed conversion ratio (FCR) = dry wt feed consumed per tank / wet wt fish gain per tank
- Hepatosomatic index (HSI, %) = (wet liver wt / final wet fish wt) × 100
- Protein efficiency ratio (PER) = fish wt gain / protein consumed
- Energy efficiency ratio (EER) = fish wt gain / energy consumed

- Apparent protein retention (PR, %) = (% protein of final body wt – % protein of initial body wt) × 100 / protein intake

- Apparent energy retention (ER, %) = (% energy of final body wt – % energy of initial body wt) × 100 / energy intake
- Apparent dietary nutrient digestibility coefficients (ADC, %) for protein, fat and energy, was calculated using the indirect method, following equation and methods described by Cho et al*.* (1982):

ADC (%) = 100 × [1 - (F/D × D_y / F_y)]

Where F = % nutrient or gross energy in faeces; D = % nutrient or gross energy in diet; D_y = % yttrium oxide in diet; F_y = % yttrium oxide in faeces.

Statistical analyses

All statistical analyses were carried out using SPSS (version 18, Chicago, Illinois, USA). The effects of dietary SESBM inclusion (4 levels: 0, 10, 20 or 30%) and water temperature (2 levels: 18 or 22 °C) on growth performance, feed efficiency, somatic parameters, compositional data, nutrient retention and digestive enzyme activities were tested with two-factor ANOVA. If a significant interaction was detected between the main effects, then the main effect was ignored and comparisons were made using Least Significant Difference (LSD) to examine the dependent relationship between the two independent factors (temperature and diet). If an interaction between the two independent factors was not significant, then the main effect was considered and different factor levels were compared using a post-hoc Tukey's HSD multiple comparison procedure with the probability of difference set at 0.05. Assumptions of homogeneity of variances were checked using Levene's equal variance test. Where necessary, data were log transformed to satisfy the assumptions of normality and homogeneity of variances, and all percentage data was arcsine transformed before analyses. The relationship between performance parameters and SESBM inclusion were measured using a second order polynomial regression analyses with the significance judged by the coefficient of determination (R^2) and *P*-value set at <0.05. All data were expressed as the mean of two replicate tanks (n = 2).

Results

Growth and survival

All diets were equally accepted by fish and there were no gross signs of disease observed during the experiment. Over the course of the feeding trial only one fish died from the 30% SESBM treatment at 22 °C. Final weight and SGR decreased at 18 °C ($P \leq 0.006$), and were significantly affected by diet ($P \leq 0.002$, Table 5.3). Second order polynomial regression analyses described a significantly negative relationship between weight gain with increasing SESBM inclusion at 18 °C (R^2=0.947, P=0.001, Fig. 5.1a) and 22 °C (R^2=0.843, P=0.010, Fig. 5.1b). Similarly, there was also a significantly negative relationship between SGR with increasing SESBM inclusion at 18 °C (R^2=0.936, P=0.001) and 22 °C (R^2 =0.854, P=0.008). At 18 °C, weight gain started to decrease at 6.5% SESBM and SGR started to decrease at 6.9% SESBM, while at 22 °C both weight gain and SGR decreased rapidly with increasing SESBM.

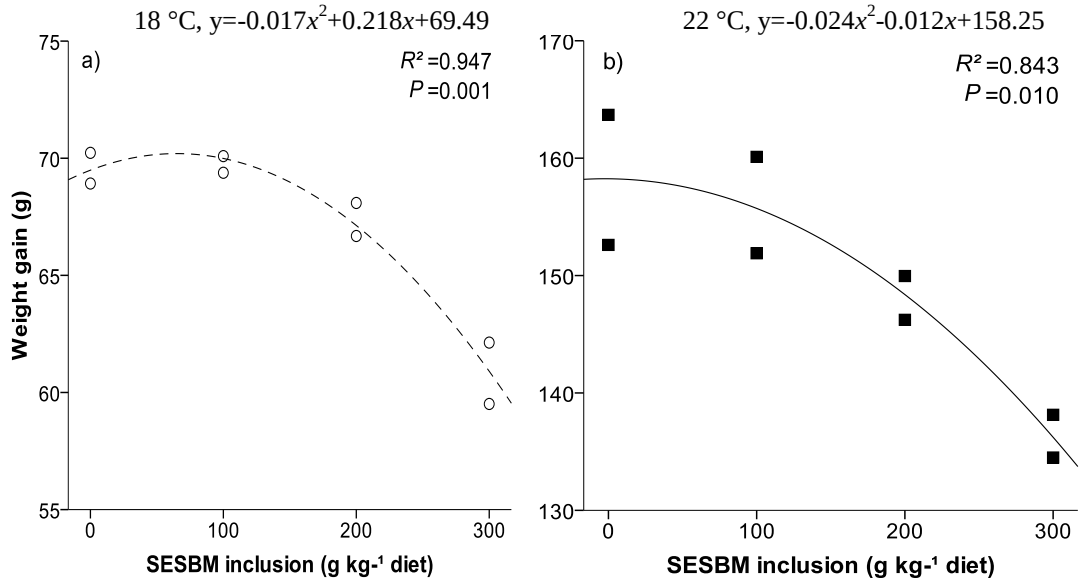


Figure 5.1. Weight gain of Yellowtail Kingfish fed increasing levels of SESBM and held at either, a) 18 °C, or b) 22 °C (mean, n = 2).

Feed efficiency

Feed intake was significantly lower at 18 than 22 °C (P<0.001) and was significantly affected by diet (P=0.003, Table 5.3). A significantly positive polynomial relationship was identified between feed intake and increasing SESBM inclusion at 18 °C (R^2=0.942, P=0.001), but this relationship was not significant at 22 °C (P=0.165) due to large variation between replicates. Apparent FCR was significantly influenced by water temperature (P=0.003), and diet (P<0.001), and there was a significant interaction (P=0.003, Table 5.3). The interaction was due to a more pronounced increased in apparent FCR in fish fed the 30% SESBM diet at 18 °C than at 22 °C (P<0.001). A significant positive polynomial relationship was demonstrated between FCR and increasing SESBM inclusion at 18 °C (R^2= 0.977, P<0.001) and 22 °C (R^2=0.948, P=0.001).

Somatic parameters

The hepatosomatic index of fish ranged from 0.9 to 1.1%, but was not affected by water temperature (P=0.059), or diet (P=0.106). The viscerosomatic index of the fish held at 22 °C (11.4%) was greater than in fish at 18 °C (6.5%) (P<0.001), but was not affected by diet (8.7-9.3%) (P=0.709).

Table 5.3. Growth performance, feed efficiency, nutrient retentions and proximate composition of Yellowtail Kingfish acclimated to two different water temperatures and fed diets differing in SESBM inclusion level[1].

Temperature	18 °C				22 °C				ANOVA[2]					
Diet (%)	0	10	20	30	0	10	20	30	Temp °C	Diet (%)				Interaction
									18 vs. 22	0	10	20	30	
Growth performance														
Initial weight (g)	22.62 ± 0.02	22.62 ± 0.02	22.64 ± 0.01	22.61 ± 0.03	22.62 ± 0.02	22.62 ± 0.02	22.60 ± 0.01	22.59 ± 0.01						
Final weight (g)	92.15 ± 0.92	92.35 ± 0.49	90.00 ± 0.99	83.40 ± 1.84	180.75 ± 7.85	178.60 ± 5.80	170.70 ± 2.69	158.90 ± 2.55	<	*z*	*z*	*z*	*y*	NS
SGR (% BW day^{-1})	4.14 ± 0.04	4.14 ± 0.02	4.06 ± 0.03	3.84 ± 0.07	6.11 ± 0.13	6.08 ± 0.09	5.95 ± 0.05	5.74 ± 0.05	<	*z*	*z*	*z*	*y*	NS
Feed efficiency														
Feed intake (g fish^{-1} day^{-1})	1.88 ± 0.04	1.91 ± 0.03	2.15 ± 0.06	2.31 ± 0.01	4.29 ± 0.07	4.21 ± 0.14	4.48 ± 0.23	4.55 ± 0.05	<	*xy*	*x*	*yz*	*z*	NS
Apparent FCR	0.92 ± 0.03^{a}	0.93 ± 0.02^{a}	1.09 ± 0.01^{b}	1.29 ± 0.04^{c}	0.92 ± 0.03^{a}	0.92 ± 0.01^{a}	1.03 ± 0.04^{b}	1.14 ± 0.01^{c}	>	*				*
Nutrient retention														
PER	2.20 ± 0.08	2.12 ± 0.05	1.83 ± 0.01	1.56 ± 0.05	2.19 ± 0.07	2.15 ± 0.01	1.94 ± 0.06	1.77 ± 0.01	<	*z*	*z*	*y*	*x*	NS
EER	4.73 ± 0.16	4.50 ± 0.10	3.71 ± 0.04	3.24 ± 0.09	4.71 ± 0.15	4.56 ± 0.01	3.91 ± 0.13	3.69 ± 0.04	<	*z*	*z*	*y*	*x*	NS
Apparent PR (%)	39.12 ± 2.84	37.06 ± 0.13	33.24 ± 3.97	27.22 ± 1.24	43.32 ± 4.02	38.36 ± 1.80	36.36 ± 1.41	34.46 ± 0.07	<	*z*	*yz*	*yx*	*x*	NS
Apparent ER (%)	32.92 ± 1.65	31.57 ± 0.35	23.63 ± 1.01	19.35 ± 0.85	34.94 ± 1.24	32.66 ± 1.38	26.20 ± 0.38	23.82 ± 0.06	<	*z*	*z*	*y*	*x*	NS
Proximate composition (% wet weight)														
Moisture	71.26 ± 0.25	71.52 ± 0.42	72.73 ± 0.14	73.93 ± 0.23	69.94 ± 0.45	69.97 ± 0.03	71.48 ± 0.54	72.41 ± 0.12	>	*x*	*x*	*y*	*z*	NS
Crude protein	17.45 ± 0.52	17.21 ± 0.25	17.69 ± 1.48	17.18 ± 0.23	19.37 ± 1.07	17.68 ± 0.69	18.50 ± 0.08	19.02 ± 0.10	<	NS				NS
Crude lipid	6.67 ± 0.36	6.78 ± 0.65	5.30 ± 0.84	4.37 ± 0.13	7.13 ± 0.22	7.08 ± 0.23	5.77 ± 0.52	5.36 ± 0.83	NS	*z*	*z*	*y*	*y*	NS
Ash	1.35 ± 0.21	1.18 ± 0.07	1.07 ± 0.08	1.03 ± 0.00	1.28 ± 0.11	1.40 ± 0.03	1.18 ± 0.11	1.17 ± 0.00	NS	*z*	*z*	*y*	*y*	NS
GE (MJ kg^{-1})	6.73 ± 0.08	6.78 ± 0.18	6.28 ± 0.25	5.99 ± 0.06	7.25 ± 0.03	7.02 ± 0.28	6.61 ± 0.11	6.40 ± 0.06	<	*z*	*z*	*y*	*y*	NS

SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; EER, energy efficiency ratio; PR, protein retention; ER, energy retention.

x, y, z For parameters with a significant effect of diet and no interaction, values without a common letter are different (*z* indicated the highest value; $P<0.05$).

a, b, c, d For parameters with a significant interaction, differences in diets are compared within each temperature (one-factor ANOVA, Tukey's HSD test), values without a common superscript are different ($P<0.05$).

[1] Mean ± SD; n = 2, three pooled fish per replicate, SD less than 0.01 are reported as "0.00".

[2] NS, non significant; *, $P<0.05$. For variables with a significant effect of temperature ($P<0.05$) and no interaction, < or > indicates whether the values measured at 18 °C was less than or greater than that measured at 22 °C.

Nutrient retentions

Nutrient retention parameters (PER, EER and apparent PR and ER) were significantly lower at 18 °C ($P \leq 0.010$), and were affected by diet ($P \leq 0.002$, Table 5.3). At 18 °C, negative polynomial relationships were demonstrated for PER (R^2=0.970, P<0.001), EER (R^2=0.957, P<0.001), PR (P=0.007, R^2=0.941) and ER (R^2=0.866, P=0.001) with increasing SESBM. Similarly, at 22 °C negative regressions were found for PER (R^2=0.942, P=0.001), EER (R^2=0.916, P=0.002), PR (R^2=0.795, P=0.019), and ER (R^2=0.939, P<0.001).

Whole body proximate composition

The whole body moisture content was higher at 18 than at 22 °C (P<0.001), and was affected by diet (P<0.001, Table 5.3). Moisture content increased in a significantly positive significant polynomial relationship with increasing SESBM inclusion at 18 °C (R^2=0.957, P<0.001) and at 22 °C (R^2=0.899, P=0.003). Whole body crude lipid and ash content were influenced by diet ($P \leq 0.028$), but not by water temperature ($P \geq 0.070$). A significantly negative polynomial relationship was observed between whole body fat as SESBM increased (R^2=0.712, P<0.001), while there was a significantly negative linear relationship for whole body ash (R^2=0.416, P=0.007) as SESBM increased. Whole body crude protein and gross energy contents were significantly lower at 18 than at 22 °C ($P \leq 0.008$). Diet affected gross energy (P<0.001), but not the crude protein content (P=0.358). There was a significantly negative polynomial relationship between whole body energy and increasing SESBM level at 18 °C (R^2=0.832, P=0.012) and at 22 °C (R^2=0.888, P<0.001).

Gut histology

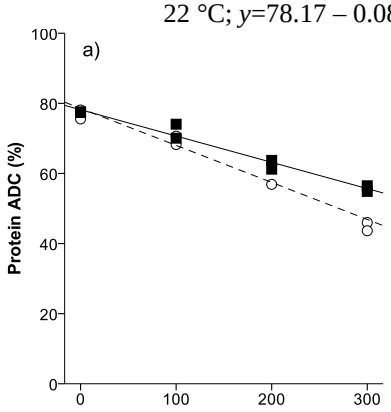
The intestinal structure of the foregut and hindgut tissues consisted of a sub-mucosal layer, sub-epithelial connective tissues and epithelial mucosa with abundant villi (mucosal folds, both simple and complex). Mucosal folds (villi) were comprised of epithelial mucosal cells, with a central distinct lamina propria containing a mixed leucocyte population. There were no detectable signs of acute enteritis in sections of the foregut or hindgut from fish fed the fish meal control diet, the SESBM diets, or in the initial fish samples. Overall, only minor differences were observed in the scores for mucosal fold height, lamina propria width, cellular infiltration of the sub-epithelial mucosa, goblet cell abundance and supranuclear vacuolisation which were similar for initial fish, fish fed the control diet, and SESBM diets. Scores for mucosal fold height, lamina propria width and cellular infiltration of the sub-epithelial mucosa ranged from 1-2 in initial fish and in fish fed increasing levels of SESBM and in fish fed the control diet. However, it must be noted that scores for goblet cell proliferation and supranuclear vacuolisation appeared to be considerably elevated, and differed between gut sections. In the foregut, the goblet cell abundance was prolific in initial fish, and in fish fed the control diet and diets containing SESBM, with the scores ranging from 3-5. The supranuclear vacuolisation for initial fish scored 1.8, but fish fed the SESBM diets and fish fed the control diet scored from 3-5. In the hindgut, the goblet cell abundance was low and there was a reduction in supranuclear vacuolisation, but there was no difference between the initial fish and fish fed the SESBM diets or control diet. Refer to Appendix 3 for a description of the results.

Apparent digestibility coefficients

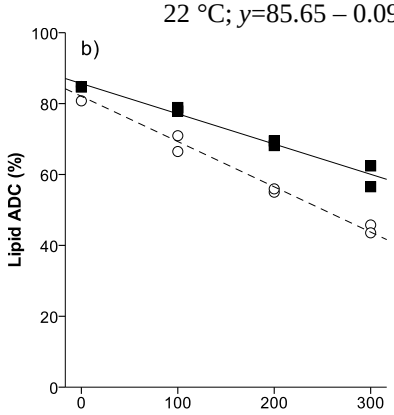
There were significantly negative linear relationships between protein, lipid and energy apparent dietary nutrient digestibility (ADC) and increasing SESBM. Apparent protein digestibility for the fish meal control diet was 76.8 ± 1.3% at 18 °C and 77.5 ± 0.1% at 22 °C, and reduced linearly with increasing SESBM inclusion, ranging from 44.8 to 69.5% at 18 °C and 55.7 to 72.1% at 22 °C (Fig. 5.2a). Lipid ADC was 82.8 ± 2.9% and 84.72 ± 0.02% in the fish meal control diet at 18 and 22 °C, respectively.

As SESBM inclusion increased the lipid ADC decreased linearly at both water temperatures, ranging from 44.7 to 68.7% at 18 °C and 59.5 to 78.4% at 22 °C (Fig. 5.2b). Energy ADC ranged from 32.7 to 64.3% at 18 °C and 38.1 to 62.7% at 22 °C and was significantly reduced by increasing SESBM inclusion (Fig. 5.2c).

Protein ADC: 18 °C; y=78.57 – 0.11x, R^2= 0.953, P<0.001.
22 °C; y=78.17 – 0.08x, R^2= 0.969, P<0.001.



Lipid ADC: 18 °C; y=82.06 – 0.13x, R^2= 0.984, P<0.001.
22 °C; y=85.65 – 0.09x, R^2= 0.968, P<0.001.



Energy ADC: 18 °C; y=62.25 – 0.10x, R^2= 0.947, P<0.001
22 °C; y=64.15 – 0.08x, R^2= 0.911, P<0.001.

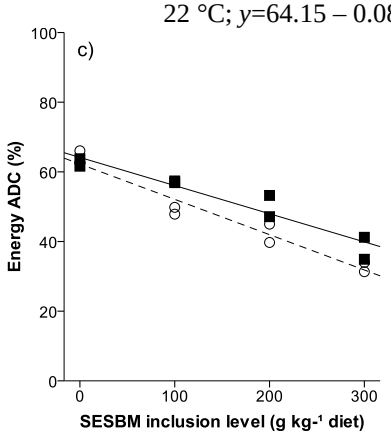


Figure 5.2. Apparent dietary nutrient and energy digestibilities (ADC) for protein (a), lipid (b) and energy (c) for Yellowtail Kingfish fed different inclusions of SESBM at 18 °C (○, dashed line) and 22 °C (■, solid line).

Digestive enzymes

Specific enzyme activities varied between the pyloric caeca, foregut/midgut and hindgut sections (Table 5.4). The trypsin activity in the digestive tract sections were influenced by temperature, with the exception of the pyloric caeca, and diet differently (Table 5.4). Water temperature did not affect the trypsin activity in the pyloric caeca (P=0.694), but the activity was higher at 18 °C in the foregut/midgut and hindgut sections than at 22 °C (P≤0.027). Diet did not affect the trypsin activities in the pyloric caeca (P=0.153), but the activity was influenced in the hindgut (P=0.042), and there was a significant interaction between temperature and diet in the foregut/midgut section (P=0.009). The interaction was due to a more pronounced increase in trypsin activity in fish fed the 10% SESBM diet at 18 °C than at 22 °C, but there was some variation between samples. In the hindgut, a significantly negative polynomial relationship was observed between trypsin activity and SESBM at 18 °C, where the activity peaked between 20-30% inclusion before decreasing (R^2=0.710, P=0.045).

Lipase activity at 18 °C in the pyloric caeca and hindgut was lower than at 22 °C (P≤0.011), but was not affected by temperature in the foregut/midgut (P=0.087, Table 5.4). The pyloric caeca and foregut/midgut were not influenced by diet (P≥0.064), but the activity was affected in the hindgut (P=0.011). There was a significantly positive linear relationship between lipase activity in the hindgut and increasing SESBM inclusion at 18 °C (R^2=0.711, P=0.045), but no relationship was observed at 22 °C due to sample variation.

The effect of temperature on α-amylase activity varied between digestive tract sections. In the pyloric caeca the activity was lower at 18 than at 22 °C (P<0.001), while the activity in the foregut/midgut was not affected (P=0.856), and the activity was higher at 18 °C in the hindgut (P=0.016, Table 5.4). The α-amylase activities in the pyloric caeca and hindgut were not affected by diet (P≥0.081), but the activity was in the foregut/midgut section (P=0.007). There was also a significant interaction between temperature and diet in the hindgut (P=0.021). The interaction was due to the more pronounced reduction in α-amylase activity in fish fed the 30% SESBM at 22 °C than at 18 °C. In the foregut/midgut, a significantly positive polynomial relationship was observed between α-amylase activity and increasing SESBM inclusion (R^2=0.636, P=0.001).

Table 5.4. Trypsin, lipase and α-amylase activity in the pyloric caeca, foregut/midgut and hindgut of Yellowtail Kingfish acclimated to two different water temperatures and fed diets differing in SESBM inclusion level[1].

Temperature	18 °C				22 °C				ANOVA[2]					
Diet (%)	0	10	20	30	0	10	20	30	Temp °C	Diet (%)				Interaction
									18 vs. 22	0	10	20	30	
Trypsin activity (U mg protein^{-1})														
Pyloric caeca	6.030 ± 0.057	6.160 ± 0.226	6.245 ± 0.318	5.755 ± 0.841	6.920 ± 0.368	5.890 ± 0.099	6.020 ± 0.000	5.705 ± 0.304	NS	NS				NS
Foregut	0.054 ± 0.016	0.092 ± 0.011	0.069 ± 0.014	0.073 ± 0.003	0.035 ± 0.005^{a}	0.012 ± 0.000^{b}	0.017 ± 0.002^{b}	0.034 ± 0.005^{a}	>	*				*
Hindgut	0.012 ± 0.004	0.028 ± 0.004	0.038 ± 0.013	0.019 ± 0.004	0.014 ± 0.002	0.017 ± 0.000	0.017 ± 0.004	0.013 ± 0.000	>	*x*	*yz*	*z*	*xy*	NS
Lipase activity (U mg protein^{-1})														
Pyloric caeca	2.324 ± 0.472	2.893 ± 0.499	2.539 ± 0.002	2.894 ± 0.209	3.960 ± 0.020	3.119 ± 0.363	3.146 ± 0.126	3.086 ± 0.795	<	NS				NS
Foregut	1.534 ± 0.117	1.464 ± 0.241	1.388 ± 0.207	1.529 ± 0.059	1.646 ± 0.206	1.330 ± 0.124	1.587 ± 0.108	1.986 ± 0.152	NS	NS				NS
Hindgut	0.356 ± 0.075	0.488 ± 0.049	0.582 ± 0.035	0.716 ± 0.214	0.688 ± 0.083	0.785 ± 0.060	0.615 ± 0.000	0.913 ± 0.012	<	*y*	*yz*	*yz*	*z*	NS
α-Amylase activity (U mg protein^{-1})														
Pyloric caeca	0.044 ± 0.007	0.038 ± 0.006	0.039 ± 0.001	0.044 ± 0.001	0.074 ± 0.008	0.061 ± 0.004	0.072 ± 0.011	0.081 ± 0.004	<	NS				NS
Foregut	0.017 ± 0.000	0.010 ± 0.004	0.021 ± 0.001	0.024 ± 0.008	0.012 ± 0.003	0.016 ± 0.005	0.017 ± 0.001	0.029 ± 0.004	NS	*y*	*y*	*yz*	*z*	NS
Hindgut	0.008 ± 0.004	0.016 ± 0.002	0.013 ± 0.001	0.016 ± 0.003	0.012 ± 0.004	0.009 ± 0.000	0.011 ± 0.004	0.006 ± 0.001	>	NS				*

x, y, z For parameters with a significant effect of diet and no interaction, values without a common letter are different (*z* indicated the highest value; $P<0.05$).
a, b, c, d For parameters with a significant interaction, differences in diets are compared within each temperature ($P<0.05$), values without a common superscript are different.
[1] Mean ± SD, n = 2, three pooled fish per replicate, SD less than 0.001 are reported as "0.000".
[2] NS, non significant; *, $P<0.05$. For variables with a significant effects of temperature ($P<0.05$) and no interaction, < or > indicates whether the values measured at 18 °C was less than or greater than that measured at 22 °C.

Discussion

Growth

The results of the present trial indicate that under the conditions tested, the substitution of fish meal with up to 20% SESBM did not significantly affect the growth performance of fish. There were also no differences detected in any of the other parameters measured between 0% and 10% SESBM inclusion. However, the regression analyses demonstrated that an increasing SESBM inclusion had a negative effect on growth performance, nutrient retention and the apparent nutrient and energy digestibility of diets for Yellowtail Kingfish, regardless of water temperature. In a related study in Chapter 6 (Bowyer et al., 2013c) we found that the more refined soy protein concentrate (SPC) (20% SPC inclusion) was a better ingredient to use in diets for Yellowtail Kingfish as a substitute for fish meal. This is in agreement with studies on Japanese Yellowtail where SPC has been reported to be a better ingredient than SESBM for dietary fish meal substitution (Shimeno et al., 1992; Shimeno et al., 1995).

Despite this, all SESBM diets were well accepted by the fish. This is of interest because, irrespective of how available and digestible the ingredient is, if the diet is not palatable and feed intake is reduced, then the ingredient is of limited value (Glencross et al., 2007). Therefore, the evaluation of SESBM on the nutritional performance of Yellowtail Kingfish was not governed by an issue of low feed intake, but instead feed intake increased with SESBM inclusion, and as expected the feed intake was lower at 18 than at 22 °C. A similar result of increased feed intake and decreased feed efficiency was found when Atlantic Salmon were fed high levels of lupin meal (Carter and Hauler, 2000). The authors suggested that the increased feed intake was influenced by factors other than macro-nutrient intake. In the current study, the levels of crude protein in the SESBM diets were all relatively similar with only 1.18% difference between the crude protein diet values, and the dietary crude lipid level decreased slightly due to the substitution of fish meal with SESBM. The dietary gross energy levels remained similar, but the level of nitrogen-free extract increased with SESBM inclusion. The level of methionine and lysine in the current diets were based on the requirements for Japanese Yellowtail (1.11 and 1.78 g 100 g^{-1} of dry diet, respectively) (Ruchimat et al., 1997a; Ruchimat et al., 1997b), and were added to excess in the current diets to 1.40 and 3.35 g 100 g^{-1} of dry diet, respectively. Many of the essential crude and available amino acid requirements for *Seriola* spp. have not yet been determined (NRC, 2011), therefore, further validation is needed to determine if there were any amino acid deficiency in the current diets. Additionally, nutritional requirements have been shown to be intra- and inter-specific in fish (NRC, 2011), and using the nutritional requirement information from a surrogate species may over or underestimate the true requirement.

Nutrient retention

Apparent protein retention has been used as a determinant of amino acid requirements (Rodehutscord et al., 1997). In the current study, apparent protein retention values were reduced with the increasing inclusion of SESBM, with significant reductions observed at >10% SESBM inclusion. However, the whole body protein level was not significantly affected by dietary SESBM. On the other hand, the whole body total fat and gross energy levels were reduced with increasing SESBM inclusion, significantly so above 10% inclusion. This suggests that fish were utilising the dietary lipid for energy purposes and the contribution/utilization of energy from the nitrogen-free extract was minimal. In addition, the change in the efficiency of lipid digestion and absorption from the diet may have contributed to the reduction in whole body total fat and gross energy level.

Nutrient digestibility and digestive enzyme activity

The apparent nutrient and energy digestibilities were all affected by diet, with reductions in digestibilities as the level of SESBM increased. The low digestibility of SESBM-based diets may be ascribed to anti-nutritional factors and non-protein compounds such as carbohydrates present in the SESBM, or possible protein damage during the ingredient processing phase (Vohra and Kratzer, 1991; Francis et al., 2001; Peres and Lim, 2008). Trypsin inhibitors are an anti-nutrient factor commonly associated with soybean meal (5-8 mg g^{-1} trypsin inhibitor). The diets in this study were subjected to extrusion processes, which should have reduced the majority of trypsin inhibitors present in the soybean meal (Anderson and Wolf, 1995). Barrows et al. (2007) examined the effect of extrusion processing on trypsin inhibitor activity in SESBM ingredient and demonstrated that the activity was 5100 trypsin inhibitor units g^{-1} (TIU g^{-1}) before processing, and 2300 TIU g^{-1} after extrusion. The trypsin inhibitor activity was not measured in the present study, therefore it cannot be quantitatively stated that trypsin inhibitors did not have an effect on the trypsin activity. A review by Francis et al. (2001) stated that if trypsin inhibitors in plant protein ingredients are below 5 mg g^{-1} of ingredient, most cultured fish species are able to compensate for the presence of trypsin inhibitors (Olli et al., 1994). For example, in rainbow trout, fish can compensate for the effects of trypsin inhibitor activity by increasing digestive enzyme secretion, enhancing the absorption of protein in the distal parts of the intestine (Krogdahl et al., 1994). In the current study, the trypsin activity in the pyloric caeca and the foregut/midgut were not influenced by diet, but the activity in the hindgut did increase up to 20% SESBM inclusion, but then the levels decreased. However, the apparent dietary protein digestibility decreased linearly with increasing SESBM inclusion at both 18 and 22 °C. This suggests that the trypsin activity in Yellowtail Kingfish did not enhance protein absorption as it did in rainbow trout, and that the lower growth and protein digestibility at ≥10% SESBM inclusion was more likely due to the presence of other anti-nutritional factors or non-protein components.

The inclusion of carbohydrates in diets for marine fish is limited, often due to their lower digestibility and influence on digestive enzyme activities (Furuichi and Yone, 1982; NRC, 2011). Two of the main compounds of carbohydrate, oligosaccharides and non-starch polysaccharides (NSP), can hinder digestion in fish, particularly protein and fat digestibility (Bureau et al., 1998; Storebakken et al., 1998). Deng et al. (2010) demonstrated in Japanese flounder fed soy protein diets, the presence of NSP were the main cause of reduced protein digestibilities rather than soybean oligosaccharides. In the current study, the level of wheat starch in the diets decreased from 81.9 to 0% with increasing SESBM inclusion, while the level of nitrogen-free extract (NFE) increased from 21.3 to 27%. This would suggest that NSP would make up the majority of the increasing levels of dietary carbohydrate associated with increasing SESBM inclusion. Therefore, the presence of NSP is likely to be the reason for the corresponding, dose-dependent reduction in apparent dietary nutrient and energy digestibilities with increasing SESBM inclusion.

Lipid is the preferred non-protein energy source for most marine fish (Masumoto, 2002). In the current study, the apparent lipid digestibility decreased with increasing SESBM levels at both 18 and 22 °C demonstrating that Yellowtail Kingfish are susceptible to soy components that disrupt the absorption of fat. A reduction in the lipid digestibility of Atlantic Salmon fed diets containing SESBM was partly due to lipase inhibitor activity (Refstie et al., 2000; Refstie et al., 2001). Lipase inhibitors, such as the undigested high molecular fraction in soybeans can prevent the function of lipase by disturbing the binding of lipid droplets with bile salts, disrupting the action of digestive enzymes or movement of digesta through the digestive tract (Gargouri et al., 1984; Nguyen et al., 2011). However, in this study, there was no clear pattern in

lipase activity throughout the digestive tract in regard to the inclusion of SESBM. Therefore, it seems unlikely that the undigested high molecular fraction of SESBM was the major cause of the significant reductions in the apparent lipid digestibility. However, Deng et al. (2010) identified that the reduction in lipid digestibility in Japanese flounder fed soy protein isolate was caused by the presence of alcohol-soluble components, soya saponins and possibly isoflavones. The level of soya saponins in SESBM is around 0.6% (Brown et al., 2008).

Gut histology

Soya saponins are known to cause growth retardation, altered intestinal morphology and reductions in mucosal enzyme activity in the distal intestine in some fish species (Bureau et al., 1998). Knudsen et al. (2008) examined whether the inclusion of soya saponin concentrate into diets for Atlantic Salmon increased gut permeability and led to the onset of enteritis. The authors suggested that the soya saponin concentrate increased the intestinal epithelial permeability, but was not solely the cause of inducing enteritis. However, in combination with other anti-nutritional factors it may be a contributing factor to intestinal damage and reduced digestibilities. According to the criteria of Baeverfjord and Krogdahl (1996) and Urán (2008) the histological results in the current study did not show any signs of SESBM inducing enteritis in Yellowtail Kingfish. Despite this, the presence of saponins in combination with other ANF in SESBM may have contributed to the reduction in dietary apparent nutrient and energy digestibilities, leading to the poorer growth performances. However, further validation is required.

At similar experimental inclusions of soybean ingredients, extensive changes to Atlantic Salmon intestinal structure have been well documented (Olli et al., 1995; Refstie et al., 2001). Studies investigating the possible onset of soybean enteritis in species other than Atlantic Salmon have often demonstrated dietary-related changes. For example, Arctic charr (*Salvelinus alpines*) fed soybean products for 28 weeks, showed some changes to the midgut and hindgut cellular structure, and similar to the current study, the changes seen were not considered pathological (Olsen et al., 2007). When soybean ingredients were fed to rainbow trout (Romarheim et al., 2008) and common carp (*Cyprinus carpio*) (Urán et al., 2008) they also demonstrated reversible changes in the cellular intestinal structure. In addition to the current study, Bansemer (2011) and Bansemer et al. (In press; Appendix 4) also collected hindgut samples from the same Yellowtail Kingfish in this study, for more in-depth histological analysis and identified a range of alterations in the mucus layer thickness (increased erosion of the mucus layer as SESBM increased from 0 to 30%) and the type and number of goblet cells in response to increasing SESBM inclusion and water temperature (Bansemer, 2011; Bansemer et al., In press; Appendix 4). Concurrently, Bansemer et al. (In press; Appendix 4) also examined the digestive tract of similar sized Yellowtail Kingfish, held under identical experimental conditions but fed increasing levels of dietary SPC (20 to 40%), and reported no sign of mucus layer erosion or goblet cell abundance. Bansemer (2011) and Bansemer et al. (In press; Appendix 4) concluded that although sub-acute enteritis was not induced in SESBM fed fish, it was evident that the intestinal barrier was compromised. As a consequence the protective efficacy of the mucus layer may have been reduced when Yellowtail Kingfish were fed diets containing SESBM, and nutrient uptake may have been compromised. In the current study, the high goblet cell proliferation and the severe reduction of supranuclear vacuolisation were identified in all diets, including the control diet and in initial fish samples taken at the beginning of the trial. This suggests that these alterations appear to be independent of the dietary factors tested, but may have interfered with the nutrient uptake and contributed negatively to the health and growth performance of Yellowtail Kingfish. Therefore, further investigation into normal baseline Yellowtail Kingfish tissue, ideally from wild-caught

Yellowtail Kingfish, is warranted for future histological assessment. In addition, if the baseline values of wild Yellowtail Kingfish differ from Atlantic Salmon, then a Yellowtail Kingfish specific intestinal scoring system should be developed for the assessment of diet-induced enteritis in Yellowtail Kingfish.

Conclusion and Recommendations

In conclusion, under the conditions tested, the substitution of fish meal with SESBM significantly decreased the growth, feed efficiencies, nutrient retentions and apparent dietary nutrient digestibilities as SESBM inclusion increased. The inclusion of SESBM did not induce any gross symptoms of gastrointestinal histological problems in this study, but supplementary work within this study by Bansemer (2011) and Bansemer et al. (In press; Appendix 4), found histological alterations in regard to mucous layer erosion and goblet cell abundance in response to SESBM inclusion and temperature. Therefore, it is suggested that more research is required to first identify the normal histology and structure of the digestive tract from wild-caught Yellowtail Kingfish. Then if necessary, develop a new scoring system for identifying the signs of enteritis in cultured Yellowtail Kingfish. The inclusion of SESBM above 20% caused reductions in growth performance, and inclusions above 10% caused reductions in nutrient retentions and digestibility and eroded and altered the mucous layer of the digestive tract; therefore, the use of SESBM in diets for Yellowtail Kingfish is not currently recommended. Future use would be dependent upon clarifying the potential long term health problems associated with feeding of this ingredient on the erosion of the digestive mucus layer. In the interim, as identified in Chapter 6; the more refined soy protein ingredient, SPC, may be used as an alternative ingredient; 20% SPC inclusion was reported to be a suitable substitution level for fish meal in diets for Yellowtail Kingfish (Bowyer et al., 2013c). In addition, Bansemer et al. (In press; Appendix 4) did not report any negative effects of feeding SPC on digestive tract mucous layer thickness and goblet cell abundance. A longer term study would be beneficial to determine the effects of SESBM on growth performance, feed efficiency and health of Yellowtail Kingfish over an extended time period.

Chapter 6. **Investigate the maximum inclusion of soy protein concentrate as a fish meal substitute in juvenile Yellowtail Kingfish diets at optimal and suboptimal water temperatures.**

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Abstract

Juvenile Yellowtail Kingfish were fed four iso-nitrogenous and iso-calorific (digestible basis) experimental diets containing 0, 20, 30 or 40% soy protein concentrate (SPC) for 34 days at optimal (22 °C) and suboptimal water temperatures (18 °C) to measure the responses of growth, feed efficiency, apparent nutrient digestibility, gut histology and digestive enzyme activity to dietary manipulation. The substitution of fish meal with 20% SPC did not significantly affect growth. However, second-order polynomial regression analyses demonstrated that a negative impact on growth with increasing inclusion of SPC. In contrast, feed intake was not affected, but apparent feed conversion ratios were significantly increased (worse) and protein and energy efficiency ratios were also reduced above 30% SPC inclusion. Whole body moisture and fat levels were affected at >20% SPC inclusion. Nutrient digestibilities were not affected. Suboptimal water temperature decreased all growth performance and feed efficiency variables. In contrast, the protein and energy efficiency ratios were higher at 18 °C, while protein and energy retentions were not affected by temperature. Protein digestibility was influenced by temperature, and its increase at 18 °C suggests the influence of a slower gut transit time at the cooler temperature. No signs of mucus layer erosion were observed in the gut. This study indicates juvenile Yellowtail Kingfish can effectively utilise 20% SPC, regardless of water temperature. Palatability may limit further inclusion. It would be beneficial to run a trial over an entire grow-out period, in conjunction with attractants, to determine the effects of SPC inclusion on the growth, feed efficiency and health of Yellowtail Kingfish.

The information in this Chapter addresses part of Objective 4 (Subproject 8B): Determine the maximum inclusion levels of alternative protein and lipid sources to replace fish meal at optimal and suboptimal temperatures. The contents of the Chapter are published in Aquaculture: Bowyer J.N., Qin J.G., Smullen R.P., Adams L.R., Thomson, M.J.S., Stone D.A.J., 2013c. 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

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Introduction

Substitutions for dietary fish meal with more economical and widely available alternative plant protein ingredients have been studied extensively for many cultured fish species (Gatlin et al., 2007). The types of alternative protein sources with the ability to substitute for fish meal include poultry by-products and animal meals, concentrates from oilseeds and grain by-products, marine proteins from processing plants and fisheries by-catch as well as marine invertebrates and single-cell proteins (Aas et al., 2006; Gatlin et al., 2007; Salze et al., 2010; Stone et al., 2011; Zhou et al., 2011). Protein sources from soybeans have received the most attention due to their low cost, high quality and high annual yields. The highly refined soy protein concentrate (SPC) has a similar protein content (minimum 65% protein) and apparent dietary protein and amino acid digestibility to fish meal, but the amino acid profile is lacking in the essential amino acids, methionine and lysine, which are required for marine carnivorous fish species (Hardy, 2008). As SPC is a highly refined ingredient, most of the anti-nutritional factors such as protease inhibitors, lectins, saponins, antigenic proteins, phenolic compounds, oligosaccharides and phytates present in soybean meal have been removed during processing. However, the inclusion of SPC, particularly at high levels has caused reductions in feed intake due to a lowered palatability of the diet (Gomes et al., 1995; Medale et al., 1998; Blaufuss and Trushenski, 2012). The partial or total substitution of fish meal with SPC in diets for marine carnivorous fish species such as cobia (*Rachycentron canadum*), red seabream (*Pagrus major*) and Japanese Yellowtail (*Seriola quinqueradiata*) have found that the inclusion of taurine into diets was necessary to significantly improve fish production characteristics (Lunger et al., 2007a,b; Takagi et al., 2008; Takagi et al., 2010). Therefore, the substitution of fish meal with SPC requires the supplementation of methionine and lysine, as well as the addition of taurine, in diets substituting high levels or the entire fish meal component.

The nutritional value of an alternative protein ingredient is dependent on the ability of the fish to digest and absorb the diet (Allan et al., 2000) along with the type, amount and location of digestive enzymes in the gastrointestinal tract (Debnath et al., 2007). The main digestive enzymes responsible for breaking down nutrients are proteases, lipases and amylases. The capacity of nutrient utilisation and transport can be limited by the production of digestive enzymes or by the nutrient transport mechanisms (Lemieux et al., 1999; Debnath et al., 2007). Studies have found that the secretion levels of proteases, lipases and amylases do change in response to the level of ingredient inclusion (Lhoste et al., 1994). In addition, it has been reported that seasonal variations in digestive enzymes are directly correlated to water temperature in species such as Japanese Yellowtail (Kofuji et al., 2005), Atlantic Salmon (*Salmo salar*) (Einarsson et al., 1996), and pike perch (*Lucioperka lucioperka*) and bream (*Abramis brama*) (Gelman et al., 1984). Reductions in apparent dietary protein digestibility at winter water temperatures has been identified in starved Japanese Yellowtail (48 h) due to reduced pepsin activity in the stomach mucus (Kofuji et al., 2005).

The culture of the marine, carnivorous Yellowtail Kingfish (*Seriola lalandi*) is currently being undertaken in many regions of the world, including Australia, New Zealand, Japan, Taiwan and North and South America (Nakada, 2002; Fowler et al., 2003). The genus of *Seriola* belongs to the Carangidae family and includes the commonly cultured Mediterranean yellowtail (*Seriola dumerili*) and Japanese Yellowtail. Yellowtail Kingfish is a good candidate for aquaculture due to its fast growth (Booth et al., 2010a), reaching market size of 3-4 kg in 15-18 months (Fernandes and Tanner, 2008), and are a high quality sushi and sashimi product in Asian countries, particularly in Japan (Jirsa et al., 2011). However, the cost-effective production of

Yellowtail Kingfish requires the ability to incorporate alternative protein ingredients into the diets to replace expensive fish meals. There is very limited knowledge on how much fish meal can be substituted with soy products to grow juvenile Yellowtail Kingfish (Bowyer et al., 2013a; Chapter 5).The high level of protein replacement by a single ingredient is unlikely to be used in practical feed formulations, but a range of SPC inclusions were used in this study to explore the physiological response of fish to this ingredient.

Aim

The aim of this study was to investigate the potential of SPC as a substitute for fish meal at 0, 43.5, 65.2 or 87.0% fish meal substitution (0, 20, 30 or 40% SPC inclusion, respectively) based on the response of Yellowtail Kingfish growth performance, feeding efficiencies, nutrient utilisation, digestive tract histology and digestive enzyme functioning, at optimal and suboptimal water temperatures.

Materials and Methods

Experimental diets

Four experimental diets were formulated to contain 0% (the control diet) and 20, 30 or 40% soy protein concentrate (Table 6.1).

Table 6.1. Ingredient formulation of the four experimental diets fed to Yellowtail Kingfish.

Ingredients[1]	Diet (g kg^{-1} dry basis)			
	0% SPC	20% SPC	30% SPC	40% SPC
Herring meal	460.0	260.0	160.0	60.0
Soy protein concentrate	0.0	200.0	300.0	400.0
Wheat 14	90.0	90.0	90.0	90.0
Wheat gluten meal	73.9	71.2	71.2	71.2
Fish oil	93.4	107.4	114.5	121.5
Soy lecithin	5.0	5.0	5.0	5.0
Wheat starch	81.9	60.3	49.1	38.1
Poultry by-product meal	60.6	60.6	60.6	60.7
Blood meal	23.6	25.6	25.5	25.1
Choline chloride	3.0	3.0	3.0	3.0
Corn gluten meal	90.0	90.0	90.0	90.0
Vitamin/ mineral premix[2]	2.0	2.0	2.0	2.0
Vitamin C (Stay C)[3]	3.0	3.0	3.0	3.0
Vitamin E	0.4	0.4	0.4	0.4
Betaine	5.0	5.0	5.0	5.0
Monosodium phosphate	4.6	6.4	7.4	8.3
Taurine	3.6	5.4	6.2	7.1
Lysine	0.0	2.6	4.0	5.5
Methionine	0.0	2.1	3.1	4.1
Total	*1000.0*	*1000.0*	*1000.0*	*1000.0*

Diets formulated on a digestible protein and lipid basis.
Yttrium oxide was added to the diets at a rate of 200 mg kg^{-1}.
[1] Supplied by Ridley Aquafeeds, QLD, Australia.
[2] A proprietary product supplied by Lienert Australia Pty Ltd, Australia.
[3] Rovimix® Stay-C® 35, DSM Nutritional Products, Basel, Switzerland.

The diets were formulated to contain 41.5% digestible protein (50% crude protein) and 14.5% digestible lipid (20% crude lipid) and a gross energy level of 22 MJ kg^{-1} as described by Booth et al. (2010a). The amino acid composition of the diets were calculated using analysed amino acid ingredient values to satisfy the nutritional requirements for a carnivorous marine fish species (NRC, 2011) (Table 6.2). The levels of lysine and methionine were balanced in all diets according to the values for Japanese Yellowtail (Ruchimat et al., 1997a; Ruchimat et al., 1997b), and to reflect their content in the fish meal control diet (0% SPC). Based on the fact that fish meal often contains taurine in excess of 0.5%, plus the diets contained some animal products which also contain taurine (Gaylord et al., 2006), it was assumed that the dietary levels of taurine were between 0.25-0.5%. Therefore, the taurine level was over supplemented to contain 0.8% across all the diets, which was based on current commercial formulations for Yellowtail Kingfish (Dr Richard Smullen, personal communication) Yttrium oxide was added to all the diets (0.02% inclusion) as an inert maker for apparent digestibility determinations.

Table 6.2. Proximate composition and calculated amino acid composition of the SPC ingredient and the four experimental diets fed to Yellowtail Kingfish.

Item	SPC ingredient	Diet			
		0% SPC	20% SPC	30% SPC	40% SPC
Analysed proximate composition (dry basis)					
Dry matter (g kg^{-1})	879.0	938.2	938.5	949.9	935.0
Crude protein (g kg^{-1})	720.6	496.2	498.5	491.6	490.2
Crude lipid (g kg^{-1})	1.0	208.8	177.4	184.0	178.1
Ash (g kg^{-1})	63.0	82.1	66.9	60.2	50.9
NFE (g kg^{-1})[1]	215.4	199.8	241.3	250.9	262.6
Starch (g kg^{-1})	0.0	151.7	127.1	114.6	102.4
NSP (g kg^{-1})[2]	*215.4	48.1	114.2	136.3	160.2
Phosphorous (g kg^{-1})	n/a	14.8	12.7	11.6	10.0
Gross energy (MJ kg^{-1})	20.6	23.1	23.3	23.2	23.0
Calculated amino acids (g kg^{-1} dry basis)					
Arginine	57.2	29.0	30.1	30.6	31.2
Histidine	19.2	15.8	14.7	14.1	13.5
Isoleucine	33.5	24.3	23.5	23.2	22.8
Leucine	56.4	48.0	47.0	46.5	45.9
Lysine	46.7	33.5	32.4	31.9	31.4
Methionine	8.9	14.0	13.7	13.5	13.3
Phenylalanine	36.4	24.6	25.4	25.8	26.1
Threonine	29.6	21.9	21.0	20.6	20.1
Tryptophan	8.9	5.5	5.5	5.5	5.5
Valine	35.0	29.3	28.2	27.6	27.0
Σ IAA[3]	*331.8*	*245.9*	*241.5*	*239.1*	*236.7*
Taurine (g kg^{-1})	n/a	8.0	8.0	8.0	8.0

Diets formulated on a digestible protein and lipid basis.
NFE, nitrogen-free extract; NSP, non-starch polysaccharides.
IAA, indispensable amino acids.
[1] By difference: NFE = (100 – crude protein – total fat – ash).
[2] By difference: NSP = (NFE – starch).
[3] Σ IAA: total indispensable amino acid.
*NSP of SPC ingredient based on the assumption that SPC contains no starch.

The diets were produced, at the SARDI Australasian Experimental Stockfeed Extrusion Centre (Roseworthy, SA, Australia), as cooked-extruded slow sinking pellets (2.5 mm) using a Wenger X-85 (Sabetha, KS, USA). The production parameters were recorded for each diet. During diet production, the maximum levels reached for the following parameters were: extruder temperature, 85 °C; cone head pressure, 200 psi; feeder screw and extruder rotations, 16 and 300 rpm, respectively, cooler retention time, 11.3 min; and dryer temperature, 75 °C. Enough feed for approximately 1 week was maintained at 4 °C. Otherwise feeds were kept frozen at (-20 °C) prior to use.

Growth experiment

Experimental work was conducted at the SARDI, Aquatic Science Centre, West Beach, Australia. Experimental protocols followed the guidelines approved by the Animal Welfare Committee of Flinders University (E286). Yellowtail Kingfish juveniles (5-10 g per fish) were obtained from Clean Seas Tuna Ltd (Arno Bay, Australia) and were on-grown in 5000-L fibreglass tanks at the SARDI Aquatic Science Centre in ambient seawater temperatures (22 ± 1 °C) until the beginning of the trial. Fish were fed a commercial diet (Skretting, NOVA, 1.8, 2 and 3 mm sinking pellet; 50% protein, 15% lipid, Cambridge, Tasmania, Australia) prior to the commencement of the trial.

At the start of the experiment, the fish were anaesthetised using AQUI-S® (AQUI-S® New Zealand Ltd., Lower Hutt, New Zealand) at a concentration of 20 mg L^{-1} of seawater and individual weights were measured. A total of 384 fish (mean weight ± SD; 22.36 ± 0.05 g) were randomly assigned to sixteen 700-L experimental tanks (24 fish per tank), and the four experimental diets were randomly assigned to two replicate tanks at each water temperature. Fish were fed the experimental diets to apparent satiation twice a day for a period of 34 days. The following water quality parameters were monitored daily (mean ± SD) for both the 22 °C (optimal, 21.9 ± 0.5 °C) and 18 °C (suboptimal, 18.0 ± 1.2 °C) water temperature treatments during the growth trial: dissolved oxygen (6.3-7.2 mg L^{-1}), pH (7.8-8.0), and ammonia (0.20-0.21 mg L^{-1}) and weekly measurements of salinity (38 ± 0.5 ppt). At stocking, initial fish were randomly selected and analysed for proximate composition (three groups of six fish) and digestive tract histology (three groups of three fish).

The re-circulating system was housed in a temperature and photoperiod (14 h light : 10 h dark) controlled room. There were two identical re-circulating system units each consisting of a 780-L sump, an electric pump, sand filter, and a 780-L moving bed bio-filter, UV light, and eight 700-L cylindrical tanks. One re-circulating system unit contained a water chiller system which maintained the temperature in the suboptimal water temperature. The temperature of the other re-circulating system unit was maintained by heating ambient air. The ambient temperature of the incoming seawater was 24 ± 1 °C. At the end of the experiment, feeding was stopped 24 h before sample collection. All fish were euthanised by an overdose of AQUI-S® and measured for weight (nearest 0.1 g) and fork length (nearest 0.1 mm). Liver weight was used to determine hepatosomatic index (HSI). Foregut and hindgut samples were collected from the gastrointestinal tract of seven fish per tank for histological analyses. One cm sections at the anterior of the midgut and hindgut, immediately posterior to the ileal valves were taken, flushed with saline. Sections were then opened longitudinally and fixed in 10% neutral buffered formalin for 24 h, before being transferred to 70% ethanol for storage before analyses. The whole gut weights from three other fish per tank were individually weighed (wet weight) and used to determine the viscerosomatic index (VSI). The pyloric caeca, foregut/midgut and hindgut (combined tissue and mucus) were then separated from the whole gut and individually weighed (wet weight), and then snap frozen in liquid nitrogen and stored

at -80 °C until analysed for the determination of digestive enzyme activity. Another three fish from each tank were randomly selected, pooled and stored at -20 °C until analysed for whole body proximate composition. After the end of the growth trial a digestibility trial was carried out to determine the apparent digestibility coefficients (ADC) of the test diets, following the exact protocol as described in Chapter 5 and Bowyer et al. (2013a).

Preparation of gut histology and enzymatic assays

Gastrointestinal sections for histological determination were processed for paraffin histology. Foregut and hindgut samples were sectioned at 5 µm and stained with haematoxylin and eosin (H & E) and the structure was examined under light microscopy. The methods for examining the histology are described in Chapter 5 (Bowyer et al., 2013a) and Appendix 3, but briefly, the sections of the foregut and hindgut were assessed and classified for signs of acute enteritis according to the methods used by Baeverfjord and Krogdahl (1996) for Atlantic Salmon and scored on a scale of 1-5 according to Urán (2008).

The total soluble protein and specific trypsin, lipase and α-amylase enzyme activities for each individual gut section, i.e., pyloric caeca, foregut/midgut or hindgut, from three pooled fish per tank were analysed in triplicate at the temperature at which the respective tissue sample had been collected (i.e. either 18 or 22 °C). Analyses were conducted using spectrophotometric techniques and commercial enzyme test kits as outlined in Chapter 5 (Bowyer et al., 2013a). The specific enzyme activities were defined as the amount of enzyme that catalysed the conversion of one micro mole of substrate per minute per mg of protein (i.e. U mg soluble protein^{-1}) at the respective acclimation temperature.

Chemical analyses

Proximate composition analyses of diets, whole body and faeces were conducted according to methods in the British Pharmacopoeia Commission (2004) or DIN 51900-1 (2000). Fish collected for whole body proximate analyses were half thawed and then blended in a food processor. A sub-sample of tissue and faeces were freeze-dried to a constant weight at -50 °C to determine moisture. Diet samples were oven dried at 105 °C for 16 h to determine moisture. Crude protein (N × 6.25) was determined by the Kjeldahl method (BP A219 H Determination of Nitrogen, 2004) (Buchi Speed Digester K439 and FjelFlex K360, Switzerland and Mettler Toledo DL22 Food and Beverage Analyser, Switzerland). Crude lipid was determined using the method of Bligh and Dyer (1959). Ash was determined by a muffle furnace at 550 °C for 16 h. Gross energy content was determined using a bomb calorimeter (Parr Instrument Company, Moline, Illinois, USA) and calibrated with benzoic acid (DIN 51900-1, 2000). Faecal samples were not measured for ash content, due to an insufficient sample size. Diet and faecal samples were also measured for yttrium oxide using ICPOES analysis (University of Queensland, School of Land, Crop and Food Analytical Services), for the determination of apparent dietary nutrient digestibility coefficients.

Calculation of performance indices

Performance indices were calculated using the following formulae (De Silva and Anderson, 1995; Hardy and Barrows, 2002) where the weight (wt) unit was in grams. All calculations using fish weight and body composition were based on wet values and feed consumption and compositions were based on dry values.

Weight gain (g fish^{-1}) = final weight – initial weight

- Specific growth rate (SGR, % day^{-1}) = (ln(final weight) – ln(initial weight)) × 100/ days

- Apparent feed conversion ratio (FCR) = dry wt feed consumed per tank / wet wt fish gain per tank
- Hepatosomatic index (HSI, %) = (wet liver wt / final wet fish wt) × 100
- Protein efficiency ratio (PER) = fish wt gain / protein consumed
- Energy efficiency ratio (EER) = fish wt gain / energy consumed
- Apparent protein retention (PR, %) = (% protein of final body wt – % protein of initial body wt) × 100 / protein intake
- Apparent energy retention (ER, %) = (% energy of final body wt – % energy of initial body wt) × 100 / energy intake
- Apparent dietary nutrient digestibility coefficients (ADC, %) for protein, fat and energy, were calculated using the indirect method, following the equation and methods described by Cho et al. (1982):

$ADC\ (\%) = 100 \times [1 - (F/D \times D_y / F_y)]$

Where F = % nutrient or gross energy in faeces; D = % nutrient or gross energy in diet; D_y = % yttrium oxide in diet; F_y = % yttrium oxide in faeces.

Statistical analyses

All statistical analyses were carried out using SPSS (version 18, Chicago, Illinois, USA). The effects of dietary SPC inclusion (four levels: 0, 20, 30 or 40%) and water temperature (two levels: 18 or 22 °C) on growth performance, feed efficiency, somatic parameters, compositional data, nutrient retention and digestive enzyme activity were tested with two-factor ANOVA. If a significant interaction was detected between the main effects, then the main effects were ignored and multiple comparisons using Least Significant Difference (LSD) were made to examine the dependent relationship between the two independent factors (temperature and diet). If an interaction between the two independent factors was not significant, then the main effect was considered and the different factor levels were compared using a post-hoc Tukey's HSD multiple comparison procedure with the probability of difference set at 0.05. Assumptions of homogeneity of variances were checked using Levene's equal variance test. Where necessary, data were log transformed to satisfy the assumptions of normality and homogeneity of variances and all percentage data was arcsine transformed before analyses. The relationship between performance parameters and SPC inclusion were measured using a second order polynomial regression analyses and judged by the coefficient of determination (R^2) and *P*-value set at <0.05. All data were expressed as the mean of two replicate tanks (n = 2).

Results

Growth, survival and feed efficiency

All diets were equally accepted by fish and only two fish died during the course of the experiment. The final weight and SGR decreased at 18 °C ($P<0.001$) and were significantly affected by diet, decreasing above 20% SPC inclusion ($P\leq0.003$, Table 6.3). Second order polynomial regression analysis identified a significantly negative relationship between weight gain with increasing SPC inclusion at 18 °C ($R^2=0.879$, $P=0.006$, Fig. 6.1a) and 22 °C ($R^2=0.723$, $P=0.040$, Fig. 6.1b). Similarly, there was also a significant negative relationship between SGR and increasing SPC inclusion at 18 °C ($R^2=0.882$, $P=0.005$) and 22 °C ($R^2=0.711$, $P=0.045$). At 18 °C feed intake was significantly lower than at 22 °C ($P<0.001$), while the inclusion of SPC had no effect on feed intake ($P=0.050$, Table 6.3). However, a significant negative polynomial relationship showed a decreasing level of feed intake with an increasing level of SPC at 18 °C ($R^2=0.780$, $P=0.023$), but not at 22 °C ($R^2=0.451$, $P=0.223$); this was due to large variation between replicates. Apparent FCR was higher at 22 °C than at 18 °C ($P=0.017$), and was affected by SPC inclusion, being significant higher at 40% compared to all other diets ($P=0.001$, Table 6.3). A positive second order polynomial

relationship was apparent between apparent FCR and increasing SPC inclusion at 22 °C (R^2=0.963, P<0.001), but not at 18 °C (R^2=0.605, P=0.098); due to large variation between samples.

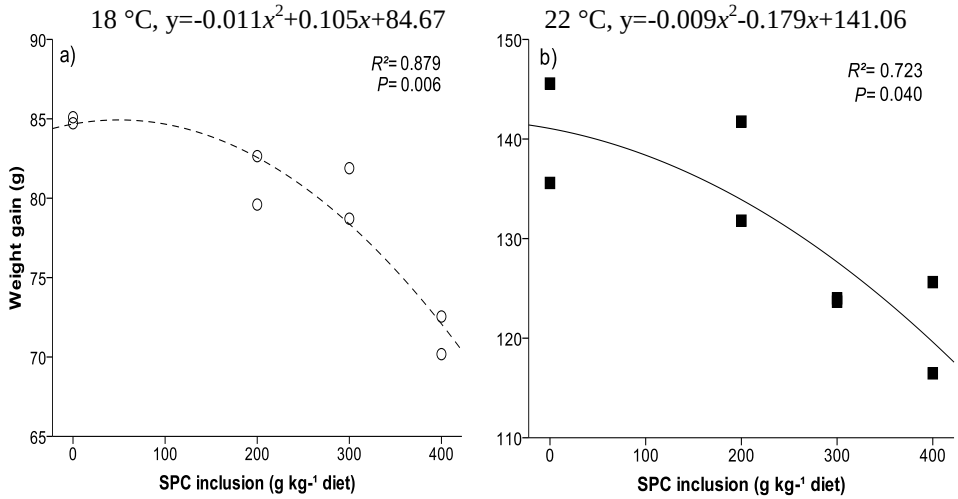

Figure 6.1. Weight gain of Yellowtail Kingfish fed increasing levels of SPC and held at either, a) 18 °C, or b) 22 °C (mean, n = 2).

Somatic parameters and nutrient retentions

The hepatosomatic index of the fish held at 18 °C (1.2%) was less than in fish held at 22 °C (1.4%, P=0.004), whereas the viscerosomatic index was affected in the opposite way, and was 7.2% at 18 °C and 6.5% at 22 °C (P=0.012). Neither of these variables was significantly affected by diet (P≥0.56). HSI values ranged from 1.1 to 1.5%, while VSI values ranged from 6.2 to 7.4%. The protein and energy efficiency ratios of fish held at 18 °C were higher than at 22 °C (P≤0.014), and were affected by SPC inclusion above 30% (P≤0.003, Table 6.3). Apparent protein and energy retentions were not affected by water temperature (P≥0.729), but were affected by SPC inclusion (P≤0.038, Table 6.3). Apparent protein and energy retentions at 30% SPC inclusion were similar to the control diet, but the energy retention was significantly reduced at 40% SPC inclusion compared to the control diet, while the apparent protein retention at 40% SPC inclusion was similar to the control diet.

Gut histology

The intestinal structure of the foregut and hindgut tissues consisted of a sub-mucosal layer, sub-epithelial connective tissues and epithelial mucosa with abundant villi (mucosal folds, both simple and complex). Mucosal folds (villi) were comprised of epithelial mucosal cells, with a central distinct lamina propria containing a mixed leucocyte population. There were no detectable signs of acute enteritis observed in sections of the foregut and hindgut from fish fed the control diet, the SPC diets, or in the initial fish samples. Normal histological appearance of the mucosal folds, lamina propria, and sub-epithelial mucosa was observed in sections of the foregut or hindgut from fish fed the control diet, the SPC diets, and the initial fish. However, in initial fish and in fish fed the control diet and diets with increasing levels of SPC there was a high infiltration of goblet cells in both the foregut, scoring 4-5, and in the hindgut, scoring 2.5-3.5. A reduction in supranuclear vacuolisation was also seen in the foregut of fish fed all dietary treatments, with scores of 3-4, which were higher than in initial fish (scoring 2.5). In the hindgut, the reduction in supranuclear vacuolisation was not as pronounced as it was in the foregut with scores of 1.5-2.5 in initial fish and fish fed all dietary treatments (including the control fish). Refer to Appendix 3 for detailed report.

Table 6.3. Growth performance, feed efficiency and nutrient retentions of Yellowtail Kingfish acclimated to two different water temperatures and fed diets differing in SPC inclusion level.

Temperature	18 °C				22 °C				ANOVA[2]				
Diet (%)	0	20	30	40	0	20	30	40	Temp °C*	Diet (%)			
									18 vs. 22	0	20	30	40
Growth performance													
Initial weight (g)	22.35 ± 0.01	22.39 ± 0.01	22.39 ± 0.05	22.42 ± 0.02	22.34 ± 0.09	22.34 ± 0.09	22.36 ± 0.06	22.30 ± 0.06					
Final weight (g)	107.25 ± 0.21	103.55 ± 2.19	102.70 ± 2.26	93.75 ± 1.63	162.90 ± 6.93	159.10 ± 6.93	146.20 ± 0.28	143.35 ± 6.43	<	*z*	*yz*	*xy*	*x*
SGR (% BW day^{-1})	4.62 ± 0.01	4.50 ± 0.06	4.48 ± 0.06	4.21 ± 0.06	5.85 ± 0.13	5.78 ± 0.13	5.53 ± 0.01	5.47 ± 0.14	<	*z*	*yz*	*xy*	*x*
Feed efficiency													
Feed intake (g fish^{-1}day^{-1})	1.64 ± 0.00	1.62 ± 0.04	1.56 ± 0.04	1.53 ± 0.00	2.84 ± 0.11	2.76 ± 0.13	2.57 ± 0.04	2.66 ± 0.13	<	NS			
Apparent FCR	0.93 ± 0.00	0.96 ± 0.06	0.94 ± 0.00	1.04 ± 0.02	0.97 ± 0.01	0.97 ± 0.00	1.00 ± 0.01	1.06 ± 0.00	<	*z*	*z*	*z*	*y*
Nutrient retention													
PER	2.17 ± 0.01	2.09 ± 0.11	2.17 ± 0.00	1.98 ± 0.05	2.08 ± 0.02	2.07 ± 0.00	2.04 ± 0.03	1.93 ± 0.00	>	*z*	*z*	*z*	*y*
EER	4.67 ± 0.02	4.48 ± 0.25	4.60 ± 0.00	4.20 ± 0.10	4.47 ± 0.06	4.43 ± 0.00	4.32 ± 0.06	4.11 ± 0.01	>	*z*	*z*	*z*	*y*
Apparent PR (%)	37.94 ± 0.86	37.39 ± 0.50	39.02 ± 1.51	33.71 ± 2.32	36.59 ± 0.13	38.07 ± 1.02	37.30 ± 2.05	35.10 ± 0.39	NS	*yz*	*z*	*z*	*y*
Apparent ER (%)	34.73 ± 0.50	31.76 ± 0.88	32.97 ± 1.90	31.52 ± 0.49	35.90 ± 1.22	35.41 ± 1.95	32.79 ± 5.83	27.20 ± 0.62	NS	*z*	*yz*	*yz*	*y*

Mean ± SD; n = 2.
Interaction non-significant for all variables.
[x, y, z] For parameters with a significant effect of diet and no interaction, values without a common letter are different (*z* indicated the highest value; $P<0.05$).
[1] SD less than 0.01 are reported as "0.00".
[2] NS, non-significant; *, $P<0.05$. For variables with a significant effect of temperature ($P<0.05$) and no interaction, < or > indicates whether the values measured at 18 °C was less than or greater than that measured at 22 °C.
SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; EER, energy efficiency ratio; PR, protein retention; ER, energy retention.

Whole body proximate composition

Whole body moisture content was greater in fish cultured at 18 °C (P=0.017) and was influenced by SPC inclusion above 20% (P=0.006, Table 6.4). A positive polynomial relationship showed that the moisture content increased with increasing SPC inclusion at 18 °C (R^2=0.927, P=0.001), but there was no significant relationship at 22 °C (R^2=0.692, P=0.052). Whole body protein level was lower at 18 than at 22 °C (P=0.049), but was not influenced by SPC inclusion (P=0.336). Whole body total fat was not affected by temperature (P=0.435), but a negative polynomial relationship showed that the crude fat level decreased as SPC inclusion increased (R^2=0.843, P<0.001). Whole body ash and gross energy were not affected by either water temperature (P≥0.172) or SPC inclusion (P≥0.366).

Apparent digestibility coefficients and digestive enzyme activities

Protein ADC was greater in fish held at 18 than at 22 °C (P≤0.001) and there was no effect of diet (P=0.581, Table 6.4). Total fat ADC ranged from 75.7 to 84.1% and gross energy ADC ranged from 57.9 to 63.0% across all treatments, but they were not affected by temperature (P≥0.494) or diet (P≥0.288).

Specific enzyme activities varied between the pyloric caeca, foregut/midgut and hindgut sections (Table 6.5). Trypsin activities in the pyloric caeca and hindgut were not influenced by water temperature (P≥0.127) or SPC inclusion (P≥0.064, Table 6.5). In the foregut/midgut the trypsin activity was higher at 18 °C compared to 22 °C (P<0.001), and was affected by SPC inclusion (P=0.004), and there was a significant interaction between temperature and SPC inclusion (P=0.012). The interaction resulted from a more pronounced increase in trypsin activity in fish fed the 20% SPC diet at 18 °C, which resulted in an unclear effect of SPC inclusion on the trypsin activity in the foregut/midgut section.

Lipase activities in the pyloric caeca and hindgut were lower at 18 °C (P≤0.001), while the activity in the foregut/midgut were not affected by temperature (P=0.089, Table 6.5). SPC inclusion had a significant effect on the lipase activities in the pyloric caeca and foregut/midgut (P≤0.003), but not in the hindgut (P=0.084). In the pyloric caeca there was a significant interaction between temperature and SPC inclusion level (P=0.014). Even though the effect of diet was significant in both the pyloric caeca and foregut/midgut, there was no clear logical pattern in the response of the lipase activity with diet.

α-Amylase activity in the pyloric caeca was lower in fish held at 18 °C (P<0.001), but water temperature did not affect the α-amylase activities in either the foregut/midgut or hindgut (P≥0.277, Table 6.5). SPC inclusion level had no effect on the α-amylase activities in the pyloric caeca or the hindgut (P≥0.232), but in the foregut/midgut as the inclusion level of SPC increased the α-amylase activity significantly increased (P=0.014).

Table 6.4. Proximate composition of Yellowtail Kingfish and apparent digestibility coefficients (ADC) for macronutrients and gross energy (GE) of diets fed to Yellowtail Kingfish acclimated to two different water temperatures and fed diets differing in inclusion level of SPC.

Temperature	18 °C				22 °C				ANOVA[2]				
Diet (%)	0	20	30	40	0	20	30	40	Temp °C	Diet (%)			
									18 vs. 22	0	20	30	40
Proximate composition (% wet weight)													
Moisture	72.01 ± 0.25	72.37 ± 0.14	72.84 ± 0.04	73.82 ± 0.43	70.70 ± 0.38	71.83 ± 1.27	72.84 ± 0.04	72.75 ± 0.37	>	*y*	*yz*	*z*	*z*
Crude protein	17.30 ± 0.28	17.60 ± 0.57	17.60 ± 0.57	16.90 ± 0.57	17.45 ± 0.07	18.10 ± 0.42	18.05 ± 0.64	17.90 ± 0.14	<	NS			
Crude lipid	6.59 ± 0.11	6.06 ± 0.05	5.35 ± 0.25	4.55 ± 0.01	7.11 ± 0.08	6.01 ± 0.82	5.25 ± 0.18	4.92 ± 0.78	NS	*z*	*yz*	*xy*	*x*
Ash	2.10 ± 0.22	2.28 ± 0.14	2.10 ± 0.02	2.06 ± 0.04	2.10 ± 0.15	2.18 ± 0.01	2.12 ± 0.17	2.25 ± 0.25	NS	NS			
GE (MJ kg^{-1})	7.04 ± 0.11	6.76 ± 0.15	6.80 ± 0.31	7.03 ± 0.04	7.68 ± 0.17	7.65 ± 0.36	7.29 ± 1.24	6.45 ± 0.13	NS	NS			
ADC (%)													
Crude protein	77.0 ± 1.4	78.0 ± 1.8	78.7 ± 0.7	77.1 ± 0.9	72.1 ± 0.9	71.5 ± 1.8	73.2 ± 0.9	72.3 ± 2.7	>	NS			
Crude lipid	76.1 ± 5.1	75.7 ± 8.9	84.1 ± 1.0	77.8 ± 2.2	77.4 ± 3.5	80.5 ± 0.5	80.5 ± 0.0	81.6 ± 2.9	NS	NS			
GE	62.1 ± 3.3	63.0 ± 1.4	61.3 ± 1.2	57.9 ± 1.2	62.2 ± 1.0	60.4 ± 0.0	60.7 ± 1.0	58.9 ± 8.6	NS	NS			

Mean ± SD; n = 2, three pooled fish per replicate.
Interaction were non-significant for all variables.
[x, y, z] For variables with a significant effect of diet and no interaction, values without a common letter are different (*z* indicated the highest value; $P<0.05$).
[1] SD less than 0.01 are reported as "0.00".
[2] NS, non-significant; *, $P<0.05$. For variables with a significant effect of temperature ($P<0.05$) and no interaction, < or > indicates whether the values measured at 18 °C was less than or greater than that measured at 22 °C.

Table 6.5. Trypsin, lipase and α-amylase activity in the pyloric caeca, foregut/midgut and hindgut of Yellowtail Kingfish acclimated to two different water temperatures and fed diets differing in SPC inclusion level[1].

Temperature	18 °C				22 °C				ANOVA[2]					
Diet (%)	0	20	30	40	0	20	30	40	Temp °C	Diet (%)				Interaction
									18 vs. 22	0	20	30	40	
Trypsin activity (U mg total protein^{-1})														
Pyloric caeca	6.050 ± 1.527	5.765 ± 0.884	7.490 ± 0.580	8.320 ± 0.3495	7.965 ± 0.092	7.230 ± 0.693	6.915 ± 0.262	8.010 ± 0.198	NS	NS				NS
Foregut	0.043 ± 0.005^{b}	0.080 ± 0.009^{a}	0.043 ± 0.015^{b}	0.061 ± 0.005^{ab}	0.018 ± 0.001^{a}	0.020 ± 0.008^{a}	0.021 ± 0.006^{a}	0.050 ± 0.006^{b}	>	*				*
Hindgut	0.012 ± 0.004	0.020 ± 0.008	0.017 ± 0.000	0.017 ± 0.009	0.008 ± 0.002	0.013 ± 0.003	0.015 ± 0.001	0.018 ± 0.002	NS	NS				NS
Lipase activity (U mg total protein^{-1})														
Pyloric caeca	2.201 ± 0.413	1.852 ± 0.195	2.532 ± 0.108	1.858 ± 0.063	4.529 ± 0.333^{a}	2.529 ± 0.596^{b}	3.044 ± 0.030^{ab}	2.801 ± 0.351^{b}	<	*				*
Foregut	1.549 ± 0.107	1.031 ± 0.074	1.216 ± 0.168	1.050 ± 0.116	1.962 ± 0.368	0.919 ± 0.019	1.653 ± 0.165	0.957 ± 0.005	NS	*z*	*y*	*z*	*y*	NS
Hindgut	0.433 ± 0.069	0.381 ± 0.017	0.367 ± 0.107	0.575 ± 0.087	0.669 ± 0.010	0.597 ± 0.068	0.578 ± 0.076	0.672 ± 0.008	<	NS				NS
α-Amylase activity (U mg total protein^{-1})														
Pyloric caeca	0.040 ± 0.011	0.057 ± 0.015	0.040 ± 0.000	0.041 ± 0.006	0.076 ± 0.010	0.074 ± 0.006	0.090 ± 0.006	0.085 ± 0.005	<	NS				NS
Foregut	0.017 ± 0.001	0.030 ± 0.001	0.026 ± 0.008	0.036 ± 0.004	0.019 ± 0.004	0.022 ± 0.004	0.034 ± 0.016	0.044 ± 0.006	NS	*y*	*yz*	*yz*	*z*	NS
Hindgut	0.009 ± 0.001	0.014 ± 0.001	0.010 ± 0.004	0.012 ± 0.002	0.010 ± 0.002	0.011 ± 0.001	0.014 ± 0.006	0.019 ± 0.007	NS	NS				NS

Mean ± SD, n = 2, three pooled fish per replicate.

[x, y, z] For parameters with a significant effect of diet and no interaction, values without a common letter are different (*z* indicated the highest value; $P<0.05$).

[a, b, c, d] For parameters with a significant interaction, differences in diets are compared within each temperature, values without a common superscript are different ($P<0.05$).

[1] SD less than 0.001 are reported as "0.000".

[2] NS, non-significant; *, $P<0.05$. For variables with a significant effects of temperature ($P<0.05$) and no interaction, < or > indicates whether the values measured at 18 °C was less than or greater than that measured at 22 °C.

Discussion

Growth performance, feed efficiency and gut health

This study has shown that SPC has the potential as a substitute for fish meal in diets for Yellowtail Kingfish. However, fish did respond differently to both water temperature and the inclusion level of SPC as reflected in the growth performance, feed efficiency, nutrient retentions and digestive enzyme activities. According to the scoring system developed for Atlantic Salmon by Baeverfjord and Krogdahl (1996) and Urán (2008), there were no signs of acute enteritis observed in the foregut or hindgut of the digestive tract of Yellowtail Kingfish fed diets containing SPC. However, an increase in goblet cell proliferation and a reduction in supranuclear vacuolisation in both regions of the digestive tract were observed in initial fish and fish fed all dietary treatments, regardless of SPC inclusion level. These histological alterations were similar to those reported in Chapter 5 (Bowyer et al., 2013a), and Appendix 3 where we investigated the dietary inclusion of solvent extracted soybean meal (SESBM) for Yellowtail Kingfish. As previously suggested these alterations may have interfered with the nutrient uptake and contributed negatively to the health and growth performance of Yellowtail Kingfish. Bansemer (2011) and Bansemer et al. (In press; Appendix 4) also examined the digestive tract of the SESBM fed Yellowtail Kingfish and reported signs of mucus layer erosion and an increase in goblet cell abundance with increasing SESBM dietary inclusion. In contrast, Bansemer et al. (In press; Appendix 4) did not report mucus layer erosion or an increase in goblet cell abundance in the digestive tract of Yellowtail Kingfish fed up to 40% SPC. Therefore, further histological assessment into normal baseline tissue from wild-caught Yellowtail Kingfish is necessary for future histological assessment. In addition, if the baseline values differ from Atlantic Salmon, then a Yellowtail Kingfish specific intestinal scoring system should be developed for the assessment of acute enteritis in Yellowtail Kingfish.

In this study, using a refined soy protein ingredient, we demonstrated that 20% SPC inclusion was a suitable substitution level for fish meal in diets for Yellowtail Kingfish. There were significant differences in the final weight and SGR of fish fed diets containing >20% SPC compared to the fish meal control diet, regardless of water temperature. The substitution of dietary fish meal with 20% SPC (43% fish meal replacement) in diets for Yellowtail Kingfish is in agreement with acceptable inclusion levels reported for Japanese Yellowtail. A study by Takii et al. (1989) found that a diet containing 20% SPC (44% fish meal replacement) with supplemental amino acids could be fed to Japanese Yellowtail (100-200 g) at 24 °C without affecting growth performance.

SPC has been reported to be a better ingredient than SESBM for dietary fish meal substitution for Japanese Yellowtail (Shimeno et al., 1992; Shimeno et al., 1995). Results from the current study, and a previously related study in Chapter 5 also suggest that this is the case for Yellowtail Kingfish. Based on the results for growth performance, feed efficiency and nutrient retentions and health concerns, reported in Chapter 5, it was concluded that SESBM was not a suitable substitute for fish meal in diets for Yellowtail Kingfish at 18 and 22 °C (Bowyer et al., 2013a). In contrast, in the current study, based on performance parameters, the inclusion of the more refined soy protein ingredient allowed for the substitution of fish meal at 20%.

The dietary feed intake by Yellowtail Kingfish in this study was significantly reduced at 18 °C, but was not significantly influenced by dietary SPC inclusion level (P=0.050). However, there was a numerical reduction in feed intake as SPC inclusion level increased. Further investigation, using non-linear regression analysis, indicated that the relationship between decreasing feed intake and increasing SPC inclusion

level was found to be significant at 18 °C, but not at 22 °C. This would suggest that it may be beneficial to include feeding stimulants into diets containing high levels of SPC for Yellowtail Kingfish to improve the feed intake, particularly at 18 °C. The inclusion of feeding stimulants such as certain amino acids (i.e. arginine, glycine, proline, valine, etc.), taurine, betaine, nucleotides and organic acids have been shown to have a positive influence on increasing feed intakes and possible improvements in digestive capabilities in marine carnivorous fish species (Kofuji et al., 2006; Kader et al., 2010; Sarker et al., 2012). Japanese Yellowtail fed a non-fish meal diet based on 58% SPC and supplemented with 4.5% taurine had similar growth rates to the fish meal control diet (Takagi et al., 2008). In the present study, the inclusion of 0.8% taurine and 5% betaine did not add any growth advantage to Yellowtail Kingfish fed the SPC diets compared to the control diet. Clearly, there is a need to further investigate the use of feeding stimulants in diets containing high levels of SPC to help improve feed intake.

Nutrient retention and body composition

Protein retention and essential amino acid retentions are the most sensitive indicators of an inadequate supply of amino acids (Rodehutscord et al., 1995). In this study, the whole body protein level was not influenced by diet. The apparent protein retention of fish fed diets containing SPC were similar to fish fed the control diet, while the PER was only affected above 30% SPC inclusion. In addition, there was also no significant effect of SPC inclusion level on apparent dietary protein digestibility in Yellowtail Kingfish. This indicates that Yellowtail Kingfish may have the ability to utilise and retain protein in diets containing moderate levels of SPC, assuming the amino acid composition is not limiting. Similarly, Day and Gonzelez (2000) found that turbot displayed constant apparent dietary protein digestibilities, but the protein efficiency ratio reduced when fish were fed diets with >50% fish meal substitution with SPC. The authors suggested that a proportion of dietary protein was used for catabolic processes instead of anabolic processes when SPC inclusion increased above 50%. This could explain the reduction of apparent protein retention in Yellowtail Kingfish fed diets containing high levels of SPC.

Reductions in the whole body crude lipid level were seen in Yellowtail Kingfish fed diets containing 30 and 40% SPC inclusion compared to the fish meal control diet. It is most likely that the fish were utilising a larger proportion of the dietary lipid for energy at the high SPC inclusion levels. As the level of SPC increased, the level of nitrogen-free extract (NFE) in the diets increased. In comparison to herbivorous or omnivorous fish, carnivorous are unable to effectively utilise and digest carbohydrates, particularly non-starch-polysaccharides (NSP) which are found in soy products (Wilson, 1994; Hemre et al., 2002; Stone, 2003). It has been reported that in diets for Japanese Yellowtail the optimum gelatinised starch level is less than 20% (Shimeno et al., 1979; Furuichi et al., 1986). Therefore, in this study it is likely that Yellowtail Kingfish were unable to utilise the dietary carbohydrate as a source of energy in the diets containing >20% SPC inclusion. This resulted in the decreased whole body lipid level and also contributed to the reductions in protein and energy efficiency ratios.

The whole body protein level was lower at 18 °C, which is reflective of the reduced final weight, SGR and feed intake at the low temperature. However, the efficiency ratio of both protein and energy was higher at 18 than 22 °C, suggesting that Yellowtail Kingfish may have the ability to utilise dietary protein more efficiently at low temperature. Reductions in water temperature can lead to significant changes to gut transit time and nutrient digestibility as it directly affects feed intake and enzyme activity (Hidalgo et al., 1999; Temming and Herrmann, 2001). In this study, the apparent dietary protein digestibility was significantly higher at 18 °C than at 22 °C. In

another study on Yellowtail Kingfish, Miegel et al. (2010) found that the gastric evacuation rate of digesta was 36-48 h at 13 °C and 12-16 h at 21 °C, which is similar to results found in Japanese Yellowtail (Nakada, 2000; Watanabe et al., 2001). Therefore, the colder water temperature subsequently decreased the feed intake in Yellowtail Kingfish, but the digestibility of the diets may have increased due to the prolonged period of food exposure to digestive enzymes and absorptive surfaces.

Digestive enzyme activity and nutrient digestibility

In this study, the impact of temperature on digestive enzyme activity was dependent on the section of the digestive tract. The activities were measured in the combined tissue and mucus of starved fish and it was found that the storage level of trypsin in the pyloric caeca was not influenced by temperature. In contrast, a study by Kofuji et al. (2005) reported that the trypsin activity in the pyloric caeca of 1 kg Japanese Yellowtail starved for 48 h was lower at 16-18 °C than at 22-25 °C. In the present study, temperature did not affect the trypsin activity in the pyloric caeca, which may be reflective of the twice daily feeding schedule. Pancreatic enzyme secretion is initiated when food arrives in the pyloric caeca or intestine and trypsin synthesis is initiated after feeding or trypsin secretion (Murashita et al., 2005). Therefore, fish may be synthesising trypsin more often due to the frequency of feeding and not allowing the storage levels of trypsin to deplete. In addition, since feed intake was reduced at 18 °C, not as much trypsin secretion is required, allowing for a similar trypsin storage level between the two temperatures. In the intestinal sections, trypsin activity in the foregut/midgut was higher at 18 °C, but there was no influence of temperature on the activity in the hindgut. In the study by Kofuji et al. (2005) and in a similar study on Yellowtail Kingfish by Miegel et al. (2010), the trypsin activity in the intestinal contents (representative of enzyme secretion) was higher at low temperatures than high temperatures. This suggests that the slower transit speed of digesta at the low temperatures led to a longer retention time of digestive enzymes in the chyme. Therefore, it is possible that the increase in protein digestibility at 18 °C is indicative of the longer exposure time of the chyme to trypsin activity.

The apparent dietary protein digestibility was not affected by the inclusion of SPC compared to the control diet. The protein digestibility of SPC could be related to the high level of processing this refined ingredient has undergone. The solvent extraction process uses ethanol and/or acidic water to remove water-soluble carbohydrates, soybean isoflavones, antigenic storage proteins and saponins, which are suggested to induce enteritis, but it does not remove phytic acid (Lusas and Riaz, 1995; Refstie et al., 2001). SPC also contains low levels of trypsin inhibitor activity with <4 mg g^{-1} crude protein, which is even lower than that found in SESBM (5-8 mg g^{-1} crude protein) (Brown et al., 2008). When trypsin inhibitors are below 5 mg g^{-1} in a variety of differently treated solvent extracted soybean meals, there is little effect on growth performance and protein digestibility in species such as rainbow trout (*Oncorhynchus mykiss*) (Rumsey et al., 1993). Trypsin activity observed in Yellowtail Kingfish in the present study showed a tendency to increase in the foregut and hindgut as SPC inclusion increased, which contrasts with the negative response reported for Japanese flounder with the inclusion of soy-based diets compared to fish meal-based diets (Deng et al., 2010). The increase in trypsin activity in Yellowtail Kingfish suggests that increasing dietary SPC inclusion may modify the digestive enzyme secretions. However, there was no observed effect on apparent protein digestibility in Yellowtail Kingfish in relation to increasing dietary SPC inclusion. In contrast, as reported in Chapter 5 the protein digestibility in Yellowtail Kingfish fed diets containing SESBM was significantly decreased compared to the control diet (Bowyer et al., 2013a). However, the reduction in digestibility in that study was suggested to be due to the level of non-starch polysaccharides interfering with digestion, not

trypsin inhibitors. Despite this, the results of the present study confirms that an SPC ingredient with a low level of protease inhibitors is a good quality protein source and is digestible at all levels of SPC inclusion tested under the conditions of this study.

Lipase is an important bile-salt activated enzyme in fish that is involved in the digestion and absorption of dietary lipids (Patton et al., 1975). In this study, the lipase activity was affected by temperature, and was lower at 18 °C in the pyloric caeca and the hindgut. In contrast, another study on Yellowtail Kingfish found the lipase activity measured in the chyme from the foregut and hindgut was higher at 13 °C than at 21 °C (Miegel et al., 2010). The reduction in lipase activity in the current study did not result in any reductions in apparent dietary lipid digestibility, and despite the increase in lipase activity found by Miegel et al. (2010) no improvements in lipid digestibility were observed. Thus, the relationship between enzyme activities and apparent digestibility in Yellowtail Kingfish is likely to be determined by multi-factors (Fountoulaki et al., 2005) and needs further investigation.

Carnivorous fish species are primarily fed on diets with high levels of protein and lipid and low levels of carbohydrates. Therefore, the role of α-amylase activity in carnivorous fish species is more ambiguous than in herbivorous and omnivorous fish (Hidalgo et al., 1999). The α-amylase activity has been detected throughout the digestive tract in a range of carnivorous fish species at low levels (Kuz'mina et al., 2003), including Asian seabass (*Lates calcarifer*) (Sabapathy and Teo, 1993), rainbow trout, gilthead seabream (Santigosa et al., 2008), turbot (Munilla-Morán and Stark, 1990) and larval Yellowtail Kingfish (Chen et al., 2006). In a comparative study by Hidalgo et al. (1999), the α-amylase activity in the digestive tract tissue of eel (*Anguilla anguilla*) was 98% less than the activity in carp (*Cyprinus carpio*). The low levels of α-amylase activity in the present study are in agreement with previous findings for carnivorous fish.

The level of α-amylase activity may be related to the amount of dietary carbohydrate (Cahu and Infante, 1994). The inclusion of plant proteins rich in crude starch (rapeseed, peas and corn meal) into the diets for rainbow trout and gilthead seabream did not induce changes in the α-amylase activity levels (Santigosa et al., 2008). However, in a study by Spannhof and Plantikow (1983), the α-amylase activity was reduced in rainbow trout fed diets containing crude starch, as the α-amylase was adsorbed to the crude starch crystal and inhibited starch hydrolysis. In the present study, the α-amylase activity in the pyloric caeca of Yellowtail Kingfish was not influenced by diet, but the levels were increased in the foregut/midgut and hindgut at 40% SPC inclusion compared to the control diet. These results agree with the findings reported for other carnivorous species such as common dentex (*Dentex dentex*) (Pérez-Jiménez et al., 2009), and gilthead seabream (Fountoulaki et al., 2005) where higher dietary carbohydrate contents increased the level of α-amylase activity. Pérez-Jiménez et al. (2009) and Fountoulaki et al. (2005) used either wheat gluten (NSP) and/or different forms of dextrin and cellulose as the sources for dietary carbohydrate. In the present study, the inclusion level of dietary starch based ingredients (wheat and wheat starch) decreased with increasing SPC inclusion, while the level of NFE increased. Therefore, it seems that NSP account for the majority of the increasing levels of dietary carbohydrate as the SPC level increases. However, further research is needed to investigate the effects of dietary carbohydrate type on digestive carbohydrase activities in Yellowtail Kingfish.

Conclusion and Recommendations

In conclusion, the substitution of fish meal with >20% SPC led to reductions in growth performance, feed efficiency and nutrient retentions. In the previous related study, reported in Chapter 5 (Bowyer et al., 2013a), it was concluded that the less refined soy ingredient, SESBM, was not a suitable substitute for fish meal in diets for Yellowtail Kingfish. In contrast, under similar conditions tested, the results reported in this Chapter have identified that the highly refined SPC ingredient is a suitable substitute for fish meal in diets for Yellowtail Kingfish at a 20% inclusion level (Bowyer et al., 2013c). Feed intake was numerically decreased above 20% SPC inclusion, particularly at the lower water temperature, so possible feeding stimulants may be beneficial to improve feed intake. No signs of acute enteritis were observed in the foregut or hindgut of the digestive tract of Yellowtail Kingfish fed diets containing SPC. Additionally, no symptoms of mucus layer erosion or changes in goblet cell abundance were observed (Bansemer et al., In press; Appendix 4). However, it would be beneficial to extend the duration of the trial to determine the effects of SPC inclusion on the growth performance, feed efficiency and health of Yellowtail Kingfish over an entire grow out period to gain a better understanding on physiological responses of fish to the inclusion of SPC in the diet.

Chapter 7. **Evaluate the enzyme activity of Yellowtail Kingfish under a range of water temperatures.**

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Abstract

A five week experiment was carried out with juvenile Yellowtail Kingfish *Seriola lalandi* to investigate the interactive effects of water temperature (21, 24, or 27 °C) and dissolved oxygen regime (normoxic vs. hypoxic) on the growth rate, feed intake and digestive enzyme activity of this species. Yellowtail Kingfish have a fairly narrow, parabolic, temperature range over which growth and FCE are optimised. Specific growth rate (SGR) was highest at 24 °C, regardless of oxygen regime, but the SGRs of the fish exposed to hypoxia at 21, 24 and 27 °C were 13, 20 and 17% lower, respectively, than the SGRs recorded for the fish reared under normoxic conditions. The digestive enzyme activity (i.e. trypsin, lipase and α-amylase) did not appear to be affected by dissolved oxygen concentration. At >24 °C, the reduction in growth rate was likely an increased metabolic oxidation of amino acids, and not the inhibition of the trypsin activity. Information on the effects of water temperature and dissolved oxygen on the growth and digestive capacity of juvenile Yellowtail Kingfish will further improve feed management decisions related to fish production in different environmental conditions.

The information presented in this Chapter addresses part of Objective 4 (Subproject 10): Determine the maximum inclusion levels of alternative protein and lipid sources to replace fish meal and fish oil, at optimal and suboptimal temperatures. The contents of this Chapter are published in Aquaculture Research: Bowyer J.N., Booth M.A., Qin J.G., Stone, D.A.J., 2013b. Temperature and dissolved oxygen influences growth and digestive enzyme activities of Yellowtail Kingfish (*Seriola lalandi*). Aquaculture Research 2013, 1–11 doi:10.1111/are.12146.

Introduction

Temperature is one of the most important extrinsic factors influencing metabolic rate in ectotherms, directly governing the rate at which biochemical and physiological processes proceed (Clarke and Johnston, 1999) as well as having a direct influence on fish activity (Jobling, 1982). The relationship between temperature and metabolic rate is strongly linked to the temperature dependence of enzymatic reactions (Hochachka and Somero, 2002). A reduction in growth rate at suboptimal temperature is often related to reduced activity or concentration of various enzyme activities at low temperature, ultimately manifesting in the reduced digestibility of feeds and availability of nutrients (Kofuji et al., 2005; Masumoto, 2005). In Yellowtail Kingfish *Seriola lalandi* (Valenciennes), digestive enzymes change rapidly in the early life stages (Chen et al., 2006). Recent work on this species indicated that the movement of digesta through the gut slowed significantly at 13 °C compared to 21 °C and there was a tendency for protease and lipase activities in the chyme to be higher at 13 °C than at 21 °C (Miegel et al., 2010). Miegel et al. (2010) considered that the elevation in enzyme activities at 13 °C was due to the slower movement of digesta along the gastrointestinal tract resulting in the accumulation of digestive enzymes. In Chapter 4, we reported the activities of trypsin, lipase and α-amylase in Yellowtail Kingfish changed significantly with water temperature (Bowyer et al., 2012b). Although temperature and dissolved oxygen can affect fish feeding and growth in captivity (Zheng et al., 2008; Nerici et al., 2012), it is unclear if the activities of digestive enzymes are affected by other abiotic factors such as dissolved oxygen.

The natural solubility of oxygen in water depends on its partial pressure ($P0_2$), water temperature and salinity (Campbell, 1990; Withers, 1992). Hypoxia is defined as any level of dissolved oxygen low enough to negatively impact the behaviour and physiology of an organism (Pollock et al. 2007), which usually occurs at $P0_2$ levels >6 kPa, while severe hypoxia occurs at $P0_2$ <6 kPa (Cook and Herbert, 2012). Most fish have some ability to cope with fluctuations in dissolved oxygen. If the hypoxic condition persists, the fish will eventually die (Fitzgibbon et al., 2007; Cook and Herbert, 2012). In the wild, fish species naturally seek out optimal environmental conditions for growth and survival (Zinichey and Zotin, 1987; Larsson, 2005). However, in an aquaculture environment, fish held in sea cages are unable to respond to suboptimal environmental conditions using their natural behaviour (Smith, 1976). For example, temporary oxygen depletion in wild environments usually results in fish moving to areas of higher dissolved oxygen (Pollock et al., 2007; Cook and Herbert, 2012). In a sea cage environment, localised depletion of dissolved oxygen which can result from heavy feeding events, overcrowding (i.e. high stocking density) or reduced water exchange must be endured. In addition, these problems become more critical for fish at higher water temperatures due to the low solubility of oxygen (Barnes et al., 2011). The long term effects of chronic hypoxia, regardless of the cause will lead to low voluntary feed intake, poor growth, high susceptibility to disease and infection and low survival (Kestemont and Baras, 2001; Fitzgibbon et al., 2007; Pörtner, 2010). High water temperature combined with hypoxia inside sea cages has caused acute mortality of juvenile Yellowtail Kingfish in SA, especially after episodes of heavy feeding (Mike Thomson, personal communication).

Yellowtail Kingfish are commercially cultured in sea cage operations in Australia and New Zealand (Jirsa et al., 2011). In SA, sea cage sites experience seasonal fluctuations in water temperature from 10 to 24 °C (Miegel et al., 2010), which expose fish to cyclical environmental stressors, leading to a possible enteritis (Sheppard, 2004). In addition, temperatures can reach up to 27 °C, which is thought to be supra-optimal with respect to the performance and health of juvenile Yellowtail Kingfish. Fluctuating water temperature is also a significant issue for culturing

Japanese Yellowtail and related species in Japan, where seasonal water temperatures range from 12 to 27 °C (Kofuji et al., 2005). Optimal or preferred growing temperatures for Yellowtail Kingfish are not well documented, but Pirozzi and Booth (2009) have suggested that the optimal temperature for growth of juvenile Yellowtail Kingfish is approximately 22.7 °C based on studies of routine metabolic rate. However, a recent study by Abbink et al. (2012) reported that the optimal temperature for this species was 26.5 °C, resulting in a dilemma on the range of optimal temperature for Yellowtail Kingfish.

Aim

The aim of the experiment was to measure the biological response of juvenile Yellowtail Kingfish to near optimal and supra-optimal water temperatures (21, 24 and 27 °C) when exposed to either normoxic or hypoxic conditions. Neither the biomass production nor economic losses associated with fluctuating environmental conditions in the Australian Yellowtail Kingfish industry are well understood. Therefore, some indication of the effect these factors have on growth and physiology of juvenile Yellowtail Kingfish would shed light on improving the management practices and feed development of this species and other similar species.

Materials and Methods

Experimental facility

Three seawater recirculating systems, described by Pirozzi and Booth (2009),were used for the different temperature treatments (21, 24 and 27 °C). Each system consisted of a 1500-L rectangular sump, a protein skimmer (Aquasonic Pty Ltd, Wauchope, New South Wales (NSW), Australia), a 500-L rotating biological filter (B-cell media) and a twin cartridge (25 µm) particle filter, a temperature control unit capable of chilling or heating the circulating water (OzSea Pty Ltd, Peats Ridge, NSW, Australia) and eight 200-L cylindrical, flat bottom, white polyethylene tanks. Water flow rate was 7–8 L min^{-1}, and top-up seawater was added to each system as required. Black plastic was wrapped around each of the tanks, a black plastic lid, which covered half the tank opening, was fitted to minimise external disturbance of fish and black 10 mm mesh was used to prevent fish escaping. Average salinity and pH were 30.7 g L^{-1} and 7.74 pH units, respectively, and total ammonia-nitrogen levels were ≤0.63 mg L^{-1}. Water temperature was recorded using data loggers submerged in each sump (Tinytag Model TG-4100 Aquatic 2, Hastings Data Loggers, Port Macquarie, NSW, Australia) and water temperatures (mean ± SD) recorded in each recirculation system were 20.55 ± 0.94 °C, 23.51 ± 0.74 °C and 27.03 ± 0.30 °C, respectively.

Management of dissolved oxygen in each system

High and low dissolved oxygen regimes were established after the temperature acclimation phase. A high dissolved oxygen saturation regime was maintained in each temperature unit by diffusing industrial grade oxygen (BOC Pty Ltd, Australia) into 4 of 8 experiment tanks within each system using a fine air-bubble diffuser. Oxygen flow was controlled using a combination of oxygen regulator (400 kPa Ezi-Flow Series O) and a flow-meter (Comweld Medical, Cigweld Pty Ltd, Preston Victoria, Australia). A low dissolved oxygen saturation regime was maintained in the remaining four tanks by reducing the flow rate of influent water. Flow rates in both high and low dissolved oxygen regimes were adjusted to ensure oxygen saturation levels remained >90% and <70%, respectively. No effort was made to control the natural variation in dissolved oxygen concentration due to feeding. Dissolved oxygen concentration and measured between 0830 h and 0900 h each day prior to feeding. To establish the minimum and maximum level of dissolved oxygen in each treatment,

the dissolved oxygen concentration was also monitored over extended periods (Table 7.1).

Table 7.1. Indicative range of dissolved oxygen and oxygen saturation concentrations recorded on two occasions in the different temperature systems over a 24 h period during the experiment.

Temperature	DO regime	Minimum	Maximum
Dissolved oxygen (mg L^{-1})			
21 °C	hypoxic	3.37 ± 0.49	5.26 ± 0.17
24 °C	hypoxic	2.89 ± 0.18	4.94 ± 0.31
27 °C	hypoxic	3.05 ± 0.11	4.91 ± 0.12
21 °C	normoxic	5.98 ± 0.35	7.32 ± 0.13
24 °C	normoxic	5.53 ± 0.45	7.27 ± 0.40
27 °C	normoxic	4.66 ± 0.17	6.16 ± 0.03
Oxygen saturation (%)			
21 °C	hypoxic	45.28 ± 5.80	68.62 ± 3.27
24 °C	hypoxic	40.91 ± 3.01	68.07 ± 4.01
27 °C	hypoxic	45.40 ± 1.48	72.54 ± 0.62
21 °C	normoxic	80.65 ± 3.39	97.04 ± 2.11
24 °C	normoxic	78.58 ± 6.86	100.96 ± 7.39
27 °C	normoxic	69.74 ± 2.53	92.04 ± 0.01

Mean ± SD; n = 4 replicate tanks per treatment.

Fish and stocking protocols

Juvenile Yellowtail Kingfish were obtained from Clean Seas Tuna Ltd (CST, Arno Bay, SA, Australia). Fish were fed a commercial aquafeed as described by (Booth et al., 2011). The measured proximate, amino acid and fatty acid methyl ester (FAME) composition of the diet is presented in Table 7.2. All diet analyses were conducted by Agri-Science Queensland Department of Employment, Economic Development and Innovation (Australia) according to specific in-house methodology or methods described by the AOAC (AOAC, 2005). The crude protein content of diet samples was determined by multiplying the measured nitrogen content (N) of each sample by a factor of 6.25.

Prior to stocking, juvenile fish were lightly sedated (10 mg L^{-1}, AQUI-S®, New Zealand Ltd. Lower Hutt, New Zealand) and individually weighed. Fish were then systematically distributed to each of 24 × 200-L tanks in groups of 15 until each tank contained 30 fish (8.7 ± 1.6 g). At this time a representative sample, consisting of 9 initial fish, were euthanised and immediately frozen (-80 °C) to provide tissue samples for digestive enzyme analysis. During the first week in the laboratory fish allocated to each system were carefully acclimated from the nursery temperature (18 °C) to their experimental temperature regimes of 21, 24 or 27 °C at a rate of 1-2 °C day^{-1}. At the end of the acclimation period, all fish were bulk-weighed to obtain a starting weight. Fish were fed to apparent satiation twice daily (0900 h and 1430 h) from Monday to Friday and once on Saturday and Sunday (0900 h). All fish were starved for 48 h prior to the end of the experiment, humanely killed with an overdose of anaesthetic (AQUI-S®) and individually weighed.

Table 7.2. Measured proximate, amino acid and fatty acid composition of a commercial aquafeed fed to Yellowtail Kingfish[1].

Proximate	(g kg^{-1}, dry basis)	Fatty acid	(g kg^{-1}, dry basis)
Dry matter	919.00	14:0	11.53
Ash	116.00	14:1n-5	-
Crude protein	556.88	15:0	0.86
Total lipid	252.37	16:0	34.92
NFE	74.75	16:1n-7	14.08
GE (MJ kg^{-1})	23.53	17:0	0.79
		17:1n-8	-
		18:0	7.08
		18:1n-9	17.66
Amino acids		18:1n-7	6.12
Alanine	26.59	18:2n-6	6.42
Arginine	30.82	19:0	-
Aspartic acid	36.42	18:3n-3	1.39
Cystine	5.73	18:4n-3	4.21
Glutamic acid	75.86	20:0	1.19
Glycine	27.10	20:1n-11	-
Histidine	15.99	20:1n-9	2.66
Isoleucine	19.39	20:1n-7	0.44
Leucine	34.14	20:2n-6	0.33
Lysine	31.35	20:3n-6	-
Methionine	11.95	20:4n-6	1.96
Phenylalanine	20.51	20:3n-3	-
Proline	25.68	20:4n-3	-
Serine	20.22	20:5n-3 (EPA)	32.89
Threonine	20.75	22:0	0.24
Tryptophan	6.34	22:1n-11	-
Tyrosine	16.05	22:1n-9	0.38
Valine	23.07	22:1n-7	-
		23:0	1.46
		22:4n-6	-
		22:5n-6	-
		24:0	-
		22:5n-3	4.52
		22:6n-3 (DHA)	20.43
		24:1n-9	0.83
		Total fatty acids	*172.37*

[1] Nutra Alpha (Skretting, Chile), 2.3 mm; ingredient list given as fish meal, fish oil, wheat, milling by-products, vegetable protein concentrates, crustacean meal, vitamins, minerals, astaxanthin.
Note: hyphen "-" indicates <0.01 g kg^{-1} fatty acid.
NFE, nitrogen free extract; GE, gross energy; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Collection and preparation of digestive enzymes

At the end, five fish were randomly chosen from every tank to obtain the whole viscera. The weight of the liver was then determined separately before the organs were recombined and stored at -80 °C. The whole viscera from all five fish from each replicate tank (n = 4) were partially thawed, pooled together and rinsed with distilled water before being blotted dry, weighed, and homogenised in four volumes of ice-cold phosphate buffered saline (PBS, pH 7.4) (*W/V*) using a hand-held homogeniser (CAT, X 120, Staufen, Germany). The suspensions were centrifuged at 3893 *g* for 30 min at 4 °C. Supernatants were kept in aliquots and stored at -80 °C until digestive enzyme analysis. The specific enzyme activity was analysed in triplicate at the temperature at which the respective tissue sample had been collected (21, 24 or 27 °C) and analysed spectrophotometrically using enzyme test kits. Trypsin (E.C 3.4.21.4) activity (Catalogue No. K771-100; Biovision, California, USA) was determined after reading the absorbance of samples at a wavelength of 405 nm at 0 and 1 h. Lipase (E.C 3.1.1.) activity was determined according to the method described by Furukawa, et al. (1982) with the absorbance of samples read at a wavelength of 412 nm after 10 and 20 min (Catalogue No. DLPS-100; QuantiChrom^{TM}, BioAssay Systems, California, USA). Amylase (E.C 3.2.1.1) activity was determined after reading the absorbance of samples at a wavelength of 412 nm at 0 and 20 min (Catalogue No. K711-100; BioAssay Systems, California, USA). Total protein was determined using a bicinchoninic acid protein assay kit (Sigma-Aldrich, Missouri, USA), with bovine serum albumin solution as the standard. The specific enzyme activities were defined as the amount of enzyme that catalysed the conversion of one micro mole of substrate per minute per mg of protein (i.e. U mg soluble protein^{-1}) at the respective acclimation temperature.

Performance criteria

Growth and related performance criteria were calculated using the average weight, length or organ weight of individual fish from each replicate tank using the following equations:

- Geometric mean body weight (GMBW, g) = (initial weight × final weight)$^{0.5}$;
- Specific growth rate (SGR, %day^{-1}) = (ln(final weight) – ln(initial weight)) × 100/ days;
- Relative feed intake (g kgBW$^{-0.8}$ day^{-1}) = g intake fish^{-1} (GMBW/1000)$^{0.8}$)/ day;
- Feed conversion efficiency (FCE, %) = weight gain × 100 / feed consumption;
- Hepatosomatic index (HSI, %) = liver weight × 100/ final fish weight

Unless otherwise noted, the average tank value for specific performance criterion was subsequently used in tables, figures and statistical analysis.

Statistical analysis

All statistical analysis was performed using SPSS (version 18, Chicago, Illinois, USA). The experiment was designed for evaluation using two-factor ANOVA. The fixed factors were temperature (three levels: 21, 24 or 27 °C) and dissolved oxygen regime (two levels: suboptimal, 50-70% saturation or optimal >90% saturation). Visceral weight was included as a covariate in the two-factor ANOVA for measuring digestive enzyme activity, but no significance was found and was therefore excluded from the final analyses. If a significant interaction was detected between the main effects, then the main effects were ignored and multiple comparisons using least significant difference were made to examine the dependent relationship between the two independent factors (temperature vs. dissolved oxygen). If there was a non-significant interaction between the two independent factors, then the main effect was separately considered and compared using a post-hoc Tukey's HSD multiple comparison procedure. The probability level of significant difference was set at 0.05. Levene's test was used to determine if the standard deviations (SD) among

treatments were similar prior to ANOVA. If treatment SDs were heterogeneous, appropriate transformations were applied to the raw data before ANOVA was conducted. The relationship between growth performance parameters and the different digestive enzyme activities (U mg protein^{-1}) were measured using either a Pearson's correlation coefficient (r) or a non-linear correlation coefficient (R). If a strong correlation was identified then a linear regression or a second order polynomial regression analysis was performed, and judged by coefficient of determination (R^2).

Results

Survival and growth

Overall survival was high in all treatments (Table 7.3) and mortality was restricted to only one or two fish. Survival was affected by temperature (P=0.004), but not by dissolved oxygen regime (P=0.734), and there was also a significant interaction (P<0.001). Multiple comparisons indicated that under normoxic conditions, survival was not influenced by temperature (P≥0.086). However, under hypoxic conditions, no mortality occurred at 21 or 24 °C (P=0.99), but at 27 °C, survival was reduced (P<0.001). Fish grew rapidly in this experiment, with SGR reaching 7.5% day^{-1} for fish at 24 °C under normoxic conditions. Two-factor ANOVA indicated that SGR was affected by dissolved oxygen regime (P<0.001), water temperature (P<0.001) and their interaction (P<0.001, Table 7.3). Multiple comparisons indicated that SGR peaked at 24 °C regardless of dissolved oxygen regime (P<0.001). However, under normoxic conditions the effect of increasing the temperature from 24 to 27 °C caused a reduction in SGR (P<0.001), whereas under hypoxic conditions there was no effect of increasing temperature on SGR (P=0.056).

Relative feed intake and apparent FCE

Relative feed intake was affected by dissolved oxygen regime (P<0.001), water temperature (P<0.001) and their interaction (P<0.001, Table 7.3). Multiple comparisons indicated that the interaction was driven by the smaller magnitude in relative feed intake between fish held under the normoxic or hypoxic regimes at 21 °C compared to the more pronounced effect at 24 and 27 °C.

Apparent FCE was affected by dissolved oxygen regime (P<0.001), water temperature (P<0.001) and their interaction (P=0.012, Table 7.3). Multiple comparisons found that under normoxic conditions the FCE was lowest at 27 °C, then improved at 21 °C, and was highest at 24 °C. While under hypoxic conditions, the same trend applied as for normoxic conditions, except there was no difference in FCE between 21 and 24 °C (P=0.576). The interaction on FCE was driven by the more worsening effect of increasing temperature from 24 to 27 °C under hypoxic conditions than under normoxic conditions.

Somatic parameters

HSI was not affected by temperature (P=0.061) nor by the interaction between temperature and dissolved oxygen regime (P=0.163), but HSI values were higher in fish held under normoxic conditions compared to those held under hypoxic conditions (P<0.001, Table 7.3). Visceral weight was affected by water temperature (P=0.001), dissolved oxygen level (P<0.001), and their interaction (P=0.037, Table 7.3). Multiple comparisons showed that the visceral weight of fish significantly increased with temperature regardless of dissolved oxygen regime, but the interaction was driven by the more pronounced increase in the visceral weight of fish held under the normoxic regime compared to the hypoxic regime at 24 °C.

Table 7.3. Growth performance, feed efficiency and hepatosomatic index, visceral weight of Yellowtail Kingfish reared at 21, 24 or 27 °C and at either hypoxic or normoxic dissolved oxygen conditions.

Temperature	21 °C		24 °C		27 °C		ANOVA[1]				
Dissolved oxygen	Hypoxic	Normoxic	Hypoxic	Normoxic	Hypoxic	Normoxic	DO	Temperature °C			Interaction
							Hypoxic vs. Normoxic	21	24	27	
Survival (%)	100.0 ± 0.0[a]	97.5 ± 1.7	100.0 ± 0.0[a]	98.3 ± 2.0	90.8 ± 3.1[b]	99.2 ± 1.7	NS	*			*
Initial weight (g)	8.75 ± 0.64	8.53 ± 0.28	8.25 ± 0.12	8.42 ± 0.31	8.43 ± 0.15	8.58 ± 0.17	NS	NS			NS
Final weight (g)	40.12 ± 1.99[b]	49.25 ± 1.34[c]	46.95 ± 1.17[a]	73.93 ± 2.68[a]	45.60 ± 0.65[a]	66.27 ± 2.69[b]	<	*			*
SGR (% BW day^{-1})	5.44 ± 0.10[c]	6.26 ± 0.07[c]	6.21 ± 0.06[a]	7.76 ± 0.23[a]	6.03 ± 0.09[b]	7.30 ± 0.18[b]	<	*			*
Relative FI (g $kgBW^{0.8}day^{-1}$)	16.49 ± 0.31[c]	19.32 ± 0.19[b]	19.49 ± 0.09[b]	25.88 ± 0.96[a]	20.52 ± 0.58[a]	26.95 ± 0.64[a]	<	*			*
FCE	131.08 ± 2.03[a]	132.38 ± 2.24[b]	132.00 ± 1.32[a]	136.98 ± 1.67[a]	111.70 ± 4.09[b]	120.80 ± 1.09[c]	>	*			*
HSI (%)	1.09 ± 0.02	1.18 ± 0.08	1.07 ± 0.09	1.34 ± 0.12	0.98 ± 0.03	1.20 ± 0.14	<	NS			NS
Visceral weight (g)	2.39 ± 0.20	3.48 ± 0.22[b]	2.78 ± 0.27	4.74 ± 0.42[a]	2.71 ± 0.14	4.27 ± 0.48[a]	<	*			*

Mean ± SD, n = 4.

[a, b, c] For variables with a significant interaction, differences in temperature are compared within each dissolved oxygen level (LSD test, multiple comparison), values without a common superscript are different (P<0.05).

[1] NS, non-significant; *, P<0.05. For variables with a significant effect of dissolved oxygen (P<0.05) and no interaction, < or > indicates whether the values measured at low DO was less than or greater than that measured at high DO.

DO, dissolved oxygen; SGR, specific growth rate; FI, feed intake; FCE, feed conversion efficiency; HSI, hepatosomatic index.

Digestive enzymes

Two-factor ANOVA indicated that trypsin activity was affected by water temperature (P<0.001) and the interaction between water temperature and dissolved oxygen regime (P=0.035), but not by dissolved oxygen regime alone (P=0.225). Multiple comparisons identified that under normoxic conditions the trypsin activity was different at each temperature, being most active at 24 °C, intermediate at 27 °C and lowest at 21 °C (P<0.001, Fig. 7.1a), whereas under hypoxic conditions the trypsin activity was highest at 24 °C (P=0.001, Fig. 7.1b) and similar at 27 °C (P=0.369) compared to fish reared at 21 °C. The reason for the interaction was that the trypsin activity was significantly more pronounced in fish held at 24 °C under normoxic conditions than under hypoxic conditions at either 21 or 27 °C.

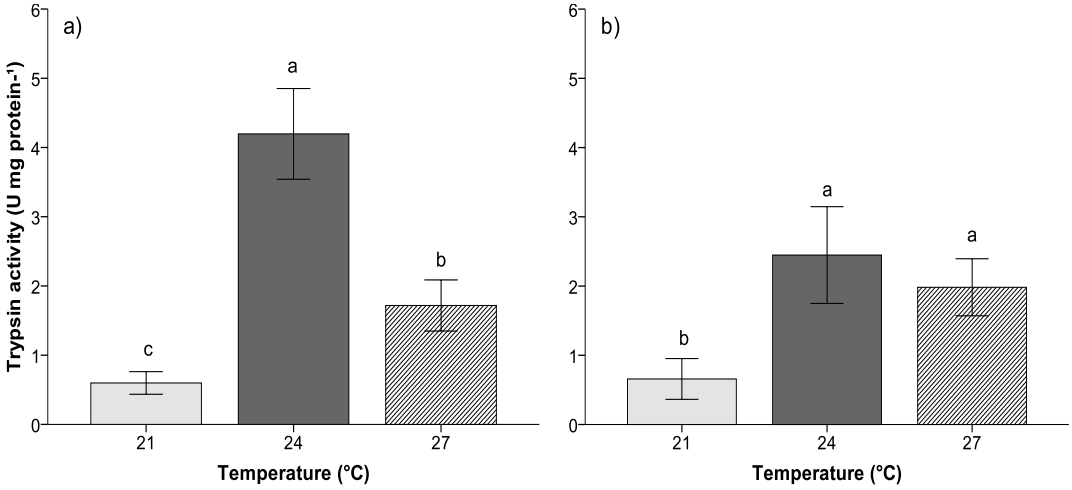

Figure 7.1. Trypsin activity in the whole gut tissue of Yellowtail Kingfish acclimated to different temperatures and held under either a normoxic (a) or hypoxic (b) regime. Values (mean ± SD, n = 4) without a common superscript are significantly different (P<0.05). *Initial trypsin activity values (mean ± SD, n = 10; pooled sample of 10 fish) at 21, 24 and 27 °C were 0.21 ± 0.11, 1.27 ± 0.29, and 1.29 ± 0.10 U mg protein^{-1}, respectively.

Two-way ANOVA indicated lipase activity was affected by water temperature (P<0.001), but not by dissolved oxygen regime (P=0.597, Fig. 7.2). Lipase activity was significantly lower at 21 and 27 °C than at 24 °C, regardless of dissolved oxygen regime. α-Amylase activity was affected by water temperature (P<0.001), and dissolved oxygen regime (P<0.001), and their interaction (P=0.006). The α-amylase activity increased with increasing water temperature, but the reason for the interaction was that there was a more pronounced increase in α-amylase activity in fish held at 24 °C under the normoxic conditions (Fig. 7.3a) than under hypoxic conditions (Fig. 7.3b). However, the α-amylase activity was statistically similar between 24 and 27 °C at normoxic conditions (P=0.543).

There was a positive linear correlation between SGR and trypsin activity at both normoxic (r=0.892, n = 11, P<0.001) and hypoxic regimes (r=0.850, n = 12, P<0.001, Table 7.4), with a higher SGR associated with higher the trypsin activity (Fig. 7.4a). There was a negative non-linear correlation between relative feed intake and trypsin activity at both dissolved oxygen regimes (Table 7.4). The relationship was best described by a quadratic equation which demonstrated that as feed intake increased the trypsin activity increased, but once feed intake reached the level corresponding to 24 °C, trypsin activity began to decrease (Fig. 7.4b).

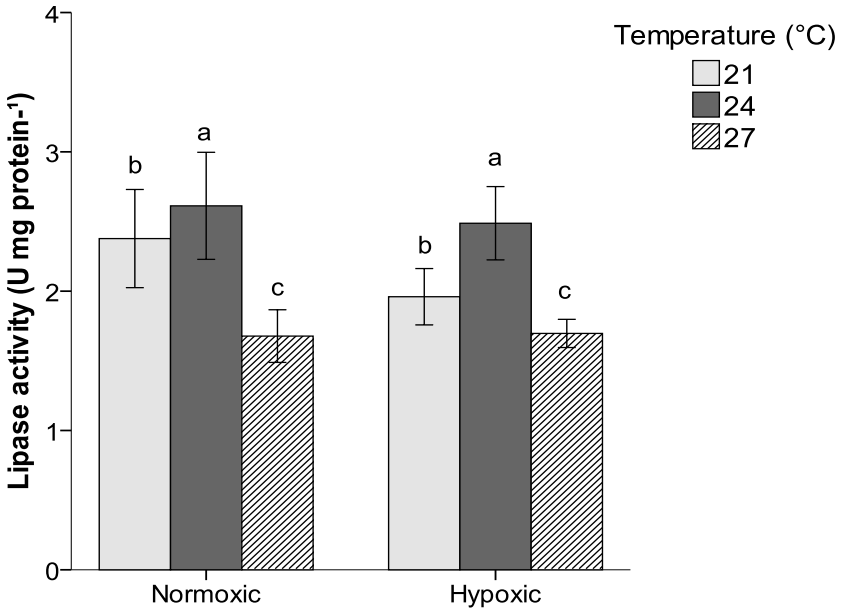

Figure 7.2. Lipase activity in the whole gut tissue of Yellowtail Kingfish acclimated to different temperatures and held under either a normoxic or hypoxic regime Values (mean ± SD, n = 4). displaying different letters within a dissolved oxygen level are significantly different (P<0.05). *Initial lipase activity values (mean ± SD, pooled sample of 10 fish) at 21, 24 and 27 °C were 1.65 ± 0.22, 2.40 ± 0.41, and 1.09 ± 0.07 U mg protein-1, respectively.

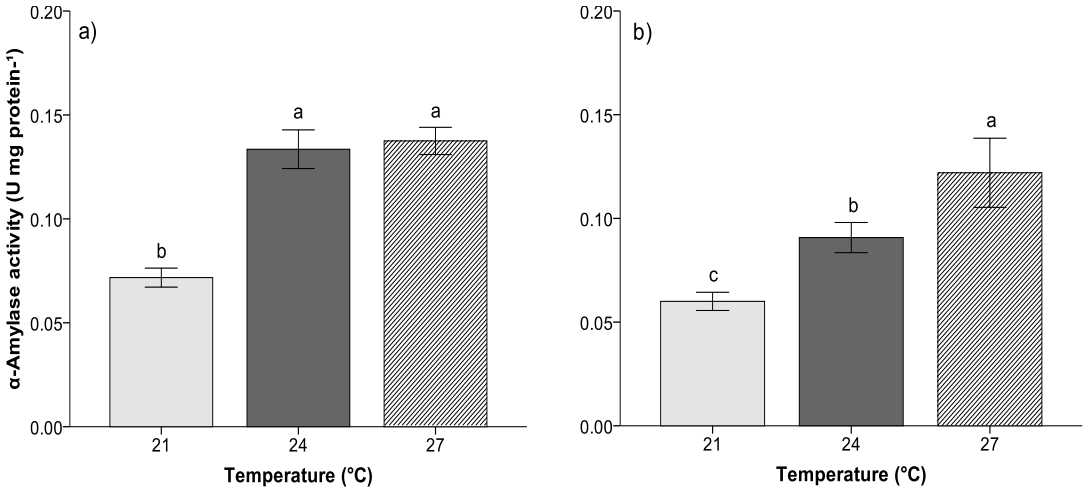

Figure 7.3. α-Amylase activity in the whole gut tissue of yellowtail acclimated to different temperatures and held under either a normoxic (a) or hypoxic (b) regime. Values (mean ± SD, n = 4) without a common superscript are significantly different (P<0.05). *Initial α-amylase activity values (mean ± SD, pooled sample of 10 fish) at 21, 24 and 27 °C were 0.059 ± 0.001, 0.125 ± 0.002, and 0.140 ± 0.004 U mg protein^{-1}, respectively.

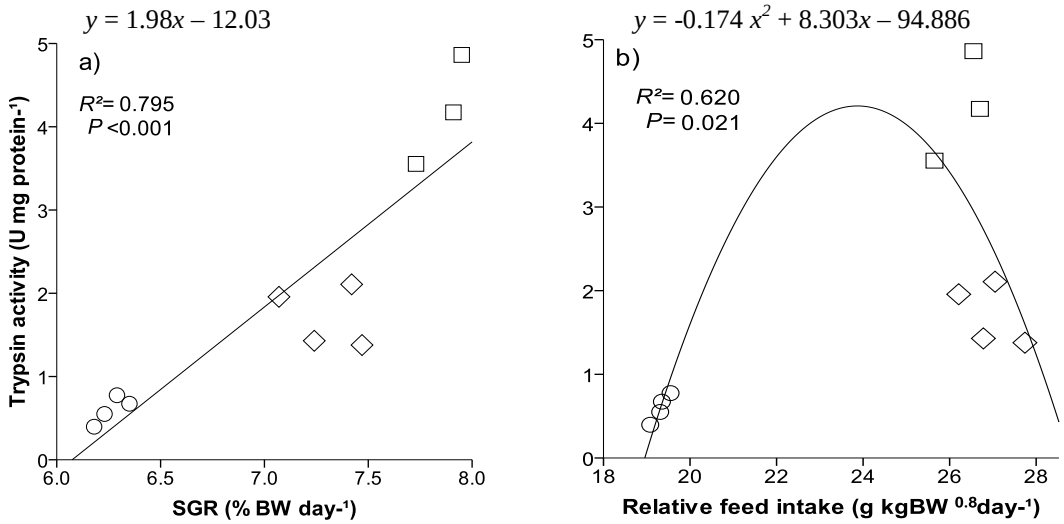

Figure 7.4. The relationship between trypsin activity and, a) specific growth rate, and b) relative feed intake in Yellowtail Kingfish under a normoxic regime. Trypsin activities were identified at the respective acclimation temperatures, 21 °C (○), 24 °C (□), and 27 °C (◊).

There was no correlation between trypsin activity and feed conversion efficiency at both dissolved oxygen regimes (Table 7.4). Under both dissolved oxygen conditions, the lipase activity was not correlated to SGR or relative feed intake. However, there was a positive linear correlation between lipase activity and feed conversion efficiency under normoxic conditions (r=0.836, n = 12, P=0.001), but there was no correlation under hypoxic conditions (R=0.701, n = 11, P=0.067) (Table 7.4). There were negative non-linear correlations between the α-amylase activity and SGR, regardless of dissolved oxygen regimes. Under hypoxic conditions, the relative feed intake and FCE were linearly correlated to α-amylase activity (r=0.887, n = 12, P=0.001 and r=-0.743, n = 12, P=0.006, respectively, Table 7.4). Under normoxic conditions, relative feed intake and FCE were non-linearly correlated to the α-amylase activity (Table 7.4).

Table 7.4. Non-linear correlation coefficient (R) between digestive enzyme activity and specific growth rate, relative feed intake and feed conversion efficiency of Yellowtail Kingfish at either normoxic or hypoxic dissolved oxygen conditions.

Enzyme	Trypsin			Lipase			α-Amylase		
	R	*P*	*n*	*R*	*P*	*n*	*R*	*P*	*n*
Normoxic									
SGR	*0.892	<0.001	11	0.545	0.205	12	-0.987	<0.001	12
Relative feed intake	-0.788	0.021	11	-0.619	0.114	12	-0.975	<0.001	12
FCE	0.665	0.097	11	*0.836	0.001	12	0.826	0.006	12
Hypoxic									
SGR	*0.850	<0.001	12	0.561	0.220	11	-0.814	0.007	12
Relative feed intake	-0.811	0.008	12	-0.691	0.075	11	*0.887	0.001	12
FCE	-0.174	0.871	12	0.701	0.067	11	*-0.743	0.006	12

*Pearson correlation coefficient (r) analyses data presented as the relationship was linear.
Data pooled for water temperature (21, 24 and 27 °C).
Enzyme activity units (U mg protein-1).
SGR, specific growth rate; FCE, feed conversion efficiency; relative feed intake (g kg BW0.8 day-1).

Discussion

Water temperature has a profound effect on the physiology of aquatic animals through regulation of biochemical reactions (Withers, 1992). In practical terms, Yellowtail Kingfish can probably be classed as eurythermal because it tolerates a wide range of rearing temperatures (e.g. 10-28 °C). However, our results indicate that the growth rate and feed conversion efficiency of Yellowtail Kingfish are maximised at 24 °C when oxygen concentrations are maintained at or near normoxic levels. Growth rate was also maximised at 24 °C when fish were held under hypoxic conditions. This suggests that 24 °C is close to the preferred or optimum temperature for this species and is in agreement with studies by Pirozzi and Booth (2009) and Abbink et al. (2012). Therefore, our results and the above literature evidence support the fact that Yellowtail Kingfish have a fairly narrow, parabolic temperature range over which growth and FCE are optimised.

Growth and feed utilisation

This study also clearly demonstrated that growth rate, FCE and relative feed intake of Yellowtail Kingfish were negatively affected under hypoxic conditions. The swimming behaviour of this species varied when held under hypoxic conditions, especially after the afternoon-feed when dissolved oxygen levels were at their lowest. At these times fish could be seen swimming rapidly in a circular motion with a wide mouth gape. This behaviour attenuated to normal swimming behaviour over time as dissolved oxygen returned to pre-feeding levels. This behaviour is different from that described by Cook and Herbert (2012), who found that swimming speed of Yellowtail Kingfish did not increase in response to inescapable hypoxia ($P0_2$ of 4 kPa ~20% air saturation), but that fish adopted a burst and rest swimming behaviour. This burst/rest behavioural response was associated with anaerobic stress, evident as increases in plasma lactate, glucose and cortisol (Cook and Herbert, 2012). The difference in behaviour of Yellowtail Kingfish in either study is probably best explained by the length or level of hypoxia. Some fish held under the 27 °C hypoxic regime refused to consume any pellets at the afternoon feeding. On these occasions the saturation levels were as low as 35-40%. To meet increased demand for oxygen many active fish such as Yellowtail Kingfish and tuna use ram ventilation as an alternate means of gill ventilation (Clark and Seymour, 2006; Fitzgibbon et al., 2008). Paradoxically for Yellowtail Kingfish, increasing levels of activity especially at high temperature result in increased respiratory demands and thus additional energy expenditure (Clark and Seymour, 2006; Booth et al., 2010a).

Critical oxygen levels can vary for species and with temperature (Dong et al., 2011). Recent research with mulloway (*Argyrosomus japonicas*, Temminck & Schlegel) showed their metabolic capacity was impaired by even mild hypoxic conditions (i.e. 75% saturation) and that their critical oxygen concentration is around 1.8 mg L^{-1}. At oxygen concentrations below this critical level mulloway became oxygen conformers and lost their ability to maintain their routine metabolic rate (Fitzgibbon et al., 2007). Barnes et al. (2011) recently investigated the effect of progressive hypoxia on Tasmanian strains of Atlantic Salmon (*Salmo salar*, Linnaeus) to assess if fish were able to regulate their metabolic rate at optimal and supra-optimal water temperatures. The authors found that critical oxygen threshold increased from 3.42 to 4.59 mg L^{-1} as temperature increased from 14 or 18 °C to 22 °C. In this study, although critical oxygen limits have not been determined for Yellowtail Kingfish, there were incidences of individual mortality in several tanks allocated to the 27 °C hypoxic regime where dissolved oxygen dropped to 2.6 mg L^{-1} (~39.5% air saturation). These fish were found with flared gill operculum and gill lamellae were anaemic. The duration of hypoxia was not accurately measured in our study and survival is likely to be affected

by duration as well as critical oxygen threshold. More research is needed to determine the critical oxygen threshold limits for juvenile Yellowtail Kingfish.

Digestive enzyme activities

Both trypsin and lipase activities in the fish gut tissue peaked at 24 °C, regardless of normoxic or hypoxic conditions. These peaks were generally well correlated with the growth and feed intake responses. Interestingly, the activity of trypsin at 24 °C was down regulated by almost 38% (i.e. 4 to 2.5 units) under hypoxic conditions compared to normoxic conditions, while the trypsin levels did not differ between oxygen conditions at either 21 or 27 °C. This suggests that there may be a link between water temperature and the level of dissolved oxygen with regard to protease enzymes such as trypsin. Such dramatic down regulation between normoxic and hypoxic conditions did not occur in lipase. The α-amylase activity in fish reared under hypoxic conditions decreased by 11.3, 32.0, and 16.4% at 27, 24 and 21 °C, respectively, compared to normoxic conditions. The α-amylase activity systematically increased with increasing temperature under hypoxic conditions, but under normoxic conditions α-amylase activity peaked at 24 °C and levelled off by 27 °C. This suggests that Yellowtail Kingfish may be up-regulating their α-amylase activity at higher temperature to ensure any energy available from glucose precursors such as dietary starch (i.e. carbohydrate) that is readily available for use in aerobic metabolic pathways. The increasing α-amylase activity with temperature is supported by the finding from Kuz'mina et al. (2003) who reported that the amylolytic enzyme complex in all six different fish species showed a high thermal stability.

The down regulation of enzymes involved in protein digestion due to seasonal variation in water temperature has been implicated in lower digestibility of feeds in Yellowtail Kingfish (Miegel et al., 2010) and Japanese Yellowtail (Kofuji et al., 2005). Indeed, Kofuji et al. (2005) showed that activities of trypsin and chymotrypsin from the pyloric caeca of starved fish (48 h) were reduced at low water temperatures (i.e. 16-18 °C vs. 22-25 °C). In contrast, the same study found that trypsin and chymotrypsin activities in the intestinal contents of fed fish were inversely related to water temperature; being more active at lower temperatures (i.e. 16-18 °C) than at higher temperatures (22-25 °C), possibly due to the slow chyme movement through the digestive system. This notion is generally supported by the results of Miegel et al. (2010) who found a water temperature of 13 °C significantly delayed the passage of chyme in 2 kg Yellowtail Kingfish compared to fish reared at 21 °C. In addition, Miegel et al. (2010) found that the protease and lipase activities in the intestinal tract of fed fish tended to be higher at the lower temperature. In this study, we did not measure enzyme activity in fed fish, but there was a strong correlation between trypsin activity and peaks in relative feed intake. These peaks coincided with temperature effects and may reflect the feeding history of each treatment (i.e. higher enzyme activity was stimulated by higher feed intake). Trypsin, but not chymotrypsin activity (both present primarily in the pyloric caeca) has increased with protein content of the diet (Kofuji et al., 2005), but as fish in this study were reared on the same feed throughout the trial, the effects of protein content *per se* on enzyme activity can likely be excluded. However, there is a reasonable difference in absolute protein intake among treatments, being higher at optimal temperature and this may explain the higher activity of trypsin and probably other proteases under these conditions. This is supported by Hardewig and van Dijk (2003) who found a correlation between feeding rate and enzyme activity in roach (*Rutilus rutilus*, L.), where fish acclimated to 4 and 12 °C had both lower feed intake and enzyme activity compared to those at the warmer temperatures of 20-30 °C.

Conclusion and Recommendations

In conclusion, the results of this study demonstrate that culture temperatures as little as ± 3 °C below or above 24 °C lead to decreases in the growth potential and aberrations in FCE, which at the cooler temperatures was possibly linked to a reduction in trypsin activity. Importantly, juvenile Yellowtail Kingfish appear to be somewhat tolerant of short term hypoxia, but chronic exposure to hypoxic conditions in sea cages will severely retard growth potential, worsen FCE and lead to increased mortality. The outcome of this trial emphasises the importance of providing normoxic conditions in sea cages for juvenile Yellowtail Kingfish and has wider implications for the water quality management and production outcomes for the sea cage farming of Yellowtail Kingfish. In addition, information on the effects of water temperature and dissolved oxygen level on the growth and digestive capacity of juvenile Yellowtail Kingfish will provide further information towards improving the feed management practices for this species under changing environmental conditions.

Chapter 8. **A literature review: the current status of knowledge of the nutritional requirements of Yellowtail Kingfish.**

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Abstract

With regards to the current status of knowledge of the nutritional requirements of Yellowtail Kingfish very little is known and we are dependent on information from related and unrelated species to formulate diets for this species. Apart from the excellent temperature and size dependent protein and energy requirement information for Yellowtail Kingfish provided by Booth et al. (2010), most of the other nutrient requirements specific to Yellowtail Kingfish have not been determined. Information for the closely related *Seriola* spp. and other marine species may be used to formulate diets for Yellowtail Kingfish in the interim. However, this must be done with caution. There have been many instances of large unexpected differences in the nutrient requirements on inter- and intra-specific levels with fish. To add to this, many of the nutrient requirement studies examined and discussed throughout the review were carried out under near optimal conditions. Suggested dietary requirements may differ considerably when fish are exposed to stressors in the challenging environment of the sea cage (Lygren et al., 2001). Taurine supplementation has recently received a great deal of attention with Yellowtail Kingfish and cobia. As pointed out, the closely related Japanese Yellowtail cannot synthesise taurine endogenously, therefore, the dietary provision of this sulphonated organic acid is conditionally essential. Levels of 5 to 8 g kg^{-1} diet have been recommended for Yellowtail Kingfish when dietary fish meal levels of at least 30% are used. More, when fish meal taurine levels are low, or when dietary fish meal substitution reduced dietary fish meal levels below 30%. Given that Yellowtail Kingfish are extremely fast growing and have an extremely high metabolic rate, their diets will need to be formulated with very high nutrient specifications using high quality, readily digestible ingredients. To add to this, without additional knowledge, and due to the current status of fish meal and fish oil supply and prices, one would assume that the diets for Yellowtail Kingfish will be more expensive than diets currently used for the culture of salmonid and other fish species that have had their nutritional requirements relatively well determined. Clearly, in the quest for more sustainable diets, more nutrition research is needed in the areas of essential amino acid requirements, supplemental taurine, levels, and ratios of DHA to EPA, and vitamins and minerals. CST is using information from this review to ensure that nutrient specifications of current Yellowtail Kingfish production diets are up to scratch, and also to formulate, and test, new diet formulations prior to their incorporation into Yellowtail Kingfish production. CST also plan to use the information provided from this review and project, in combination with previous work, as the foundation to attempt to push on-farm whole cycle feed conversion ratios (FCR) from above 2:1 to 1.7:1 for Yellowtail Kingfish.

The information presented in this Chapter addresses part of Objective 4 (Subproject 11). The literature review has been critically reviewed by Assoc. Prof. Masashi Maita (Tokyo University of Marine Science and Technology, Japan).

Introduction

Feeding contributes significantly to the cost of an aquaculture operation. In intensive aquaculture systems the cultured fish is dependent solely on the provision of an external dietary source for all life processes including growth, product quality, reproduction and health. Therefore, for the successful culture of a chosen species, whether it is in land-based or sea cage culture systems, it is essential to have a thorough understanding of its nutritional requirements, unfortunately, this is rarely the case for aquaculture. The nutrient requirements for some salmonid species such as Atlantic Salmon, *Salmo salar*, and Rainbow trout, *Oncorhynchus mykiss,* have been established reasonably well, however, this is not the case for most other marine species, such as Yellowtail Kingfish, *Seriola lalandi*. There has been limited research to establish the nutritional requirements of the closely related Japanese Yellowtail, *Seriola quinqueradiata*.

This review will summarise the current status of knowledge of the nutritional requirements for Yellowtail Kingfish. In the advent of knowledge gaps, which were vast, nutrient requirement information for the closely related Japanese Yellowtail or other non-related marine species was used as a guide. This is not an ideal situation, and while the approach may be useful in the short term, further detailed research is required to establish the nutrient requirements for Yellowtail Kingfish in relation to age, water temperature and the use of alternative protein sources to ensure the successful culture of this species.

Protein

Protein plays an important role in the growth, development and health of fish (Hepher, 1988; NRC, 2011). The protein requirement of fish can vary depending on the bioavailability of the protein source, its amino acid profile, the dietary energy level, and water temperature (Oliva-Teles, 2012). Protein bioavailability refers to the ability of an animal to metabolise and utilise the feedstuff. For example, in comparison to herbivorous fish, a carnivorous fish is unable to utilise plant proteins due to a lack of enzymes necessary for plant digestion (Oliva-Teles, 2012). It is also important to consider the ratio of indispensable to dispensable amino acids in feedstuffs, as a diet with a poor amino acid profile can result in reduced growth and anorexia (Tacon, 1992; Roberts, 2002). Providing fish with the optimal dietary energy level is necessary to ensure their energy requirements are met, avoiding deficiencies, which may result in incorrect protein metabolism (Masumoto, 2002).

Protein to energy ratio

Carnivorous fish such as Japanese Yellowtail (Masumoto, 2002), red sea bream (*Pagrus major*), and European sea bass (*Dicentrarchus labrax*) (Chou et al., 2001) require dietary protein levels in the range of 40-55%. A diet with deficient levels of energy will result in decreased growth due 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 excessive fat deposition due to an oversupply of protein (Masumoto, 2002; Oliva-Teles, 2012). The protein requirement is therefore based on a balanced protein-to-energy ratio (P:E). Yellowtail Kingfish, in comparison to other carnivorous species such as mulloway (*Argyrosomus hololepidotus*), barramundi (*Lates calcarifer*) and white grouper (*Epinephelus aeneus*) grown under similar conditions, have been shown to require a higher digestible protein (DP):digestible energy (DE) ratio, and a higher proportion of energy for maintenance (Booth et al., 2010a). Table 8.1 shows P:E values for Japanese and Mediterranean yellowtail (*Seriola dumerili*), and Yellowtail Kingfish under various temperature and growth stages.

Booth et al. (2010a) investigated the estimation of digestible (D) protein and energy requirements of Yellowtail Kingfish using two commercial diets (average of 339 g DP kg diet^{-1}, 81g D lipid kg diet^{-1}, 3.8 g D phosphorus, 11 MJ DE kg^{-1}). Booth et al. (2010a) reported the daily amount of dietary protein required for growth was up to five times greater for smaller 50 g Yellowtail Kingfish (22.8 g DP. kg^{-1} BW day^{-1}) in comparison to the DP maintenance requirements (4.2 g DP. kg^{-1} BW day^{-1}; Table 8.1). The results also showed as Yellowtail Kingfish grow, their dietary DP:DE ratio for optimal growth declines (Table 8.1).

Table 8.1. Estimate of daily digestible protein requirements of Yellowtail Kingfish at 21-24 °C[1].

Dietary protein requirements (g DP kg^{-1} BW day ^{1})	Fish weight (g)							
	50	100	250	500	750	1000	1500	2000
Maintenance req. (g DP. kg^{-1} BW day^{-1})	4.2	3.4	2.6	2.1	1.9	1.7	1.5	1.4
Growth req. (g DP. kg^{-1} BW day^{-1})	22.8	16.5	10.8	7.8	6.5	5.6	4.7	4.1
Total daily req. (g DP. kg^{-1} BW day^{-1})	27.0	19.9	13.4	9.9	8.4	7.3	6.2	5.5
DP:DE (g DP: MJ DE^{-1})	43.3	38.8	33.6	30.1	28.3	27.1	25.4	24.3

[1] Data from Booth et al. (2010a).

Table 8.2. Protein to energy ratio for Yellowtail Kingfish and other marine species.

Species	Temp (°C)	Fish size (g)	P:E ratio (g DP: MJ DE)	Reference
Yellowtail Kingfish	20-25	< 200	38	Booth et al. (2010a)
Yellowtail Kingfish	20-25	200-1000	31	Booth et al. (2010a)
Yellowtail Kingfish	20-25	>1000	24	Booth et al. (2010a)
Japanese Yellowtail	19	400-500	17-20	Watanabe et al. (1998, 1999, 2000 a,b,c)
Japanese Yellowtail	21-24	400-500	20-26	Watanabe et al. (1998, 1999, 2000 a,b,c)
Mediterranean yellowtail	21-26	490	28.9	Vidal et al. (2008)
Cobia[1]	28.9	5-80	34	Webb et al. (2010)
Atlantic Salmon[2]	4-13	100-600	15	Hillestad & Johnsen (1994)
Atlantic Salmon	13.9-9.5	1000-2500	19	Einen & Roem (1997)
Atlantic Salmon	13.9-9.5	2000-5000	16-17	Einen & Roem (1997
European sea bass	18	200	19-20	Dias et al. (1998)
Asian sea bass [1]	28-29	1.34-40	30.5	Catacutan & Coloso (1995)

[1] Energy = metabolisable energy.
[2] Protein = crude protein, Energy = gross energy.

Booth et al. (2010a) also investigated the predicted DP:DE ratio with varying fish size, feed intake and FCR for diets containing levels of 12, 15 or 18 MJ DE kg^{-1} which represent common digestible energy levels used in commercial aquafeeds in Australia (Pirozzi, 2010) and New Zealand (Moran et al., 2009). Booth et al. (2010a) recommended an intermediate DP:DE ratio for the three important growth stage; <200 g, 200-1000 g and >1000 g at 38, 31 and 24 g DP MJ-DE^{-1}, respectively. Booth et al. (2010a) also recommended practical dietary DP levels based on achievable DP levels for manufactured feed of ≤550 g kg^{-1}. For fish weighing up to 200 g, and based on FCR and the type of diet best suited to their feed intake constraints, was 456 g DP kg^{-1} and 12 MJ DE kg^{-1}. For fish growing between 200 and 1000 g, a diet containing 465 g DP kg^{-1} and 15 MJ DE kg^{-1} was recommended, while a diet containing 432 g DP kg^{-1} and 18 MJ DE kg^{-1} was recommended for fish >1000 g. Booth et al. (2010a) also suggested diets up to 570 g DP kg^{-1} could be manufactured to improve FCR for fish, however, these diets would need to contain expensive, highly digestible protein and energy rich ingredients and cost benefit analysis of growth, survival and fish health may need to be considered.

The optimal dietary protein to energy ratio for Japanese and Mediterranean yellowtail, two closely related species to Yellowtail Kingfish, is lower than those estimated for Yellowtail Kingfish (Table 8.2). Under similar conditions to the study by Booth et al. (2010a) the results from Watanabe et al. (1998, 1999, 2000 a, b, c) demonstrated Japanese Yellowtail, weighing 400-500 g, required a ratio of 20-26 g DP MJ-DE^{-1} at 21-24 °C. While the reported optimum levels of dietary DP for Japanese Yellowtail were reported to be more than 44% for juveniles, and dropped to 40-42% and 35 to 38% for 1 kg and larger adult fish, respectively (Watanabe et al. 1999). Vidal et al. (2008) reported the growth performance of Mediterranean yellowtail was best when fed a diet containing 28.9 g MJ-DE^{-1}. The observations of Watanabe et al. (1998, 1999, 2000 a, b, c) and Vidal et al. (2008) support the statement by Booth et al. (2010a) that compared to closely related species, Yellowtail Kingfish expend more energy on maintenance and growth. From the above findings by Booth et al. (2010a), we would recommend for CST to use similar protein to energy ratios to maximise the growth of Yellowtail Kingfish. However, as the study of this aspect of nutrition is limited for Yellowtail Kingfish, and as fish health has not been taken into account, further research may be required.

Similar species to Yellowtail Kingfish, such as cobia (*Rachycentron canadum*), European sea bass and red sea bream have also been shown to have similar protein requirements. When fed a diet containing 33.5 MJ-kg^{-1}, juvenile cobia (initial weight 33 g) have been shown to require a crude protein concentration of 44.5% for maximum growth (Chou et al., 2001), which is similar to Japanese Yellowtail (40-55%) (Masumoto, 2002). European sea bass and red sea bream require similar levels of protein to cobia, with 45-46% protein requirement for maximum growth (Oliva-Teles, 2000). This requirement has also been shown to increase to 55% for fry in species such as sea bass and sea bream (Vergara et al., 1996). Webb et al. (2010) estimated a level of 34 g protein MJ-ME^{-1} on a dry basis was necessary for maximum growth in juvenile cobia with an initial fish weight of 5 g. This is only slightly lower than the levels reported for Yellowtail Kingfish of a similar size range (Booth et al., 2010a; Table 8.1). The protein requirement for Atlantic Salmon is considerably lower than that for Yellowtail Kingfish and cobia (Table 8.2). Hillestad and Johnsen (1994) reported values as low as 15 g protein MJ-GE^{-1} on a dry basis for Atlantic Salmon growing from 100-600 g. Differences may be due to the fat and carbohydrate content in the diet, digestibility of nutrients, differing demands for nutrients for different life stages and differences in metabolic rate.

The level of protein necessary for optimal larval growth is not well understood. A study by Hilton et al. (2008) showed protein was not an important source of energy during embryogenesis in Yellowtail Kingfish. However, other fish species such as Atlantic cod (*Gadus morhua*) (Finn et al., 1995) and sea bass (Sivaloganathan et al., 1998) have shown increases in total protein during embryogenesis. It is thought that during larval development free amino acids (FAA) present in the yolk are the major energy source and for protein synthesis (Ronnestad et al., 1993, 1998, 1999). However, as marine finfish larvae have a relatively small yolk sac, amino acid reserves may be limited following absorption. Differences in the necessity for protein in larval diets may also vary due to broodstock nutrition prior to spawning (Hilton et al., 2008). Therefore, it is important to ensure the protein demands of the broodstock are adequate before the spawning season. And further research is warranted to investigate the protein requirements of broodstock and larval Yellowtail Kingfish.

Amino Acids

Amino acids are the building blocks for protein, and are necessary for numerous structural and metabolic functions. Fish are unable to synthesis all amino acids and,

therefore, these must be supplied in their diet (NRC, 2011). Fish have an absolute requirement for 10 amino acids, which are considered essential (Table 8.3) and two other amino acids cysteine and tyrosine, which are considered semi-essential (Wilson, 2002, Oliva-Teles, 2012). Recent studies have shown semi-essential amino acids or related compounds, such as taurine, may be essential or conditionally essential for some fish species, including Japanese Yellowtail (NRC, 2011). The amino acid requirement of a given fish species usually represents 30% of total protein requirement (Cowey, 1995). Ideally, a ratio of indispensable amino acids to dispensable amino acids of 50:50 is optimal for growth; however, ratios of up to 60:40 are still considered adequate for optimal growth performance for a range of marine species (Peres and Oliva-Teles, 2006, Oliva-Teles, 2012). There are values reported for Japanese Yellowtail (Table 8.3). Ruchimat et al. (1997a,b, and 1998) reported values for arginine histidine, lysine and methionine. Watanabe (2009) reported a more extensive list of amino acid requirements, as estimated from amino acid accretion in body protein, for Japanese Yellowtail. However, to our knowledge there is no published information on the amino acid requirements of Yellowtail Kingfish.

Table 8.3. Amino acid requirements of Japanese Yellowtail, and other marine and freshwater species.

Amino acid	Species	IBW	% diet	% crude protein[1]	Reference
Arginine	Japanese Yellowtail	-	1.43-1.63	3.4-3.88/42	Ruchimat et al., (1998)
	Japanese Yellowtail	-	-	3.7	Watanabe (2009)
	European sea bass	2.1	1.8	3.9/46	Tibaldi et al. (1994)
	Cobia	14	2.38	5.17/44	Zhoa et al. (2007)
Histidine	Japanese Yellowtail	-	0.65-0.85	1.49-1.95/43	Ruchimat (1998)
	Japanese Yellowtail	-	-	2.7	Watanabe (2009)
Isoleucine	Japanese Yellowtail	-	-	2.5	Watanabe (2009)
	Rainbow trout	47	0.7-1.4	1.5-2.8/33	Rodehutscord et al. (1997)
Leucine	Japanese Yellowtail	-	-	4.4	Watanabe (2009)
	Rainbow trout	49	1.1-1.4	2.3-2.9/34	Rodehutscord et al. (1997)
Lysine	Japanese Yellowtail	-	1.78	4.13/43	Ruchimat et al. (1997b)
	Japanese Yellowtail	-	-	5.0	Watanabe (2009)
	Cobia	1.25	2.33	5.3/44	Zhou et al., (2006)
Methionine	Japanese Yellowtail	-	1.11	2.56/43	Ruchimat et al. (1997a)
	Japanese Yellowtail	-	-	2.3 (met+cys)	Watanabe (2009)
	Cobia	11.6	1.19	2.64/44	Zhou et al. (2006)
	European sea bass	13.4	0.8-0.9	1.8-1.9/44	Tulli et al. (2010)
Phenylalanine	Japanese Yellowtail	-	-	4.3 (phen+tyro)	Watanabe (2009)
	Rainbow trout	12.7	0.7	2.0/35	Kim (1993)
Threonine	Japanese Yellowtail	-	-	2.7	Watanabe (2009)
	European sea bass	7.5	1.1-1.3	2.3-2.6/49	Tibaldi & Tulli (1999)
Tryptophan	Japanese Yellowtail	-	-	0.6	Watanabe (2009)
	Rainbow trout	50	0.1-0.2	0.3-0.4/33	Rodehutscord et al. (1997)
Valine	Japanese Yellowtail	-	-	2.9	Watanabe (2009)
	Rainbow trout	49	0.8-1.6	1.7-3.4/34	Rodehutscord et al. (1997)

Dash indicates data were not available. IBW = Initial body weight (g fish^{-1}).
[1] The values in the numerators are requirements as percentage of dietary protein and the denominators are the percentage of crude protein in test diets.

In other related species, such as cobia and European sea bass, the requirements for several amino acids have been shown to be higher than Japanese Yellowtail. In

juvenile cobia (initial weight 14 g) the optimal requirement for arginine for maximum weight gain was 2.38% dry basis (db) in the diet, or 5.17% of the dietary protein (Table 8.2) (Zhao et al., 2007). In Atlantic Salmon the requirement of arginine was also slightly higher than Japanese Yellowtail at 1.6% db (4.1% of dietary protein) when fed a diet containing 40% crude protein (Lall et al., 1994). Zhou et al. (2007) reported the lysine requirement for juvenile cobia was 2.33% db of the diet (5.30% db dietary protein). The lysine requirement for optimal growth of fingerling Atlantic Salmon has been reported to be 1.99% of the diet, or 3.98% of the dietary protein (CP, 50%) (Anderson et al., 1993), and are similar to the lysine requirement for as Japanese Yellowtail. The dietary requirement for methionine for juvenile cobia (initial weight 11.61 g, 44% CP and 16% lipid diet) was 1.19% of the diet (db), in the presence of 0.67% cysteine (2.64% of dietary protein (db)) (Zhou et al., 2006). In contrast, Tulli et al. (2010) showed European sea bass (initial weight 13.4 g, dietary CP 44%) required 0.8 to 0.9% methionine in the diet in the presence of 0.4% cysteine (1.8-1.9% of dietary protein). The methionine requirement for fish can be influenced by the amount of cysteine present in the diet (NRC, 2011). Dietary cysteine has been estimated to replace 40 to 60% methionine in various species (NRC, 2011; Wilson, 2002). Estimates for replacement of methionine with cysteine include 60% for channel catfish (*Ictalurus punctatus*) (Harding et al., 1977), 42% for rainbow trout (Kim et al., 1992), and 40% for red drum (*Sciaenops ocellatus*) and hybrid striped bass (*Morone saxatilis)* (Moon and Gatlin, 1991). Similarly, tyrosine has been shown to replace between 40 and 60% phenylalanine in various fish species (NRC, 1993; Guillanme et al., 1999).

In the absence of any specific amino acid data for Yellowtail Kingfish, and with references to the data contained in Table 8.3 and based on existing knowledge for Japanese Yellowtail, we would cautiously recommend the essential amino acid requirements (as a % of dietary protein) reported by Watanabe (2009) in Table 8.3 Alternately, the ideal amino ratio for teleost fish (NRC, 2011) could be used to calculate the requirements (Table 8.4).

Table 8.4. Ideal amino acid profile for teleost fish[1].

Amino acid	Teleost Fish Ideal amino acid profile (Referenced to lysine %)	Yellowtail king fish amino acid requirements [1] (% dietary protein)
Lysine (Reference)	100	4.13
Arginine	82	3.39
Histidine	35	1.45
Isoleucine	54	2.23
Leucine	70	2.89
Methionine	38	1.57
Methionine + Cysteine	54	2.23
Phenylalanine	55	2.27
Tyrosine + Phenylalanine	90	3.72
Threonine	56	2.31
Tryptophan	14	0.58
Valine	61	2.52

[1] Table adapted from NRC (2011). The suggested Yellowtail Kingfish amino acid requirements were calculated using the ideal amino acid profile for teleost fish in combination with the reported lysine requirement (4.13% as a percentage of dietary protein) for Japanese Yellowtail.

The majority of the studies investigating the amino acid requirements previously mentioned have used juvenile fish. Therefore, as the fish grows the need for these amino acids will reduce as their metabolic rate will decrease. Clearly further research is needed to determine the amino acid requirements of Yellowtail Kingfish.

Taurine

Animal proteins are rich sources of taurine, a sulphonated organic acid essential for important physiological roles such as gut development, mucus production, cellular osmoregulation, anti-oxidative defence, bile production and fat digestion and development of visual, muscular and neural systems in fish (Fang et al., 2002; Oliva-Teles, 2012). Taurine is a derivative of cysteine, and is synthesised by the transsulfuration pathway (Goto et al., 2001).

Fish meal is the preferred protein sources in aquafeeds. However, with the price of this commodity continuing to rise, more sustainable and economical alternatives such as plant proteins are being used (Naylor et al., 2000). The replacement of fish meal with plant proteins can reduce dietary taurine and interfere with normal growth, feed efficiency and induce abnormal physiological conditions, such as anaemia and discoloration of the liver, termed green liver syndrome in certain aquatic animals (Takagi et al., 2008; Takagi et al., 2010). Fish meal replacement is a common practice in Yellowtail Kingfish diets and fish meal levels may be as low as 20% in some circumstances. In Japan, fish meal levels in diets for Japanese Yellowtail are maintained at levels of 30% dietary inclusion (Dr Masashi Maita, personal communication).

Taurine levels in a range of plant, animal and microbial ingredients are displayed in Table 8.5. Taurine is not present in common plant ingredients, and levels are extremely variable in the animal ingredients displayed in Table 8.5. Levels of taurine in fish meal and meat meal can be in excess of 5 g kg^{-1} (Gaylord et al., 2006). However, this is highly variable and the concentration of taurine may be lower than this. The majority of studies investigating taurine supplementation have been carried out during fish meal replacement with plant protein sources. There are limited studies investigating taurine supplementation in diets rich in fish meal. This is due in part to the fact that fish meal typically contains high levels of taurine, and supplementation is thought to be unnecessary. However, due to the high variability of taurine in fish meal, this may not be the case.

Table 8.5. Levels of taurine in various feedstuffs used in Aquaculture.

Source	Level (g kg^{-1} dry weight)
Fish meal [1]	>5
Fish meal [2]	3.56
Salmon meal [2]	3.49
Shrimp meal [2]	0.76-1.85
Tuna meal [2]	1.08-1.53
Low ash poultry meal [2]	3.76
Regular ash poultry meal [2]	2.91
Poultry by-product meal [2]	1.89-5.35
Porcine blood meal [3]	0.08
Meat and bone meal [2]	0.09-1.11
Meat meal [2]	1.12
Yeast [2]	0.12
Soybean meal [1,2]	0
Soy protein concentrate [1,2]	0
Wheat gluten [1,2]	0

[1] Gaylord et al. (2006); [2.] Spitze et al. (2003); [3.] Daka a.m.b.a, Ringsted, Denmark.

Taurine is not considered to be essential, as it can be synthesised by some fish, but is present in large quantities in various tissues of fish (Lunger et al., 2007a,b). Carnivorous fish, such as rainbow trout, in their natural habitat consume mainly insects that contain high levels of taurine (0.34-0.46 g 100 g^{-1} tissue for the house

cricket) (Finke, 2002; Gaylord et al., 2006). In the absence of sufficient levels of dietary taurine, rainbow trout have been shown to synthesis taurine from cysteine (Yokoyama et al., 1997). However, L-cysteine sulphinate decarboxylase is rate-limiting and activity varies significantly between fish species (Jacobsen and Smith, 1968; Lunger et al., 2007a,b). In species such as Japanese Yellowtail, Atlantic bluefin tuna (*Thunnus thynnus*) and skipjack tuna (*Katsuwonus pelamis*), activity of this enzyme is not present, whereas, low activity is reported in Japanese flounder (Yokoyama et al., 2001) and red sea bream (Takagi et al., 2006b). Given that Japanese Yellowtail lack this enzyme, it is highly likely that Yellowtail Kingfish do as well; however, this needs to be investigated. Therefore, taurine supplementation may be necessary for species that are unable to biosynthesise taurine from cysteine when high levels of plant proteins are used in the diets. Given that Yellowtail Kingfish are carnivorous and may lack the enzymes, it is also likely that when fed a diet that is not composed of 100% fish meal, taurine deficiencies may occur. Also if the dietary fish meal is low in taurine, supplementation will also be necessary. Therefore, we would recommend it is essential to test for the level of taurine in dietary ingredients prior to formulation and manufacture to avoid deficiencies.

Green liver syndrome in fish can occur due to a number of causes. These include; physiological disorders due to low water temperatures (Sakaguchi and Hamaguchi, 1979), parasitic infections occluding the bile duct (Maita et al., 1997), starvation causing a reduction of bile secretion (Ozaki, 1971), bacterial infection resulting in increased haemolysis (Wada et al., 1989) and oxidative stress caused by phenyl hydrazine (Endo and Sakai, 1990; Ito et al., 1999). Moreover, the increased use of high levels of plant proteins in diets for carnivorous fish has resulted in an increased incidence of green liver syndrome (Watanabe et al., 1998; Takagi et al., 1999, 2000 a and b; Aoki et al., 2000). This syndrome has most recently been described in Japanese Yellowtail (Takagi et al., 2005, 2006a) and red sea bream (Takagi et al., 2010) fed high levels of soy protein concentrate and soybean meal, and has been linked to a taurine deficiency. Takagi et al. (2005) showed mortality, associated with green liver syndrome, to be as high as 66.3% when Japanese Yellowtail were fed a diet containing 58% soybean protein concentrate for 41 weeks. Takagi et al. (2005, 2006a, and b, 2010) reported that taurine supplementation of 10-20 g kg^{-1} in non-fish meal diets resulted in a reduced incidence of green liver syndrome and an improvement in growth in both Japanese Yellowtail and red sea bream (Table 8.6).

Recent studies have indicated supplementing taurine into diets containing high levels of plant proteins can promote growth and improve feed conversion ratio in carnivorous fish, such as Japanese flounder (*Paralichthys olivaceus*) (1.5 g kg^{-1} taurine) (Kim et al., 2003, 2005) and rainbow trout (5 g kg^{-1} taurine) (Gaylord et al., 2006). In contrast, Kim et al. (2008) showed taurine supplementation had no effect on growth or feed utilization of common carp. This supports the theory that the synthesis of taurine is highly species specific. Age can also influence the dietary requirement for taurine in fish. For example, taurine supplementation at 1.5 g kg^{-1} dry diet to non-fish meal diets in juvenile Japanese flounder (initial weight 0.3 g) improved growth performance (Kim et al., 2003). However, in a follow-up trial no improvements were observed in fingerling Japanese flounder supplemented with taurine when fed a diet of fish meal with the taurine removed through ethanol washing (Kim et al., 2003). Matsunari et al. (2005) also reported taurine supplementation of 10 g kg^{-1} diet improved growth performance of juvenile Japanese Yellowtail (initial weight 0.5 g) over a 3-week period, but no significant improvement was evident after 6 weeks. However, in this study while the results were significant statistically, the weight gains between the trial groups was only ~1 g and for all practical purposes, biologically insignificant. Therefore, it may be suggested the requirement of taurine is less for fingerlings in comparison to juveniles. However, as

the trial was only 6 weeks long, an increased trial length would be needed to verify this conclusion. Effective supplementation levels of taurine for Yellowtail Kingfish to our knowledge, has not been described previously. Several trials by Takagi et al. (2006, 2008, 2010, 2011) show supplementing taurine into diets containing high levels of plant proteins, solvent extracted soybean meal (SE SBM) and soy protein concentrate (SPC), for Japanese Yellowtail and red sea bream increased percentage weight gain and reduced the incidence of green liver syndrome (Table 8.6). Supplementation of taurine at levels greater than 30 g kg^{-1} diet significantly reduced the incidence of green liver in Japanese Yellowtail when fed a diet containing 58% SE SBM or SPC (Takagi et al., 2006, 2008, 2010, 2011). The incidence of green liver syndrome was also dramatically reduced with the inclusion of fish meal at 58% (Takagi et al., 2008). In Chapter 5 (Bowyer et al., 2013a) and Chapter 6 (Bowyer et al., 2013c) 22 g Yellowtail Kingfish were grown for 5 weeks on diets that contained SE SBM or SPC at levels of up to 30 and 40%, respectively, and no visual symptoms of green liver were observed. However, in both studies the taurine in all diets were fortified to 8 g kg^{-1}.

Replacing fish meal with high levels of SBM in excess of 50% is not realistic for carnivorous fish such as Japanese Yellowtail and Yellowtail Kingfish. SE SBM has a lower protein content and carbohydrate digestibility with a large number of anti-nutritive factors present in comparison to fish meal (Tacon, 2008). Inclusion of SE SBM above 30% has been shown to induce sub-acute enteritis in Atlantic Salmon (Uran et al., 2009). Preliminary data on the inclusion of SE SBM over 20% in Yellowtail Kingfish diets has also shown early signs of soybean meal induced-enteritis (Bansemer, 2011; Bellgrove, 2011; Bansemer et al., In press; Appendix 4). Inclusion levels of >50% of SPC may, however, be possible as this has a similar protein level to fish meal, as long as the amino acids, vitamins and minerals are balanced. However, the price of soy protein concentrate may be at times higher than fish meal, therefore using this alternative at levels >50% is unsustainable for the aquaculture industry (Gatlin et al., 2007) and palatability issues were identified when dietary levels of SPC exceeded 20% for Yellowtail Kingfish (Bowyer et al., 2013c; Chapter 6). Therefore, further studies are necessary to determine the requirement of taurine in the diet of Yellowtail Kingfish with more realistic and economically viable diets. Also in the studies by Takagi et al. (2006, 2008), the levels of taurine supplementation were graded with large increments (0, 30, 45, 60 g kg^{-1}) resulting in considerable gaps in the knowledge. Therefore, we would suggest that more tightly graded levels of taurine supplementation would be necessary in future studies. Diets for Yellowtail Kingfish in the USA are routinely supplemented with 10 g taurine kg^{-1}; however, this level of supplementation is precautionary rather than being based on actual research findings (Dr Gibson Gaylord, personal communication). Practical Japanese Yellowtail diets containing 30% fish meal are supplemented with 5 to 10 g taurine kg^{-1} (Dr Masashi Maita, personal communication). However, as previously mentioned taurine contents in FM are variable, and utilization of synthesised taurine by Japanese Yellowtail is lower than that from fish meal (Dr Masashi Maita, personal communication). Therefore, it may be wise to maintain dietary free taurine at levels of more than 5 g taurine kg^{-1}; in diets for Yellowtail Kingfish (Dr Masashi Maita, personal communication). Based on studies on Japanese Yellowtail and red sea bream, we would recommend a level of 10 to 30 g taurine kg^{-1} in diets where fish meal substitution is high, and lower levels in line with the recommendation of 5 g free taurine kg^{-1} diet by Masashi Maita when dietary fish meal levels are ≥ 30%. To ensure these levels of dietary taurine are met it is essential that the taurine level of fish meal, and other animal protein meals, used in diets for Yellowtail Kingfish are measured prior to formulation and manufacture. Further studies are necessary to investigate the effect of addition stressors, such as sea cage culture on the requirement for taurine.

Table 8.6. Levels of dietary taurine supplementation for Japanese Yellowtail, red sea bream and cobia.

Species	Duration (weeks)	Temp (°C)	Diet type	Dietary taurine supplemented (g kg^{-1} diet)	Dietary taurine measured (g kg^{-1})	Initial Wt (g)	Wt Gain (%)[2]	Green liver incidence (%)	Reference
Japanese Yellowtail	40	14.3-26.6	58% SBM	0	0	250	18.2	100	Takagi et al. (2006)
				30	33.9	250	188.2	50	
				45	52.8	252	247.9	40	
				60	71.6	246	239.9	20	
Japanese Yellowtail	39	13.9-26.6	58% SPC	0	-	470	128.0	100	Takagi et al. (2008)
				45	-	482	266.2	0	
Red sea bream	36	14.3-26.6	55% SPC	0	0.03	581	158.2	70	Takagi et al. (2010)
				5	5.62	582	190.2	10	
				10	12.4	575	182.4	20	
				15	18.4	577	181.6	20	
				20	28.2	584	179.0	10	
Red sea bream	20	17.4-25.7	55% SPC	0	1.01	72	190.3	70	Takagi et al. (2011)
				10	9.79	72	300.3	0	
				20	20.8	72	307.8	0	
Cobia	7	26.5	50% NuPro[1]	6.3	15	10	1350	-	Lunger et al. (2007b)

Dash indicates data were not available.
[1] NuPro is a yeast-based protein.
[2] 0% supplementation data was from samples taken at week 21.

Energy

The energy requirements for Yellowtail Kingfish have been described by Booth et al. (2010a) (Table 8.7). Booth et al. (2010a) reported that on a kJ DE kg^{-1} BW day^{-1} basis the requirement for dietary energy decreased with an increased body weight. Similar to Booth et al., (2010a), Masumoto et al. (1997) reported Japanese Yellowtail (initial weight 12 g, final weight 120 g) at 23-26 °C required 134 kJ kg^{-1} BW day^{-1} for maintenance. In contrast, Masumoto et al. (1997) reported that the energy requirement for maximum growth of Japanese Yellowtail (initial body weight 12 g, final body weight 120 g) to be 773 kJ BW kg^{-1} day^{-1} which is much higher than the energy requirement for maximum growth of Yellowtail Kingfish reported by Booth et al. (2010a) (Table 8.7).

Table 8.7. Estimate of daily digestible energy requirements of Yellowtail Kingfish at 21-24 °C[1].

Dietary energy requirements (g kJ BW kg^{-1} day^{-1})	Fish weight (g)							
	50	100	250	500	750	1000	1500	2000
Maintenance (kJ DE. kg^{-1} BWday^{-1})	159.2	138.6	115.4	100.4	92.6	87.4	80.6	76.1
Growth (kJ DE. kg^{-1} BW day^{-1})	464.2	375.3	283.5	229.2	202.5	185.4	163.7	149.9
Total daily req. (kJ DE. kg^{-1} BW day^{-1})	623.4	513.9	398.8	329.7	295.1	272.8	244.3	226.0

[1] Data from Booth et al. (2010a).

The difference in energy requirements may be due to the difference in water temperatures between these two studies. Booth et al. (2010a) reared Yellowtail Kingfish at 21-24 °C, whereas, Masumoto et al. (1997) reared Japanese Yellowtail at 23-26 °C. A study by Watanabe et al. (2000a) reported Japanese Yellowtail required 861.9 kJ kg^{-1} BW day^{-1} for fish weighing 31 g at 29.8 °C, 11145.9 kJ kg^{-1} BW day^{-1} for fish weighing 94 g at 27.1 °C, and 344.3 kJ kg^{-1} BW day^{-1} for fish weighing 506 g at 18.8 °C. Therefore, the energy requirement for maximum growth of Japanese Yellowtail at 18.8 °C reported by Watanabe et al. (2000a) was similar to the findings by Booth et al. (2010a). Temperature plays an important role in the energy requirements for marine species. Species similar to Yellowtail Kingfish, such as European sea bass, have been shown to require 209.0, 154.9, and 128.5 kJ kg^{-1} BW day^{-1} for fish weighing 50, 150 and 300 g respectively, when reared at 20 to 25 °C and fed a diet containing 460 g CP kg^{-1} and 20.5 MJ GE kg^{-1} (Lupatsch et al., 2001). This is significantly lower than values reported for Yellowtail Kingfish by Booth et al. (2010a).

Lipids and Essential Fatty Acids

Lipids are important for dietary energy, cell membrane structure and physiology, digestive capacity and health (Storebakken, 2002; Tocher, 2010). Dietary lipids contain essential fatty acids (EFA) that must be provided in the diet. The level and quality (EFA profile) of dietary lipid is not only dependent on the provision of exogenous sources; lipids are also present as endogenous components in many of the commonly used ingredients (NRC, 2011). Depending on the ingredients selected, the level of endogenous lipid may be significant, and may contribute up to 8 to 10% of the total dietary lipid. For example, a typical Yellowtail Kingfish grow out diet (45% crude protein) containing 20% total lipid may only require the addition of 10-12% exogenous lipid. The EFA profile of dietary ingredients may differ considerably. Fish meal and fish oil (fish oil) contain high levels of long chain omega-3 highly unsaturated fatty acids (LC n-3 HUFA) such as eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA), and the LC n-6 HUFA, arachidonic acid (20:4n-6, ARA) (Bowyer et al., 2012a; Chapter 3). Marine fish species are unable to

convert linoleic acid (18:2n-6) to ARA and α-linolenic acid (18:3n-3) to EPA and DHA at appreciable levels. These fatty acids are essential for marine carnivorous fish for growth, health and development (Tocher, 2010). Therefore, species such as Yellowtail Kingfish will require the dietary provision of LC-PUFA with 20 or more carbon atoms (Yone, 1978, Masumoto, 2002).

Dietary lipid levels of 15-20% crude lipid are considered ideal for optimal growth for species such as European sea bass, Japanese Yellowtail, Mediterranean yellowtail, and gilthead sea bream (Alvarez et al. 1998; Peres and Oliva-Teles, 1999; Koven, 2002; Masumoto, 2002). Dr Masashi Maita (personal communication) suggested larger adult Japanese Yellowtail may be fed diets containing 23 to 28% crude lipid, in conjunction with low crude protein levels, in order to obtain higher growth rates. Temperate species have the capacity to utilise high levels of dietary lipid, e.g. gilthead sea bream (initial weight 70 g) held at 20-24 °C and fed a high quality fish meal diet containing 48% CP and either 22 or 27% crude lipid (CL) (24 or 25 MJ kg^{-1} gross energy (GE), respectively) had significantly higher growth rates than fish fed 15% CL (22 MJ kg^{-1} GE) (Caballero et al., 1999). However, unlike cold-water salmonid species, temperate water marine fish species can be less tolerant to diets with high lipid contents when cultured in cold water temperatures. This was demonstrated in Mediterranean yellowtail (initial weight 95 g) fed Atlantic Salmon diets (low protein, high lipid) at 18 °C (Talbot et al., 2000). This study showed that diets high in lipid (18 g CP MJ^{-1} GE; 30% CL) did not affect growth performance, feed conversion efficiencies or liver size. However, lipid deposition within the muscle tissue and visceral cavity was significantly increased with increasing dietary lipid inclusion, 16% visceral fat (low dietary CL; 18%) to 23.5% visceral fat (high dietary CL; 30%). A high level of visceral fat is undesirable, as it contributes to losses in product yield during processing. Therefore, feeding high dietary fat levels at low water temperatures may impact on feed conversion ratios, product yield and ultimately production costs.

Lipid source may also impact growth and health for fish. Canola and poultry oil are rich sources of monounsaturated FAs and contain large quantities of oleic acid, 18:1 n-9. However, these alternatives, as well as all of the terrestrial animal and plant oils, are devoid of the essential EPA and DHA (Higgs et al., 2006). When Yellowtail Kingfish (initial weight 95 g) were fed a diet containing 45% CP, and ~25% CL, at optimal (22 °C) and suboptimal (18 °C) water temperatures, Bowyer et al. (2012a; Chapter 3) reported that 100% replacement of fish oil with poultry oil did not reduce growth or feed efficiency. However, the replacement of fish oil with 100% canola oil significantly reduced growth performance and signs of green liver were apparent at both 18 °C and 22 °C. Bowyer et al. (2012a) also reported the FA composition in the muscles tissue changed considerably when fish oil was substituted. The results reported by Bowyer et al. (2012a) could impact the dietary formulation of aquafeeds for Yellowtail Kingfish by reducing the cost of aquaculture diets. However, the results were from a short term study and further research is required with Yellowtail Kingfish to test this hypothesis.

Fish have an absolute requirement for both LC n-3 HUFA and LC n-6 HUFA. However, the requirement for EFAs is species specific (Table 8.8) (Sargent et al., 2002; Oliva-Teles, 2012). Previous research in marine fish such as Japanese Yellowtail has shown that these species have a higher requirement for n-3 fatty acids such as EPA and DHA, than for n-6 series of FA (ARA) (Furukawa et al., 1966; Tsukahara et al., 1967). However, there are limited studies available on the requirements of LC n-6 HUFA in marine species, therefore, further investigations are necessary. This will be discussed in more detail further on in this Chapter.

For species such as turbot, red sea bream and European sea bass the EFA requirement has been shown to be met by levels of LC n-3 HUFA of ≤ to 1% dry weight of the diet. In contrast, other species including Japanese Yellowtail and gilthead sea bream demonstrate a high demand for LC n-3 HUFA at levels of >1% (Table 8.8) (Deshimaru et al., 1982; Tocher, 2010). In fact, Japanese Yellowtail weighing 45-80 g have been reported to require 2% dietary LC n-3 HUFA on a dry weight basis (Deshimaru et al., 1982). Therefore, in the absence of further research, we would consider that Yellowtail Kingfish would have a comparable n-3 LC-HUFA requirement to Japanese Yellowtail. However, the requirements for LC n-3 HUFA can be further complicated by the ratio of DHA to EPA, with higher levels of DHA being preferable.

Table 8.8. Quantitative essential fatty acid requirement of juvenile and sub-adult marine fish species[1].

Species	Essential fatty acid	Requirement (% dry diet)	Reference
Turbot	n-3 HUFA	0.8	Gatesoupe et al. (1977)
	ARA	~0.3	Castell et al. (1994)
Red sea bream	n-3 HUFA or EPA	0.5	Yone (1978)
	EPA	1.0	Takeuchi et al. (1990)
	DHA	0.5	
Gilthead sea bream	n-3 HUFA with a; DHA:EPA ratio of	0.9 1.0	Kalogeropulos et al. (1992)
	n-3 HUFA with a; DHA:EPA ratio of	1.9 0.5	Ibeas et al. (1997)
European sea bass	n-3 HUFA	1.0	Coutteau et al. (1996)
Japanese Yellowtail	n-3 HUFA	2.0	Deshimaru et al. (1982)

[1] Table adapted from Tocher (2010).

DHA has generally been reported to have a higher EFA value than EPA for fish (Tocher, 2010). Studies by Kalogeropulos et al. (1992) and Ibeas et al. (1994) have shown that the actual LC n-3 HUFA requirement of gilthead sea bream may be reduced by increasing the ratio of DHA to EPA in the diet (Table 8.8). For example, when the DHA:EPA ratio was increased from 0.5 to 1.0, the LC n-3 HUFA requirement was reported to reduce from 1.9 to 0.9 for gilthead sea bream (Kalogeropulos et al., 1992; Ibeas et al, 1994). Tocher (2010) has also reported for this response to hold true for other larval marine fish. Therefore, the levels of DHA and EPA have to be considered when determining the LC n-3 HUFA requirement for Yellowtail Kingfish. Until additional information for Yellowtail Kingfish is available, we would suggest the level of 2% LC n-3 HUFA in the diet, as recommended by Deshimaru et al. (1982), for the Japanese Yellowtail (Table 8.8). With regards to fish oil substitution and the supply of essential n-3 HUFA at the practical level, several factors need to be considered to guard against a potential deficiency. The level of essential n-3 HUFA may differ significantly between fish oil types (Stone et al., 2011a and b) and batches (Young, 1986). Terrestrial animal and plant oils are also devoid of essential n-3 HUFA. Therefore, even though in the short term Yellowtail Kingfish may grow well at high levels of fish oil substitution; careful attention to the essential n-3 HUFA profile of the lipid sources will be required to ensure nutrient deficiencies do not arise as fish oil substitution levels are pushed to extremes.

The LC n-3 HUFA requirements for larval and early juvenile fish are considerably higher (~twice as high) than for sub-adults (Table 8.9). Determining the nutrient requirements in the diets of marine larvae and fry can be difficult as they have poorly developed digestive systems that cannot digest formulated feeds. Commonly, larvae

and fry are fed live rotifers and *Artemia*, however, these can have poor LC n-3 HUFA levels and fatty acid profiles (Tocher, 2010). Feeding larvae and fry rotifers, *Artemia* or artificial diets low in EFA will result in reduced growth and poor survival (Izquierdo, 1996). Therefore, enrichment of live feeds with EFAs is necessary to ensure maximum growth and survival (Tocher, 2010).

Table 8.9. Quantitative essential fatty acid requirements of larval and early juvenile fish[1].

Species	Essential Fatty acid	Requirement (% dry diet)	Reference
Red seabream	n-3 HUFA	2.1	Fururita et al. (1996a)
	DHA	1.0-1.6	
	EPA	2.3	
Gilthead sea bream	n-3 HUFA with a DHA:EPA ratio	5.5 0.3	Rodriguez et al. (1994a)
	n-3 HUFA with a DHA:EPA ratio	1.5 2.0	Rodriguez et al. (1998)
Japanese Yellowtail	n-3 HUFA with a DHA:EPA ratio	3.9 0.5	Furuita et al. (1996b)
	DHA	1.4-2.6	
	EPA	3.7	

[1] Table adapted from Tocher (2010).

Larvae have been shown to have a higher requirement for EPA rather than DHA, which is consistent with sub-adult red sea bream (Table 8.8 and 8.9) (Tocher, 2010). The n-6 HUFA, ARA has been shown to be important for larval development in gilthead sea bream (Bessonart et al., 1999) and Japanese flounder (Estevez et al., 1997). Levels of 1.0-1.5% of ARA (dry basis) of the diet improved growth in larval sea bream (Bessonart et al., 1999). Survival was also increased in larval gilthead sea bream with the addition of dietary ARA to rotifers (12.5% ARA-phospholipid) through inclusion of heterotrophically grown fungus, *Mortierella alphina* (52% total FA), fed before handling stress (Koven et al., 2001). However, some species such as yellowtail flounder (*Limanda ferruginea*) have been shown to require a higher requirement for DHA in the larval stages, with ARA inclusion at 0.8% of the diet on a dry weight basis (7% total FA) resulting in inhibited growth, increased mortality and negative effects of pigmentation in comparison to the fish fed the rotifer DHA enriched diet (43.3% total FA) (Copeman et al., 2002).

Japanese Yellowtail larvae, which grow about 3.8 times faster than sea bream, are reported to require high levels of DHA in comparison to other species (Table 8.9). This EFA has been shown to play an important role in the function of the nervous and visual systems (Takeuchi, 2008). Ishizaki et al. (2001) reported rotifers enriched with DHA (2% mass of *Artemia*) fed to larval Japanese Yellowtail (12 mm in length) significantly affected the ontogeny of schooling behaviour through increased brain growth, specifically the tectum opticus, in comparison to larvae fed non enriched *Artemia*. The EFA requirements for larvae and fry are still relatively unknown for many species, with results varying due to differences in growth, dietary lipid level and survival. As previously mentioned, in the absence of further research, data for Japanese Yellowtail should be used with caution for Yellowtail Kingfish (Table 8.9).

Cholesterol

Fish oil and animal fats are rich in cholesterol, while plant ingredients are deficient (Cheng and Hardy, 2004). Cholesterol is a known essential dietary nutrient (Hernández et al., 2004), and although most vertebrates have the ability to synthesis cholesterol from sterol precursors (NRC, 2011), the rate at which Yellowtail Kingfish synthesise cholesterol *de novo* is unknown. Cholesterol levels need to be considered

when fish oil is substituted with plant oils, and to a lesser extent with animal fats as it has many important biological functions including disease resistance and taurine metabolism (Moschetta et al., 2005; Maita et al., 2006). Among the many important functions of cholesterol in the liver, one of great significance to nutrition and health is that it is converted by cholesterol-7α hydroxylase via cytochrome P450-mediated oxidation, to bile acids, which can be conjugated with taurine, before storage as bile in the gallbladder (Moschetta et al., 2005).

Yellowtail Kingfish fed a diet where 50 or 100% of fish oil had been replaced with canola oil had inferior growth performance, feed efficiency and nutrient retention, and showed higher incidences of green liver syndrome and lower plasma cholesterol levels than fish fed the fish oil and poultry oil diets (Bowyer et al., 2012a; Chapter 3). Bowyer et al. (2012a) suggested that the green liver syndrome may be due to an interaction of low dietary cholesterol levels and taurine levels, resulting in the production of less bile salt for the conjugation with taurine, which in turn may have caused the accumulation of bile acids, and subsequently the bile pigments in the liver causing the observed green colouration of the liver. Reduced plasma cholesterol levels have also been reported for marine species such as European sea bass and black sea bream (*Acanthopagrus schlegeli*) when fish oil was replaced with plant oils (Richard et al., 2006; Peng et al., 2008) due to the fact that diets containing plant oils rich in 18:1n-9, 18:2n-6 and 18:3n-3 can reduce cholesterol levels (Fernandez and West, 2005).

Plant oils also contain phytosterols (Phillips et al., 2002), which are known to reduce the levels of total cholesterol in some fish species by decreasing the intestinal cholesterol absorption (Gilman et al., 2003). Refined fish oils may also contain lower levels of cholesterol (Young, 1986). An additional implication of reduced cholesterol level is the subsequent reduction in bile for digestive processes. Therefore, it is 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. The levels of cholesterol in common oils and ingredients used in fish feeds are displayed in Table 8.10. While no actual cholesterol requirement is available, we would suggest taking into consideration the level of cholesterol that has been lost during the fish oil substitution. Further research is necessary for Yellowtail Kingfish.

Table 8.10. Cholesterol content of common aquafeed ingredients.

Source	Cholesterol level (g kg^{-1})
Bone meal	0.78
Blood meal	2.20
Canola meal	0.09
Fish meal, 68% CP	4.20
Fish meal, Herring	3.00
Meat and bone meal, 43% crude protein	1.00
Meat and bone meal, 56% crude protein	1.00
Poultry by-products meal, 65% crude protein	1.70
Soybean meal	0.01
Squid meal	7.90
Fish oil (South American)	3.20
Cod liver oil	3.1
Squid oil	13.00
Soy bean meal oil	0.05
Wheat gluten	0.08

[1] Adapted from Nates and Swisher (2010).

Carbohydrates

Fish do not have a specific requirement for dietary carbohydrates. In the wild, natural fish food usually contains low amounts of carbohydrates (Stone, 2003; Oliva-Teles, 2012). Plants ingredients such as grains, oilseeds and legumes are increasing being used as energy sources in fish diets to decrease costs associated with feeding fish meal and fish oil and also improving sustainable production. However, these alternatives are typically high in carbohydrates which are poorly utilised by many species of fish (Alexis and Nengas 2001; Glencross, et al., 2003; Stone, 2003). Providing energy by feeding fish carbohydrates may reduce the use of other nutrients such as protein and lipids for energy and ensures their maximum use for tissue growth and maintenance (Stone, 2003). However, the success of this approach is limited and highly species dependent.

Dietary inclusion of carbohydrates is species specific, typically with fish in warmer water temperatures being able to utilise much higher levels of dietary carbohydrates than cold-water or marine fish (Wilson, 1994). For example, Japanese Yellowtail (Furuichi and Yone, 1981; Masumoto, 2002) and salmonids (Hilton and Atkinson, 1982) have been shown to utilise a level of ≤ 20% digestible carbohydrate for optimal growth (Table 8.11; Wilson, 1994). Whereas, channel catfish (Garling and Wilson, 1977), common carp (Takechi et al., 1979; Furuichi and Yone, 1980), red sea bream (Furuichi and Yone, 1980) and tilapia (Luquet, 1991) are able to tolerate dietary levels of up to 40% digestible carbohydrate.

In comparison to common carp and red sea bream, Japanese Yellowtail are unable to utilise carbohydrates due to lower endogenous amylase and carbohydrase enzyme activity, and insulin secretion (Masumoto, 2002). Similarly, Bowyer et al. (2012c; Chapter 4) reported Yellowtail Kingfish have a low α-amylase activity (0.066 U mg protein^{-1}). Japanese Yellowtail fed diets containing diets >10% digestible carbohydrates have been shown to exhibit unregulated blood glucose uptake (Shimeno, 1979; Shimeno, 1991; Brauge et al., 1994; Wilson, 1994; Stone, 2003) (Table 8.11). Shimeno (1979) fed fish meal based diets supplemented with gelatinised levels of starch at 0, 10, 20 and 40% and reported inclusion of 10 and 20% starch increased growth, feed efficiency and energy retention in comparison to the diets containing 0 and 40% gelatinised starch. Other species including rainbow trout and Atlantic Salmon have been reported to exhibit hyperglycaemia, increased liver size and glycogen content when fed diets with levels of dietary starch > 10% (Brauge et al., 1994; Hauler, 1995). However, more thorough investigations are necessary to understand the dietary requirement of carbohydrate for Yellowtail Kingfish.

Table 8.11. Optimum or recommended dietary levels of digestible carbohydrate for salmonid and marine fish[1].

Species	Digestible carbohydrate (%)	Reference
Asian sea bass	<20	Boonyaratpalin (1991)
Atlantic Salmon	<20	Helland et al. (1991)
Pacific salmon	<20	Hardy (1991)
Rainbow trout	<20	NRC (1981)
Japanese Yellowtail	<10	Shimeno (1991)

[1] Table adapted from Wilson (1994).

Feeding carnivorous fish high levels of dietary carbohydrate has also been shown to affect disease status and stress tolerance (Oliva-Teles, 2012). For example, Waagbo et al. (1994) reported an effect of dietary carbohydrate inclusion on immunity and

bacterial disease resistance in Atlantic Salmon. Fish were fed dietary carbohydrate levels ranging from 0 to 30%. The results showed a decrease in blood haemoglobin concentration, serum cortisol and serum haemoglobin. However, diets with less than 10% carbohydrate inclusion resulted in reduced mortality after a disease challenge with *A. salmonicida*. Other species, such as rainbow trout, showed no change in immune responses after long term feeding with diets high in dietary carbohydrates (Page et al., 1999).

Carbohydrates have also been suggested to be important for early larval development for several species including Yellowtail Kingfish (Finn et al., 1995; Moran et al., 2007). The common carbohydrates, glucose and glycogen, are thought to be important for fuelling the first hours of development. Non-protein bound free amino acids (FAA) are also known to be significant energy substrates for developing embryos and newly hatched larvae of marine fish with pelagic eggs (Ronnestad et al., 1999). Moran et al. (2007) measured the changes in oxygen consumption and metabolite concentrations in particular FAA, glucose and glycogen levels, of larval Yellowtail Kingfish at different temperatures. This study reported leucine, valine, serine, isoleucine and alanine, accounted for 50-85% of the FAA pool of the eggs. Studies from Atlantic cod (Finn et al., 1995), European sea bass (Ronnestad et al., 1998) and turbot (Finn et al., 1996) showed 60-90% of the energy requirements of embryos and early larvae are met through FAA catabolism (Moran et al., 2007). Therefore, it is suggested that the predominant energy source for marine embryos is FAAs rather than carbohydrates, however further investigation is necessary to understand the role of carbohydrates in embryogenesis.

To our knowledge there have been no published reports on dietary carbohydrate utilization for Yellowtail Kingfish. Therefore, based on data available for similar species we would recommend that the level of digestible dietary carbohydrate should not exceed 5 to10% to ensure that growth and survival are not compromised. This amount is typically provided with the inclusion of 10-15% wheat that is used to assist pellet binding during the extrusion process.

Minerals

Minerals are inorganic elements or compounds required for skeletal formation, homeostatic regulation and for biologically important compounds such as hormones and enzymes (Watanabe et al., 1997). Typically the dietary supplementation of minerals is relatively inexpensive, therefore, enabling relatively cheap additions to dietary formulations to avoid mineral deficiencies (Hardy, 2001). Relatively little information is available on the mineral requirements of marine fish species, and none specifically for Yellowtail Kingfish. Leaching of minerals can occur during feeding, causing problems for quantifying the mineral requirement. Macro- and micro-minerals can also be obtained directly from the sea water. This further complicates the determination of specific requirement for certain minerals. Therefore, this presents a potential problem to feed formulators as the mineral content of the culture water may vary significantly, both geographically and seasonally, between sites. Due to the current lack of mineral requirement information for Yellowtail Kingfish we have provided Table 8.12 listing the recommended levels of relevant macro- and micro-minerals, based on information from a range of marine and freshwater species. We will also discuss the requirements for the macro-nutrient, phosphorus and the micro-nutrient, selenium, in more detail as CST has indicated particular interest in these two minerals.

Macro-minerals including calcium, chlorine, magnesium, phosphorus, potassium, and sodium, are important for the formation of bones and other hard tissues (e.g. fins,

scales and exoskeleton etc.), and osmoregulation. Little information is available on the dietary requirement of these minerals due to their abundant presence in the aquatic environment (NRC, 2011).

The dietary requirement for phosphorus has been reported to range from 3 to 15 g kg^{-1} in various species (Lall, 2002). Shimeno et al. (1991) reported small Japanese Yellowtail require 6.7 g available phosphorus kg^{-1} dry diet (Table 8.12). Dietary supplementation of phosphorus is important as it is poorly utilised by fish and its presence in sea water is limited. Phosphorus deficiencies are characterised by poor growth and feed efficiency due to metabolic impairment. Skeletal deformities may also occur in phosphorus deficient diets (Sullivan et al., 2007).

Table 8.12. Requitements for macro- and micro- minerals for various marine and freshwater fish[1].

Mineral	Species	Requirement (units kg^{-1} dry diet)	Reference
Macro-minerals			
Calcium	Red sea bream	Dispensable	Sakamoto and Yone (1976a)
	Rainbow trout	Dispensable	Ogino and Takeda (1976)
	Chum salmon	Dispensable	Watanabe et al. (1980)
Phosphorus	Atlantic Salmon	6.0 (available P) g	Ketola (1975)
	Red sea bream	6.8 g	Sakamoto and Yone (1976a)
	Red drum	8.6 g	Davis & Rodinson (1987)
	European sea bass	6.5 g	Oliva-Teles and Pimenrel-Rodrigues (2004)
	Japanese Yellowtail	6.7 g	Shimeno et al. (1991)
Potassium	Red sea bream	Dispensable	Sakamoto and Yone (1976b)
Sodium/chloride	Rainbow trout	Dispensable	Salman and Eddy (1988)
	Red drum	Dispensable	Gatlin et al. (1992)
Sodium	Red sea bream	Dispensable	Sakamoto and Yone (1976b)
Magnesium	Rainbow trout	0.5-0.7 g	DaBrowska et al. (1989)
Micro-minerals			
Copper	Rainbow trout	3.5 mg	Julshamn et al. (1988)
	Atlantic Salmon	5-10 mg	Lorentzen et al. (1998)
Iron	Red sea bream	199 mg	Sakamoto and Yone (1976b)
Zinc	Rainbow trout	20-80 mg	Satoh et al. (1987)
	Red drum	20 mg	Gatlin et al. (1991)
Manganese	Rainbow trout	12-13 mg	Ogino and Yang (1980)
Selenium	Rainbow trout	0.15-0.38 mg	Hilton et al. (1980)
Iodine	Chinook salmon	0.6-1.1 mg	Woodall & LaRoche (1964)

[1] Table adapted from NRC (2011).

The majority of studies on phosphorus in fish diets focus on effluent output and environmental impact (Satoh et al., 2004). An important point to consider when providing phosphorus to fish is that phosphorus is the second limiting nutrient to phytoplankton growth in seawater, and excess phosphorus discharge may contribute to blooms of potentially toxic species of microalgae in the vicinity of the culture environment (Satoh et al., 2004). This may become a more important issue as Yellowtail Kingfish production intensifies on existing sites. Diets high in fat and low in protein are increasingly being used to reduce the discharge of nitrogen and phosphorus into the environment (Talbot and Hole, 1994). However, the use of poor quality (low P) fish meal-based diets has been reported to result in phosphorus deficiencies in post juvenile stages of Japanese Yellowtail (Makino, 1990). Recent studies, however, suggest that phosphorus supplementation may not be necessary when good quality low ash fish meal is fed. High ash fish meals may contain a relatively high proportion of bone (Makino, 1990).

Phosphorus tied up in bones in fish meal is not readily bio-available to fish. For example, Shearer et al. (1992) fed juvenile Atlantic Salmon (initial weight 3.9 g) fish meal that ranged from 10.5 to 17.5% ash and reported increasing levels of dietary ash reduced feed efficiency. The level of phosphorus excreted also increased. Therefore, feeding fish high levels of ash can have both economic and environment impacts, and supplementation with phosphorus may be necessary. However, Satoh et al. (2004) reported phosphorus supplementation was not necessary for Japanese Yellowtail (initial weight 346 g, final weight 912 g) when fed a fish meal based diet (total P 14.5 g kg^{-1}, 8.5 to 10.7% ash) containing 50% jack mackerel meal.

Care must also be taken when feeding diets with increasing levels of plant proteins as the availability of phosphorus may be reduced by the presence of phytic acid (phytate). This substance is a phosphorus rich complex that is found in appreciable quantities (60-80% of total P) in plants and acts as an inbuilt anti-nutrient defence mechanism against insects and other foraging animals. This also presents problems when feeding plant ingredients rich in phytate to fish (Storebakken et al., 1998). The bioavailability of phytate phosphorus in plant ingredients is very low for fish and monogastric animals, with reports of phosphorus availability in SBM ranging from 0 (Richie and Brown, 1996) to 22% for fish (Sugiura et al., 1998).

Phytic acid has not only been demonstrated to interfere with phosphorus uptake in animals, it has also been shown to inhibit the uptake of protein, minerals, notably zinc, and other micro- and macro-nutrient (Gatlin et al., 2007). For example, Sullivan et al. (2007) reported spinal deformities in Atlantic Salmon (final weight 63 to 85.7 g) fed three commercial diets (available P 4.1 to 6.4g kg^{-1}) for 30 weeks. The phosphorus availability in the commercial diets was significantly lower than the recommended levels of 9.0 g available P kg^{-1} for 1.4 g Atlantic Salmon fry (Asgard and Shearer, 1997). Sullivan et al. (2007) attributed the low phosphorus availability to the presence of phytic acid. Effective strategies to reduce the effects of dietary phytic acid include formulating using ingredients low in phytate, removal of phytate through processing, the addition of enzymes specific to hydrolysing phytate, such as phytase, or gene manipulation of the plant.

The use of commercial enzyme supplements containing phytase have been demonstrated to be beneficial in reducing the negative effects of phytic acid and increasing phosphorus and other nutrient uptake and utilisation in a range of aquatic species. For example, Storebakken et al. (1998) reported Atlantic Salmon (initial weight 100 g) had improved protein digestibility and retention, feed conversion, and reduced metabolic nitrogen excretion when soy protein concentrate was treated with phytase (phytase treated soy concentrate 480 g kg^{-1} wet weight). Similarly, in rainbow trout (initial weight 57 g) fed a soy ingredient (Soycomil P and Hamlet Protein 300) pre-treated with phytase (Hamlet Protein microbial phytase; Natuphos 5000L, BASF) showed a positive weight gain, improved feed efficiency and increased protein, phosphorus, calcium, magnesium and zinc utilization in comparison to fish fed diets with untreated soy fortified with phosphorus (total P, 9 g kg^{-1}; un-supplemented diet total P, 6 g kg^{-1}) (Vielma et al., 2002). Vielma et al. (2002) also reported the retention of phosphorus increased from 33% for fish fed the regular soy protein to 72% for fish fed the dephytinised soy protein. Therefore, it would be worthy to consider the use of phytase in diets containing moderate to high levels of plant ingredients for Yellowtail Kingfish. Further research in this area is warranted.

Variations in the requirement for phosphorus cannot only result from nutrient bioavailability shortfalls; they may also be related to fish size or age. Sarker et al. (2009) showed the minimum requirement for phosphorus was 4.4 g available P kg^{-1} dry diet for Japanese Yellowtail weighing 1 kg. This study also showed that no

additional supplementation was necessary for 1 kg fish fed fish meal-based diets (containing 467 g kg^{-1} anchovy meal), and a 2 g kg^{-1} supplementation with phosphorus resulted in a 34.5% increase in P excretion in the effluent water which was unfavourable.

Given the lack of P requirement information for Yellowtail Kingfish we would recommend 7 g available phosphorus or 15 g total P kg^{-1} diet for ≥1 kg fish based on that provided by Japanese Yellowtail as outlined above; with higher levels recommended for smaller fingerlings. However, further studies are necessary to understand the phosphorus requirement during different growth stages for Yellowtail Kingfish. Additionally phosphorus availability in relation to fish meal replacement with plant proteins rich in phytate also requires investigation.

Micro-minerals, which are required in much lower concentrations than macro-minerals, are necessary for a number of biochemical processes and are important components of enzymes and hormones. The common micro-minerals include copper, iodine, iron, manganese, selenium and zinc. Similar to the macro-minerals, and due to knowledge gaps in the literature, the majority of studies focus on freshwater species rather than marine species. However, the importance of mineral supplementation has been described in the juvenile stages of Japanese Yellowtail. Satoh et al. (2001) fed diets excluding phosphorus, magnesium, zinc and manganese from the mineral mixture to Japanese Yellowtail (initial weight 1.7 g) for eight weeks. The results showed that growth and mineral composition of the carcass were significantly reduced in diets without phosphorus, magnesium, manganese or zinc supplement.

There is limited recent information available on iron and selenium requirements in Japanese Yellowtail and related species. Iron (Fe) is an important component of haemoglobin and is also important for cellular respiration and oxidation/reduction reactions (Watanabe et al., 1997). Iron requirements in fish have been reported between 30 and 199 mg Fe kg^{-1}. Fish meal and meat meal are rich sources of iron containing about 400-800 mg Fe kg^{-1}, whereas, oil seeds contain only 100-200 mg Fe kg^{-1} and cereals contain considerably less (30-60 mg Fe kg^{-1}) (Watanabe et al., 1997). Early studies in Japanese Yellowtail (Ikeda et al., 1973) and red sea bream (Sakamoto and Yone, 1978) reported diets deficient in iron may have induced anaemia. However, growth depression was not observed in these studies. Andersen et al. (1996) determined the dietary iron requirement for fingerling Atlantic Salmon (initial weight 5 g) was between 60 and 100 mg Fe kg^{-1} in the diet based on haematology and hepatic iron concentration. While, Sakamoto and Yone (1976b) reported an iron requirement of 199 mg Fe kg^{-1} for red sea bream. As there is no published literature on the specific dietary requirement of iron in Yellowtail Kingfish or Japanese Yellowtail, we would assume they are similar, or slightly higher due to the higher growth rates and metabolic requirements for Yellowtail Kingfish, to that of other cultured marine fish such as Atlantic Salmon and red sea bream. As previously mentioned Japanese Yellowtail have been observed to grow 3.8 times faster than red sea bream (Furuita et al., 1996a, b), therefore, it may be wise to fortify iron levels ≥200 mg Fe kg^{-1} in diets for Yellowtail Kingfish.

Selenium (Se) is essential for fish as it plays an important role in cell and membrane protection against oxidative damage (Watanabe et al., 1997). Selenium is a component of the enzyme glutathione peroxidase, which converts hydrogen peroxide and lipid hydroperoxides into water and lipid alcohols, respectively (NRC, 2011). Glutathione peroxidase also acts with vitamin E to function as an antioxidant to protect lipids in cells and membranes against peroxidative damage (Lovell, 1989). Diets deficient in selenium can result in reduced growth and a reduction in the activity

of glutathione peroxidase (NRC, 2011). A recently study by Halver et al. (2004) reported that stress confinement for 30 h during transport reduced carcass selenium of Chinook salmon by 20%, while liver glutathione peroxidase activity increased. Therefore, this study indicated increasing the selenium from 5 to 8 mg kg^{-1} diet prior to stress may be beneficial for Chinook salmon. The toxicity of selenium has been described in various species with levels of 13 to 15 mg Se kg^{-1} diet from sodium selenite resulting in reduced growth and elevated mortality (Hilton et al., 1980; Gatlin and Wilson, 1984; NRC, 2005). As Yellowtail Kingfish are fed diets high in lipid, the importance of selenium may be heightened. Growth of juvenile Yellowtail Kingfish increased with increasing levels of selenium from 3.3 to 5.34 mg kg^{-1} when fed in a diet deficient in vitamin E, however these high levels of selenium had no benefit to growth within a diet containing adequate levels of vitamin E (180 mg kg^{-1}) (Le et al., In press). Le et al. (In press) recommended further studies to determine the effect of the low levels of dietary selenium at varying levels of vitamin E inclusion on Yellowtail Kingfish growth and health. Selenium requirements for fish range from 0.15 to 0.5 mg Se kg^{-1} in the diet (Watanabe et al., 1997). Diets containing >15% fish meal should supply the adequate levels of selenium necessary for optimal growth in most fish species. However, for diets containing low quality fish meal, plant proteins or fish oil, supplementation may be necessary. We would suggest that the level of 0.5 mg Se kg^{-1} in diets containing adequate amounts of vitamin E would be suitable for Yellowtail Kingfish. However, further research is needed.

With respect to the requirements of other dietary mineral (Table 8.12), calcium, potassium and sodium/chloride have been reported to be dispensable as they may be acquired from the seawater. Whereas, magnesium (0.7 g kg^{-1} diet), copper (3.5 mg kg^{-1}), zinc (20 mg kg^{-1}), manganese (12 mg kg^{-1}) and iodine (1.1 mg kg^{-1}) should be provided in the diet (Table 8.12). Further studies are necessary to understand the dietary requirements of macro- and micro-minerals for Yellowtail Kingfish.

Vitamins

Vitamins are organic compounds and are necessary for normal growth, reproduction and health and need to be provided in the diet in trace amounts (NRC, 2011). Vitamin deficiencies are the most common cause of deficiency in commercial aquaculture (Hardy, 2001). The deficiencies signs include: abnormal swimming, skin disease, skeletal deformities, oedema, eye disease, and alterations in gill pathology, haemorrhaging, liver disease and reduced growth (NRC, 2011). The important vitamins in fish nutrition include the fat-soluble vitamins: A, D, E and K and; the water soluble vitamins: vitamin B complex and vitamin C (NRC, 2011). The dietary vitamin requirements optimal growth and maximum liver storage for Japanese Yellowtail established by Shimeno (1991) and Hosokawa (1999) (Table 8.13). Maximum liver storage levels of vitamins are beneficial for immune defences. Therefore, suggestions for vitamin requirements of Yellowtail Kingfish provided here are based on maximum liver storage levels of Japanese Yellowtail or unrelated species, when available. Additionally, due to the current lack of vitamin requirement information for Yellowtail Kingfish, we will also provide additional information listing the recommended levels of vitamins for a range of other marine and freshwater species for comparison (Table 8.14).

Table 8.13. Japanese Yellowtail vitamin requirements based on growth and maximum liver storage levels[1].

Vitamin	Requirement for optimal growth (mg kg^{-1} dry diet)	Requirement for maximum liver storage (mg kg^{-1} dry diet)
A (Retinol (as acetate)	5.6	-
B1 (Thiamine hydrochloride)	1.2	11.2
B2 (Riboflavin)	2.9	11
B3 (Nicotinic acid)	12	-
B4 (Choline chloride)	2100	2920
B5 (Pantothenic acid)	13.5	35.9
B6 (Pyridoxine hydrochloride)	2.5	11.7
B8 (Inositol)	190	423
B9 (Folic acid)	0.8	1.2
B12 (Cyanocobalamin)	0.053	-
C (Ascorbic acid)	122	-
E (A-Tocopherol)	119	-
H (Biotin)	0.22	0.67

Dash indicates data were not available.
[1] Table adapted from Masumoto (2002) and NRC (2011) using data from Shimeno (1991) and Hosokawa (1999).

Table 8.14. The recommended requirement levels of vitamins for a range of marine and freshwater species based on weight gain[1].

Vitamin	Species	Requirement (mg kg^{-1} diet)	Reference
A (Retinol)	Rainbow trout	0.75	Kitamura et al. (1967)
	European sea bass	31	Villeneuve et al. (2005a,b)
B1 (Thiamine)	Pacific salmon	10-15 (MLS)	Halver (1972)
	Rainbow trout	1-10	McLaren et al. (1947)
B2 (Riboflavin)	Pacific salmon	7	Leith et al. (1990)
	Rainbow trout	2.7 (MLS)	Amezaga and Knox (1990)
	Hybrid striped sea bass	4.1-5.0	Deng and Wilson (2003)
B3 (Niacin)	Pacific salmon	150-200 (MLS)	Halver (1972)
	Rainbow trout	10	Poston and Wolfe (1985)
B4 (Choline)	Pacific salmon	600-800 (MLS)	Halver (1972)
	Rainbow trout	714-813	Rumsey (1991)
	Red drum	588	Craig and Gatlin (1996)
B5 (Pantothenic acid)	Pacific Salmon	17	Leith et al. (1990)
	Rainbow trout	20	Cho and Woodward (1990)
B6 (Pyridoxine hydrochloride)	Atlantic Salmon	5	Lall and Weerakoon (1990)
	Pacific salmon	6	Leith et al. (1990)
	Rainbow trout	2	Woodward (1990)
B9 (Folic acid)	Pacific salmon	2	Leith et al. (1990)
	Rainbow trout	1	Cowey and Woodward (1993)
B12 (Cyanocobalamin)	Pacific salmon	0.015-0.02 (MLS)	Halver (1972)
C (Ascorbic acid)	Atlantic Salmon	10-20	Sandnes et al. (1992)
	Rainbow trout	40-100	Halver (1982)
D (Cholecalciferol)	Rainbow trout	0.040-0.060	Barnett et al. (1982)
E (Tocopherol)	Atlantic Salmon	35-60	Lall et al. (1988)
	Pacific salmon	40-50	Halver (1972)
	Rainbow trout	30	Woodall et al. (1964)
	Red drum	31	Peng and Gatlin (2009)
K (Phylloquinone)	Atlantic Salmon	<10	Krossoy et al. (2009)

[1] Table adapted from NRC (2011); MLS: maximum liver storage.

Vitamin A (retinol) is necessary for cell differentiation and thus plays an important role in embryonic development. It is also essential for vision (NRC, 2011). A diet deficient in vitamin A has been shown to cause anaemia, dark pigmentation of the skin, haemorrhaging in the eyes and liver, twisted gill opercula, and increased mortality in fingerling Japanese Yellowtail (Hosokawa, 1999; Shimeno, 1991). Vitamin A toxicity can also occur. Ornsrud et al. (2002) reported reduced growth, abnormal vertebral formation and increased mortality in Atlantic Salmon (initial weight 0.17 g) fed a diet containing 938 mg retinol kg^{-1} diet. We would recommend a level of 5.68 mg kg^{-1} diet for Yellowtail Kingfish (Table 8.13).

Relatively few studies have investigated the requirement of vitamin D for marine fish species. Vitamin D is important for the development, growth and maintenance of the skeletal structure (NRC, 2011). Deficiencies reported in fish are associated with poor growth and hypocalcaemia (George et al., 1981). Requirement values reported for vitamin D for rainbow trout were 40 to 60 µg kg^{-1} diet (Table 8.14). Darias et al. (2010) reported larval European sea bass grew well with a vitamin D_3 supplemented at 68 µg kg^{-1} diet, compared to 8.4 µg kg^{-1} diet. The values reported by Darias et al. (2010) are 12 times those recommended for juvenile European sea bass (NRC, 1993). We recommend ≥60 µg vitamin D kg^{-1} diet for Yellowtail Kingfish (Table 8.14).

In comparison to other fat-soluble vitamins, the dietary requirement of vitamin E has been well documented. Fish deficient in vitamin E show signs of muscular dystrophy including atrophy and necrosis of white muscle fibres, oedema of the heart, muscle and other tissues and anaemia and nervous disorders (NRC, 2011). Vitamin E has been shown to play an important role in broodstock nutrition, particularly during reproduction. Feeding broodstock diets deficient in vitamin E has been associated with a reduction in fertilisation capacity of sperm in gilthead sea bream (Fernandez-Palacios et al., 2005) and yellow perch (Lee and Dabrowski, 2004). An interaction between vitamin E and selenium has also been described in Atlantic Salmon (Poston et al., 1976), rainbow trout (Bell et al., 1985) and Yellowtail Kingfish (Le et al. (In press), with the symptoms of deficiency becoming more apparent when both vitamin E and selenium are deficient in the diet at the same time. Feeding diets with high concentrations of PUFAs have been associated with an increased requirement for vitamin E in rainbow trout (Cowey and Woodward, 1993), hybrid tilapia (Shiau and Shiau, 2001) and grouper (Lin and Shiau, 2005). Hosokawa (1999) also described this interaction in Japanese Yellowtail with an increase in dietary lipid content from 8% to 15% and 23%, requiring an increase of the vitamin E (α-Tocopherol) concentrations from 35 to 93 mg kg^{-1} and 160 mg kg^{-1}, respectively. Therefore, consideration to vitamin E level must be taken into account when formulating diets high in PUFAs, as the vitamin E concentration may be too low resulting in a reduction in growth and health.

Limited information is available on the dietary requirement of vitamin E in marine fish species. A study by Shimeno (1991) showed a requirement of 119 mg kg^{-1} for Japanese Yellowtail when measuring maximum liver storage. In comparison, Hamre and Lie (1995) found Atlantic Salmon at first feeding (initial weight 0.165 g, final weight 2.84-5.05 g, diet: 5.6% PUFAs) required a minimum of 60 mg dl-α-tocopheryl acetate kg^{-1} dry diet. Hamre and Lie (1995) also reported feeding 60 and 120 mg α-tocopheryl acetate supported growth and survival. More recently, Lygren et al. (2000) fed Atlantic Salmon (initial weight 64 g) 40, 300 or 1100 mg kg^{-1} all-rac-α-tocopheryl acetate under normoxic and moderate hyperoxic conditions for 12 weeks, and reported increased levels of dietary vitamin E did not protect the fish against oxidative stress. We would recommend a level of 120 mg kg^{-1} dry diet vitamin E for Yellowtail Kingfish (Shimeno, 1991; Hosokawa, 1999; Table 8.13). Further studies

are necessary to investigate the dietary vitamin E requirements for different growth stages of Yellowtail Kingfish.

The health effects of dietary vitamins C and E are commonly discussed together due to their antioxidant properties. These properties include, improving stress tolerance, immunological response and disease resistance in fish (Koshio, 2007). A recent study by Hamre et al. (2004) investigated the effect of feeding high levels, below toxic levels, and low levels, above deficient levels, of vitamins C and E, astaxanthin, lipid, iron, copper and manganese to post-smolt Atlantic Salmon (initial weight, 148 g), and reported vitamins C and E supplemented at levels of 30 and 1000 mg kg^{-1}, and 70 and 430 mg kg^{-1}, respectively, showed only minor effects on growth feed conversion and fillet quality. This led Hamre et al. (2004) to suggest that large variations in micro-nutrients have only minor effects on gross fish performance. To measure the health benefits of these vitamins, the actual requirement can be 10 to 100 times greater than for the minimum suggested dietary requirement (Sealy and Gatlin, 2002; Koshio, 2007). Diets deficient in vitamin C are association with immunosuppression and an increased risk to infectious diseases (Lim et al., 2001). Structural deformities such as scoliosis (sideways curvature) and lordosis (dorsal or ventral curvature) have been observed in several species of fish fed diets deficient in vitamin C including; Japanese Yellowtail (Sakaguchi et al., 1969), Japanese sea bass (Ai et al., 2004) and red drum (Aguirre and Gatlin, 1999). Shimeno (1991) and Hosokawa (1999) reported a vitamin C (ascorbic acid) requirement of 122 mg kg^{-1} dry diet was necessary for optimum growth of Japanese Yellowtail. We would recommend the same level for Yellowtail Kingfish (Shimeno 1991; Hosokawa 1999; Table 8.13); however, further research is needed for Yellowtail Kingfish.

Choline, a water soluble vitamin (B_4), has also been shown to play important roles in metabolic functions including: as a component of phosphatidylcholine, which has structural functions in membrane and tissue lipid utilization; as a precursor of the neurotransmitter acetylcholine; and as a precursor of betaine, which acts as a methyl donator (NRC, 2011). Choline deficient fish have been reported to show signs of anaemia, reduced growth and altered liver lipid metabolism (NRC, 2011). Fingerling Japanese Yellowtail have been reported to require 210-290 mg of choline chloride 100 g^{-1} of diet when fed nine levels of choline chloride (0, 10, 25, 50, 100, 200, 300, 400 and 800 mg per 100 g of diet) for a period of 20 days (Hosokawa et al., 2001). Hosokawa et al. (2001) also reported that deficiency signs of anorexia and reduced growth developed within 5 days, and disappeared within 3 days after re-feeding the fingerlings with a diet containing 800 mg choline chloride 100 g^{-1} of diet. The requirement for chlorine can differ greatly between species (Tables 8.13, 8.14 and 8.15. Rumsey (1991) suggested that 50% of the choline requirement for rainbow trout can be met from dietary betaine. Table 8.15 outlines the differences in choline requirements in both freshwater and marine species.

Table 8.15. A comparison of choline requirements based on weight gain for various freshwater and marine species[1].

Species	Requirement (mg kg^{-1} dry diet)	Reference
Pacific salmon	600-800 (MLS)	Halver (1972)
Rainbow trout	50-100	McLaren et al. (1947)
Japanese Yellowtail	2, 920 (MLS)	Shimeno (1991)
Hybrid striped bass	500	Griffin et al. (1994)
Red drum	588	Craig and Gatlin (1996)
Cobia	696	Mai et al. (2009)

[1] Table adapted from NRC (2011); MLS: Maximum liver storage.

Shimeno (1991) and Hosokawa (1999) reported a choline (vitamin B_4) requirement of 3 g kg^{-1} dry diet was necessary for maximum liver storage in Japanese Yellowtail. We would recommend the same level for Yellowtail Kingfish (Shimeno 1991; Hosokawa 1999; Table 6.1). Clearly, further investigation is necessary to understand the vitamin requirements for Yellowtail Kingfish.

Apparent Nutrient Digestibility and Availability for Common Feed Ingredients

There is increasing pressure to grow fish on diets containing low levels of marine protein and oils. This will be a difficult proposition in Yellowtail Kingfish given the shortage of information for nutritional requirements, coupled with the limited knowledge of ingredient nutrient digestibility and availability for fish of different sizes cultured at different water temperatures. Table 8.16 lists information specifically pertaining to proximate composition and energy nutrient digestibility for a range of ingredients commonly used in diets for the culture of Japanese Yellowtail (NRC, 2011) and Yellowtail Kingfish (Booth et al., 2010b). With the increased use of plant protein sources to replace fish meal, specific information will be required to formulate feeds balanced in available essential amino acids. Unfortunately, there is no information available for the availability of amino acids for Yellowtail Kingfish, and comparative information for the Japanese Yellowtail is limited, i.e. casein, corn gluten meal, fish meal, meat meal, soy protein concentrate (NRC, 2011). At this point in time, the formulation of diets for Yellowtail Kingfish can only be undertaken using proximate composition data and the limited information available for closely related *Seriola* spp. To further compound the problem, information concerning the effects of the anti-nutrients present in many of the alternative plant protein and oil ingredients are not known for Yellowtail Kingfish, and is also limited for related species.

Table 8.16. Apparent digestibility of common feed ingredients for Japanese Yellowtail and Yellowtail Kingfish.

Ingredient	Ingredient apparent digestibility coefficient (%)			
	Dry matter	Crude protein	Energy	Fat
Fish meal, not specified [1]	-	89	-	-
Fish meal, Peru [2]	79.4	87.3	86.9	94.2
Fish meal, Ecuador [2]	85.5	91.6	90.4	92.1
Casein [1]	-	95	-	-
Fish oil [2]	98.9	-	95.9	99.4
Poultry oil [2]	94.1	-	97.3	99.1
Canola oil [2]	94.2	-	97.3	98.1
Squid meal [2]	73.2	77.5	77.9	98.4
Krill meal [2]	84.2	88.9	89.3	96.6
Meat meal [1]	-	80	-	-
Meat meal [2]	71.5	77.5	82.0	94.7
Poultry meal [2]	79.2	76.4	83.1	90.3
Feather meal [2]	70.8	59.6	73.6	96.9
Blood meal [2]	80.9	86.6	84.2	-
Soybean meal [2]	70.3	79.2	75.5	69.2
Soy protein concentrate [1]	-	87	-	-
Corn, gluten meal [1]	-	50	-	-
Dehulled lupin [2]	69.1	85.5	74.2	67.6
Whole field peas [2]	61.2	79.3	66.6	-
Maize gluten [2]	34.7	50.7	37.4	62.3
Vital wheat gluten [2]	75.7	87.3	73.0	64.2

Dash indicates data were not available.
[1] Japanese Yellowtail data from NRC (2011).
[2] Kingfish data from Booth et al. (2010b).

Low or No Fish Meal Diets for Yellowtail Kingfish

This topic is an entire literature review within itself. Therefore, this section will only briefly discuss this topic in relation to the culture of Yellowtail Kingfish. There is considerable pressure to reduce the use of fish meal in diets for all marine species, including Yellowtail Kingfish. The two most pressing reasons are associated with ingredient cost and also sustainability. The majority of diet formulation for Yellowtail Kingfish in Australia has been based on the knowledge of nutrient requirements of salmonid fish. To a certain extent this is understandable, especially in the early stages of diet development due to the lack of species specific information. However, in the long term this is not a satisfactory approach. As pressure for fish meal substitution in Yellowtail Kingfish diets has gradually increased; initially due to the rise in price of this commodity, and more recently due to sustainability issues, dietary fish meal levels have been reduced dramatically from ~50% to below 30% over a relatively short period of two to three years. Concurrently, on-farm growth performance has been declining and problems associated with nutrient deficiencies and fish health have been escalating as fish meal has been taken out of the diets.

Experience in Japan has shown that to culture Japanese Yellowtail successfully, diets should not contain less than 30% fish meal and should be supplemented with taurine and other essential nutrients (Dr Masashi Maita, personal communication). In Japanese Yellowtail, lower growth and hypocholesteremia were induced by feeding extruded pelleted diets containing less than 30% fish meal without taurine supplementation (Aoki et al., 2000). As previously mentioned, the supplementation of taurine in diets for Yellowtail Kingfish where fish meal has been replaced by alternative protein sources would be essential based on the research in Japanese Yellowtail. The balance of essential amino acids and other vitamins and minerals should also be considered with fish meal substitution. Watanabe (2009) suggested the use of various plant proteins, together in Japanese Yellowtail feed, to reduce nutrient shortfalls. This practice is commonly used for a range of fish species. In Japanese Yellowtail, a combination of soy bean meal, corn gluten meal and meat meal is effective for improving growth performance by compensating for essential amino acid shortages of ingredient (Watanabe, 2009). Consideration must be given to inclusion levels of plant ingredients such as corn gluten meal, corn distiller's grains and dried algal meals etc. as they contain pigments which impact on flesh colour.

Information for the suggested limitation levels of alternative ingredient to replace fish meal in Yellowtail Kingfish diets is presented in Table 8.17. Certain ingredients commonly used to replace fish meal in aquafeeds may also impact on growth and health of Yellowtail Kingfish. For example, SESBM has been used as an alternative ingredient for a range of fish species with varying success. The main limitation of SESBM is that it contains a range of anti-nutritional factors that may have deleterious effects on fish health and growth, depending on the fish species and susceptibility (Francis et al., 2001). Recent studies by Bowyer et al. (2013a, Chapter 5; 2013c; Chapter 6) provided information that would suggest that SESBM was not suitable substitute for fish meal in diets for Yellowtail Kingfish, whereas, levels of up to 20% of solvent extracted soy protein concentrate may be safely used. Further to this, taking digestive tract samples from Yellowtail Kingfish from the Bowyer et al. (2013a, Chapter 5; 2013c, Chapter 6) studies, Bansemer (2011) and Bansemer et al. (In press; Appendix 4) reported that the mucous layer, the first line of defence against bacteria invasion, of the digestive tract tissue was progressively and significantly eroded and goblet cell numbers increased as 10 to 30% SESBM was included in the diet. Subsequent research has shown that the same alterations do not occur as the more refined solvent extracted soy protein concentrate is included in Yellowtail Kingfish diets at levels of up to 40% (Bansemer et al., In press; Appendix 4).

Table 8.17. Suggested ingredient limitations for Yellowtail Kingfish diets[1].

Ingredient	Suggested dietary inclusion level (% of diet)	Ingredient limitations for dietary inclusion
Fish meal	≥35%	Quality varies widely depending on the source, preferably use low ash meals (<10) (need to quantify taurine); rancidity a problem
Poultry meal	20%? Suitable for fish but level needs to be determined for Yellowtail Kingfish	Diet palatability (need to quantify taurine), lysine damage due to production processing, variable quality and nutrient composition
Meat meal (low ash)	20%	Amino acid quality (lysine) dependent on drying and processing method (Browning reaction); BSE concerns, especially for overseas market access
Meat and bone meal	10-20%? Suitable for fish but level needs to be determined for Yellowtail Kingfish	High ash, amino acid quality (lysine) dependent on drying and processing method (Browning reaction); BSE concerns
Feather meal	5-10% ? Suitable for fish but level needs to be determined for Yellowtail Kingfish	Nutritional composition and quality is variable; amino acids variable with low lysine levels compared to higher quality meat meals; High serine content
Blood meal	≤5%	High protein and good source of lysine, however, palatability issues, amino acid imbalance high levels of leucine, valine and phenylalanine (need to quantify taurine)
Soy protein concentrate	≤20	Cost, palatability issues, taurine reduction, lysine low
Wheat gluten	≤8%	Cost, pellet hardness issues
Corn gluten meal	≤10%	Very low nutrient availability, potential to colour fillets yellow, lysine low
Soybean meal (solvent extracted)	≤5%	Enteritis issues, taurine reduction, lysine low.
Lupins (de-hulled)	1-15%? Suitable for fish but level needs to be determined for Yellowtail Kingfish	Relatively low protein, non-starch polysaccharide (NSP) levels high and relatively indigestible, lysine low
Wheat starch	≤15%	Limitations on carbohydrate utilisation. Starch and fat digestibility reduced with increasing inclusion level
Wheat	<20%	Excellent for diet binding but carbohydrate utilisation limitations (potential starch and fat digestibility limitations if levels too high; NSPs not well digested), lysine low
Fish oil	100% of fish oil added to diet	Preferably high EPA and DHA. Low peroxide value (<5meq kg^{-1} fat), prone to rancidity
Poultry oil	≤50% fish oil replacement	Energy source, deficient in essential fatty acids, must ensure EPA and DHA levels in diet are adequate; quality may vary depending on proceeding method; when using this product
Canola oil	0%	Deficient in essential fatty acids, growth reduced, green liver concerns

[1] Please note: there has been limited work specifically done on Yellowtail Kingfish so the information presented here is only a guide.

The freshness of ingredients is important, particularly if the ingredient contains high levels of oxidisable fats. Rancidity of the oil in the fish meal or of the fish oil alone may impact on the performance and health of Yellowtail Kingfish. The peroxide value (PV) measures the rancidity of oil in the diet or ingredient (NRC, 2011). A PV value of <5 meq kg^{-1} has been suggested for the quality standards of fish meal and fish oil for salmonid diets (NRC, 2011), while fresh herring oil has been reported to have a PV value of 5 meq kg^{-1} fat (Hung et al, 1981). Hung et al. (1981) also reported PV values for slight, moderate, high and extreme levels of rancidity in rainbow trout diets containing 8% of herring oil of 25, 51, 120 and 314 meq kg^{-1} fat, respectively. It is important to appreciate that oxidative rancidity of fats in feed ingredients is a complex process that occurs in three phases: 1, initiation; 2, auto-oxidation; and 3, termination. As the rancidity reaction is ongoing in fats, higher PV levels will be found in feeds as time progresses. However, PV alone is not a reliable indicator for controlling the quality of product as the value is transient and may peak and then decline (i.e. you may not know what side of the PV peak the sample is on without sensory evaluation and other tests). Additional tests, including TBARS, are required to monitor product quality of feeds. It is worth noting that if feed tastes or smells rancid, it is reasonable to accept that it is rancid.

In order to optimise diets containing low levels of fish meal for Yellowtail Kingfish there is an urgent need to move away from using salmonid nutrient requirement data. More research is required to determine the specific nutrient requirements and the safe dietary inclusion levels of alternative protein and oil sources for optimum for growth and health of Yellowtail Kingfish.

Conclusion and Recommendations

A summary of suggested nutrient requirements are presented in Table 8.18 which is based on the limited knowledge presently available for Yellowtail Kingfish, closely related *Seriola* spp. and other non-related species of marine and freshwater fish. In conclusion, very little is currently known of the actual nutrient requirements of Yellowtail Kingfish. Feed companies are dependent on using information for other species to formulate diets for this Yellowtail Kingfish. The information presented in this review demonstrates that there are large knowledge gaps in our understanding of the nutritional requirements for Yellowtail Kingfish. Apart from the excellent information on temperature and size dependent protein and energy requirements for Yellowtail Kingfish up to 2 kg provided by Booth et al. (2010), other nutrient requirement information specific to Yellowtail Kingfish has not been determined. Information for closely related *Seriola* spp. and other marine species may be used to formulate diets for Yellowtail Kingfish in the interim. However, this must be done with caution as there have been many instances of differences in the nutrient requirements on inter- and intra-specific levels with fish. To add to this, many of the nutrient requirement studies discussed throughout the review were carried out under optimal conditions and dietary requirements may differ considerably when fish are exposed to stressors in the challenging environment of the sea cage (Lygren et al., 2001).

Taurine supplementation has recently received a great deal of attention for Yellowtail Kingfish and cobia. As previously noted, Japanese Yellowtail cannot synthesise taurine, therefore, the provision of this sulphonated organic acid is conditionally essential. Levels of 5 to 8 g kg^{-1} diet have been recommended for Japanese Yellowtail when dietary fish meal levels are> 30%. More may be required when fish meal taurine levels are low, or when dietary fish meal levels fall below 30%. As a precautionary measure, Yellowtail Kingfish diets in the USA are now routinely supplemented with 10 g taurine kg-1 diet. It is recommended that diets for Australian Yellowtail Kingfish are supplemented with dietary taurine at similar levels until more is known about the conditional requirement of this nutrient for this species.

Table 8.18. The summary of suggested nutrient requirements for Yellowtail Kingfish[1].

Nutrient	Fish size	Requirement	Units	Species	Reference
Digestible Protein (DP)	<200 g	456	g/kg dry diet	Yellowtail Kingfish	Booth et al. (2010)
	200-1000 g	465	g/kg dry diet	Yellowtail Kingfish	Booth et al. (2010)
	>1000 g	432	g/kg dry diet	Yellowtail Kingfish	Booth et al. (2010)
Digestible Energy (DE)	<200 g	12	MJ/kg dry diet	Yellowtail Kingfish	Booth et al. (2010)
	200-1000 g	15	MJ/kg dry diet	Yellowtail Kingfish	Booth et al. (2010)
	>1000 g	18	MJ/kg dry diet	Yellowtail Kingfish	Booth et al. (2010)
DP : DE	<200 g	38	g DP/MJ DE	Yellowtail Kingfish	Booth et al. (2010)
	200-1000 g	31	g DP/MJ DE	Yellowtail Kingfish	Booth et al. (2010)
	>1000 g	24	g DP/MJ DE	Yellowtail Kingfish	Booth et al. (2010)
Essential Amino Acids					
Arginine		3.7	% protein	Japanese Yellowtail	Watanabe (2009)
Histidine		2.7	% protein	Japanese Yellowtail	Watanabe (2009)
Isoleucine		2.5	% protein	Japanese Yellowtail	Watanabe (2009)
Leucine		4.4	% protein	Japanese Yellowtail	Watanabe (2009)
Lysine		5.0	% protein	Japanese Yellowtail	Watanabe (2009)
Methionine		2.6	% protein	Japanese Yellowtail	Ruchimat et al. (1997a)
Methionine+Cysteine		2.3	% protein	Japanese Yellowtail	Watanabe (2009)
Tyrosine+Phenylalanine		4.3	% protein	Japanese Yellowtail	Watanabe (2009)
Threonine		2.7	% protein	Japanese Yellowtail	Watanabe (2009)
Tryptophan		0.6	% protein	Japanese Yellowtail	Watanabe (2009)
Valine		2.9	% protein	Japanese Yellowtail	Watanabe (2009)
Taurine[2] (diet FM >30%)		5	g free/kg diet	Japanese Yellowtail	Maita (per. comm.)
Taurine[2] (diet FM <30%)		10 to 30	g free/kg diet	Japanese Yellowtail	Takagi et al. (2006)
Lipids					
Crude lipid		15 to 20	% diet	Japanese Yellowtail	Masumoto (2002)
	>1.5kg	23 to 28	% at high temp low CP	Japanese Yellowtail	Maita (per. comm.)
LC n-3 HUFA	juvenile	2.0	% dry diet	Japanese Yellowtail	Deshimaru et al. (1982)
LC n-3 HUFA	larval	3.9	% dry diet	Japanese Yellowtail	
DHA		1.4 to 2.6	% dry diet	Japanese Yellowtail	Furuita et al. (1996b)
EPA		3.7	% dry diet	Japanese Yellowtail	Furuita et al. (1996b)
Digestible carbohydrate		<10	% diet	Japanese Yellowtail	Shimeno (1991)
Minerals					
Phosphorus		6.7	g available P/kg dry	Japanese Yellowtail	Shimeno (1991)
Magnesium		0.7	g/kg dry diet	Rainbow trout	DaBrowska et al. (1989)
Copper		5-10	mg/kg dry diet	Atlantic Salmon	Lorentzen et al. (1998)
Zinc		20	mg/kg dry diet	Red drum	Gatlin et al. (1991)
Manganese		12	mg/kg dry diet	Rainbow trout	Ogino & Yang (1980)
Iodine		1.1	mg/kg dry diet	Chinook salmon	Woodall & LaRoche (1964)
Selenium		0.38	mg/kg dry diet	Rainbow trout	Hilton et al. (1980)
Vitamins					
A		5.6	mg/kg dry diet	Japanese Yellowtail	Shimeno (1991)
D		>60	µg/kg dry diet	Rainbow trout	Barnett et al. (1982)
E		120	mg/kg dry diet	Japanese Yellowtail	Shimeno (1991)
C		122	mg/kg dry diet	Japanese Yellowtail	Shimeno (1991)
H		0.67	mg/kg dry diet MLS	Japanese Yellowtail	Shimeno (1991)
B1		11.2	mg/kg dry diet MLS	Japanese Yellowtail	Shimeno (1991)
B2		11	mg/kg dry diet MLS	Japanese Yellowtail	Shimeno (1991)
B3		12	mg/kg dry diet	Japanese Yellowtail	Shimeno (1991)
B4		2.92	g/kg dry diet MLS	Japanese Yellowtail	Shimeno (1991)
B5		36	mg/kg dry diet MLS	Japanese Yellowtail	Shimeno (1991)
B6		11.7	mg/kg dry diet MLS	Japanese Yellowtail	Shimeno (1991)
B8		423	mg/kg dry diet MLS	Japanese Yellowtail	Shimeno (1991)
B9		1.2	mg/kg dry diet MLS	Japanese Yellowtail	Shimeno (1991)
B12		0.053	mg/kg dry diet	Japanese Yellowtail	Shimeno (1991)

CP = crude protein; MLS = maximum liver storage levels

[1] Data for taurine supplementation is dependent on dietary fish meal (FM) substitution.

Given that Yellowtail Kingfish are extremely fast growing and have an extremely high metabolic rate, as a precaution their diets will need to be formulated with high nutrient specifications using high quality, readily digestible ingredients to meet their nutritional requirements. To add to this, without additional knowledge, and due to the current status of

fish meal and fish oil supply and prices, one would assume that the diets for Yellowtail Kingfish will be more expensive than diets currently used for the culture of salmonid and other fish species that have had their nutritional requirements relatively well determined. Clearly, when replacing dietary fish meal and fish oil in the quest to manufacture sustainable diets more nutrition research is needed in the areas of essential amino acid requirements, supplemental taurine, adequate levels, and ratios, of DHA to EPA, fortification with vitamins and minerals, particularly, vitamin A, D, C and E, iron, selenium, and available phosphorous levels to ensure optimum growth and reduce effluent.

CST are currently using the information from this review to ensure that nutrient specifications of current Yellowtail Kingfish production diets are adequate, and also to formulate, and test, new improved diet formulations prior to their incorporation into production. CST also plan to use the information provided from this review and project, in combination with previous work, as the foundation to attempt to push on-farm whole cycle feed conversion ratios (FCR) from above 2:1 to 1.7:1 for Yellowtail Kingfish.

Chapter 9. **Project benefits and adoption, and outcomes**

Benefits and Adoption

This project has delivered the Australian aquaculture industry and feed companies essential information to improve the formulation of sustainable diets for Yellowtail Kingfish at optimal and suboptimal water temperatures. The project has also provided a range of specific outcomes and outputs that will further enhance the industry's knowledge of nutrition and health issues and what is needed to address them in the future. The extremely close interaction between CST industry participants, Yellowtail Kingfish feed manufacturers and scientists for all aspects of this project has resulted in rapid information flow both ways, with the vast majority of results and recommendations of interest being adopted. A list of future research and recommendations based on the findings arising from this project are also presented in Chapter 10.

Specific areas of benefits and adoption by CST, producers and feed companies have included:

- New information regarding the use of canola oil and poultry oil as a fish oil substitute to aid in the development of sustainable diets has been developed (Chapters 3 and 4).
    - This information has been adopted by CST and the Australian feed companies to limit the inclusion of these ingredients when formulating new sustainable diets for the healthy production of Yellowtail Kingfish at optimal (22 °C) and suboptimal (18 °C) water temperatures.
- New information regarding the exclusion of SESBM as a fish meal substitute in Yellowtail Kingfish diets has been developed (Chapter 5).
    - This information has been adopted by CST and the Australian feed companies to exclude this ingredients inclusion when formulating new sustainable diets for the healthy production of Yellowtail Kingfish at optimal (22 °C) and suboptimal (18 °C) water temperatures.
- New information regarding the use of SPC as a fish meal substitute has been developed (Chapter 6).
    - CST and the Australian feed companies have adopted this information to limit the inclusion of this ingredient when formulating new sustainable diets for the healthy production of Yellowtail Kingfish at optimal (22 °C) and suboptimal (18 °C) water temperatures.
- New information has been developed (Chapter 7) in relation to the growth, feed utilisation and digestive enzyme physiology of Yellowtail Kingfish in response to hypoxic conditions at a range of water temperatures.
    - This information has been adopted by CST to develop and improve on-farm feeding practices, especially in relation to periods of high water temperature and low dissolved oxygen levels.
- The literature review reporting on the current status of knowledge of the nutrient requirements of Yellowtail Kingfish (Chapter 8) provides benefits to CST, Yellowtail Kingfish producers, feed companies and R & D providers. The suggested nutrient requirements for Yellowtail Kingfish from the review are also summarised in Table 8.18.
    - CST's management has adopted this information to:

        - Ensure that commercial feeds are formulated specifically for Yellowtail Kingfish;
        - Use as a baseline for formulating commercial production diets for Yellowtail Kingfish;
        - Formulate new diets with nutrient specifications designed specifically for Yellowtail Kingfish for pilot scale testing, either in-house at using their Arno Bay facilities, or externally at SARDI Aquatic Sciences, prior to implementation in on-farm yellowtail production systems.
        - Formulate diets to reduce disease incidents and severity within their production systems when fish are exposed to high stress during times of low or high water temperatures; and;
        - CST plan to use the information provided from this review and project, in combination with previous work, as the foundation to attempt to push on-farm whole cycle feed conversion ratios (FCR) from above 2:1 to 1.7:1 for Yellowtail Kingfish.
    - Other Yellowtail Kingfish producers may also use this information in a similar manner to CST.
    - The feed companies may also adopt information from this review to formulate diets specific to Yellowtail Kingfish.
    - R & D providers may use the information provided in the literature review to identify areas for further studies to improve our knowledge of Yellowtail Kingfish nutrient requirements and ultimately improve dietary formulations, farm productivity, fish health and sustainability.
- The project has provided a great deal of benefit to the future of the aquaculture industry by providing high quality relevant training in the field of fish nutrition, fish physiology and health to a large number of undergraduate and post graduate students. The project directly trained one visiting Post Docoral Research Fellow, one PhD student, six Honours students and several undergraduate students. The names and projects of the students are listed in Appendix 1. Several of these students have been either employed directly by companies participating within the aquaculture community, or have gone on to do post graduate studies on other AS CRC or industry related projects.

Planned Outcomes

Yellowtail Kingfish is the main closed cycle finfish cultured in SA and this industry has great potential to expand in other states of Australia. One of the major inefficiencies identified was feeds and feed management in CST's Yellowtail Kingfish production. Henceforth, the newly formed CST RMAG identified improving FCRs in Yellowtail Kingfish operations as an urgent priority. Upon reflection, the production efficiency of Yellowtail Kingfish by CST has been hindered by the poor understanding of feeding strategies, sea cage biomass determination, growth performance and the nutrient requirements of Yellowtail Kingfish at different life stages and water temperatures. It was also apparent that in order for CST to gain market acceptance for their Yellowtail Kingfish products on a global basis, the issue of sustainable use of marine ingredients, such as fish meal and fish oil, for the production of Yellowtail Kingfish needed to be addressed. In order to address the issue of sustainability detailed changes to current dietary formulations for Yellowtail Kingfish, which contained high levels of fish meal and fish oil, had to be made. Species specific information regarding nutrient

availability, growth performance, fish health and maximum dietary inclusion levels of alternative sustainable ingredients for Yellowtail Kingfish cultured at fluctuating water temperatures were required in order to make these changes.

Overall, the outcomes that should arise from the commercialisation of research outputs from this project will be an improved feed management system contributing to a reduction in FCRs from above 2:1 to 1.7:1, and revised and more sustainable Yellowtail Kingfish diet formulations (i.e. 25-30% lower proportions of marine based proteins and lipids).

Due to AS CRC approved variations, this project specifically addressed the topics of improving the sustainable use of marine ingredients, and growth performance and health of Yellowtail Kingfish cultured at optimal (22 °C) and suboptimal water temperatures (18 °C). This information is presented in Chapter 2 to 7 in this report. The project also took the initial steps toward improving our understanding of the nutrient requirements of Yellowtail Kingfish (Chapter 8). Steps were taken throughout this project to ensure the rapid extension of knowledge to CST management, feed company participants and fish health groups (Chapter 2).

Outcomes Achieved

Public Benefit Outcomes

The majority of the research described in this project has been extended to the broader scientific community. Apart from the extension of results to CST, numerous presentations were given by project participants to extend the information arising from this project. Project information was extended to other members of the aquaculture and feed industry, government departments, the general public and members of the scientific communities. Information was disseminated at domestic and international scientific conferences, AS CRC and industry workshops, directly to feed company representatives and the general public. Thirteen scientific publications, seven theses, three reports and eleven conference abstracts, and numerous presentations containing information specifically targeting ways to enhance the sustainable production of Yellowtail Kingfish, arose from this project.

Additionally, the information generated from this project is being used by CST and feed companies, both domestically and internationally, to reduce our reliance on fish meal and fish oil from the marine environment for the production of fish. This equates to a significant public benefit as it will directly reduce our reliance and impact on the marine environment for the production of Yellowtail Kingfish and related *Seriola* spp.

There are also a considerable number of outputs arising from this project that will directly benefit the public.

The education and training component of the project has also achieved a significant public benefit outcome. The project has produced a large number of undergraduate and post graduate students who have been provided with high quality relevant training in the field of fish nutrition, feed technology, physiology and health. The student details are provided in Appendix 1.

Private Benefit Outcomes

The major private outcome of this project is that CST management have acted on the knowledge and recommendations provided within this report, in collaborations with feed companies, to formulate and manufacture improved diets for the seasonal production of Yellowtail Kingfish in their sea cage operations. The production diets have been formulated to:

- Contain nutrient specifications to meet the animal's nutritional requirements to ensure improved health, growth and FCRs.
- Safely utilise greater levels of two dietary ingredients to produce cost effective and sustainable diets:
    - SPC at dietary inclusion levels of up to 20%, to replace up to 20% of dietary fish meal; and
    - Poultry oil at dietary inclusion level of up to10-15%, to replace up to 50 to 75% of dietary fish oil.
- Improve market access by a 33% improvement in the sustainable production of Yellowtail Kingfish by reducing the inclusion levels of dietary marine ingredients from ~60% down to 40%.
- Exclude or limit the inclusion of dietary SESBM and canola oil. Both of which may be detrimental to the health, growth and FCRs of cultured Yellowtail Kingfish, especially at the lower suboptimal water temperature of 18 °C.
- Be used in conjunction with information pertaining to Yellowtail Kingfish growth and feeding behaviour at optimal and suboptimal temperatures in normoxic and hypoxic conditions (Chapters 3 to 7) to improve on-farm feeding practices, and attempt to reduce on-farm whole cycle FCRs from above 2:1 to 1.7:1 for Yellowtail Kingfish.

There are also a considerable number of outputs arising from this project that will directly benefit CST.

All information from this project has been extended to CST management (Chapter 2), as it has come to hand, and they have acted on it to improve the sustainable production of Yellowtail Kingfish in their sea cage operations. Additionally, all information from Chapters 3 to 7 have been scrutinised and published in international peer reviewed scientific journals, and has been very well received when presented, both domestically and internationally, to the broader scientific community. CST also plan to use the information provided from this review and project, in combination with previous work, as the foundation to attempt to push on-farm whole cycle FCR from above 2:1 to 1.7:1 for Yellowtail Kingfish.

The project provided new information for the inclusion of two practical alternative oil sources to replace fish oil (poultry and canola oil), and two alternative plant protein sources, solvent extracted soybean meal (SESBM) and soy protein concentrate (SPC) as potential substitutes for fish meal protein at optimal and suboptimal water temperatures. These ingredients were selected as they were being used by the Australian feed companies in commercial Yellowtail Kingfish diets, with little or no Yellowtail Kingfish specific information regarding growth performance or fish health.

The results from the short-term studies with juvenile fish indicate that SPC and poultry oil are excellent candidates to use in the production of sustainable diets for Yellowtail Kingfish. In contrast, under the conditions tested in this project, SESBM and canola oil appeared to less favourable, and did appear to produce some health related problems with Yellowtail Kingfish, especially at the lower suboptimal water temperature of 18 °C. SESBM dietary inclusion sustained poor growth and produced early signs of enteritis in the digestive tract, while canola oil inclusion also produced poor growth and symptoms of green liver. It is recommended that further long-term studies are required to validate the fish meal and fish oil findings from this project.

A large amount of information was also generated with regards to Yellowtail Kingfish growth and feeding behaviour at optimal and suboptimal temperatures in normoxic and hypoxic conditions (Chapters 3 to 7). This information is essential in developing feeding practices on-farm, especially in relation to times of high water temperatures when oxygen levels are at their lowest.

A comprehensive literature review reporting on the current status of knowledge of the nutritional requirements of Yellowtail Kingfish (Chapter 8). The suggested nutrient requirements for Yellowtail Kingfish from the review are also summarised in Table 8.18. The review points our many shortfalls in our knowledge base for this species, but it also provides a large body of information gleaned from research with other closely related *Seriola* spp. and other marine fish. This comprehensive review contains nutrient requirement information that is now being used by CST to improve the nutritional profile of their production diets, which will result in improvements in feed efficiency and fish health outcomes.

Linkages with CRC Milestone Outcomes

The outputs and outcomes from this project address the following AS CRC Outputs and Milestones:

- Outputs 1.3 - Removal or reduction of key production constraints in selected aquaculture systems;
- Milestones 1.3.4 - New low-cost aquaculture diets targeting improved feed conversion developed and evaluated; and
- Milestones 1.3.5 - Production efficiency gains from genetic, health management and nutritional interventions quantified to inform long-term strategies and estimate commercial benefits.

Chapter 10. **Further development, conclusions and recommendations**

Further Development

This research has resulted in a preliminary understanding of some baseline nutritional requirements to further the development of sustainable cost-efficient diets for the culture of healthy Yellowtail Kingfish. The baseline results generated from the current research may be incorporated into follow-on studies in future projects. However, in the current research there were a few limitations and unsolved issues, or areas that were not covered in the trials, which require further investigation. Therefore, these limitations and suggested approaches have been outlined below:

1. The experimental trials in this research were carried out within a limited time frame and in a laboratory setting, which could have presented limitations to the validity of results. It is suggested to use this baseline information and run longer-term, more commercial scale dietary studies, to 1) obtain more realistic research data within a sea cage setting using commercial scale stocking densities and under the normal daily and seasonal environmental conditions; and 2) may enable the full manifestation of some of the health aspects identified in this research and provide further insight on the precise causes and possible prevention and management of these nutrition/health problems.

2. Further exploration into the green liver syndrome would be beneficial. The occurrence of this syndrome in the lipid trial was unexpected, therefore more sample collections and analyses would aid in further knowledge on this condition and possible prevention techniques. Particular attention should be given to the interaction between taurine and cholesterol and the potential development of the green liver syndrome.

3. The main research area requiring further validation is a more in-depth histological analysis of the gastrointestinal tract. Despite the significant increase of goblet cell abundance (responsible for mucus production) and reduction of supranuclear vacuolisation of the absorptive enterocytes (primary site of digestion) in the gastrointestinal tract, the changes appeared to be independent of the dietary factors as these changes appeared in initial fish samples. Therefore, it is recommended that histological samples are collected from wild Yellowtail Kingfish gastrointestinal tracts to ascertain normal baseline histological scores for this species to validate the scoring system for diagnosing enteritis. In addition, if the baseline normal values are different, then the scoring system needs to be updated from an Atlantic Salmon based assessment, to a Yellowtail Kingfish specific one, for a more accurate assessment of future histological data.

4. Histological examination of the morphology of the liver when fish were fed diets containing alternative lipids was not covered in this research as the main focus was on the histology of the gastrointestinal tract. Therefore a more comprehensive analyses of the liver to dietary and temperature effects would be recommended.

Conclusions

Extension of this work to industry fish producing participants and feed companies has been very successful. The results described in each of these chapters were communicated directly and rapidly to Michael Thomson, and Trent D'Antignana R & D Managers, CST as the work was undertaken, and in many instances also provided to other relevant CST employees (e.g. fish production and health managers) as soon as they became available and subsequently as draft reports and scientific publications. A large amount of nutritional information was provided to CST R & D and management staff at the technology transfer meeting held at Port Lincoln Marine Science Centre in March 2011. The details of this meeting were reported in Chapter 2. In response to this research and the directives of CST, the feed companies in Australia have altered their production diets for Yellowtail Kingfish to exclude canola oil and SESBM. Information produced after this meeting has also been extended rapidly. CST has used the information provided from the fish meal and fish oil substitution research (Chapters 3 to 6) and nutrient requirement data from the literature review (Chapter 8), to specify and request dietary nutrient and ingredient inclusion/exclusion from the commercial feed manufacturers for their production diets. CST are using the information provided in the literature review, which is summarised in Table 8.18, to ensure that nutrient specifications of current commercial diets used in their Yellowtail Kingfish production are up to scratch. CST have used, or plan to use the information provided from this project to test diet formulations on a pilot scale in their tuna hatchery facilities at Arno Bay, SA, prior to incorporation into commercial Yellowtail Kingfish production systems. CST also plan to use the information provided from this review and project, in combination with previous work, as the foundation to attempt to push on-farm whole cycle feed conversion ratios (FCR) from above 2:1 to 1.7:1 for Yellowtail Kingfish. Ridley aquafeed and Skretting Australia have also used the information arising from this project to aid in the formulation of their commercial Yellowtail Kingfish diets.

With regards to fish oil substitution (Chapter 3) the complete replacement of fish oil with 100% poultry oil and only 50% replacement with canola oil, had no detrimental effects on the growth of Yellowtail Kingfish under our test conditions. However, complete replacement of fish oil with 100% canola oil resulted in poor fish growth compared with the 100% fish oil diet, regardless of temperature, and lead to a pronounced occurrence of green liver. These results are certainly useful in dietary formulation to reduce feed cost in Yellowtail Kingfish farming. Complete substitution of fish oil with the oils tested in this study is not desirable, as essential fatty acid requirements for EPA, DHA and ARA would not be met, and over the long term, symptoms of fatty acid deficiency would become apparent. However, a longer term study over an entire production cycle of Yellowtail Kingfish, using high levels of poultry oil substitution or a maximum of 50% canola oil substitution' while ensuring essential fatty acid requirements are met, is recommended to confirm the application of our finding to commercial production. In particular, changes in the FA composition of the muscle tissue, specifically n-3 LC-PUFA and the n-3:n-6 ratio, induced by the replacement of 100% fish oil with alternative lipid ingredients may influence the market value of these fish. Therefore, the flesh lipid composition and fish flavour should be evaluated once fish are nearer to market size. If necessary, we would recommend a feeding strategy, which includes the use of 'finisher diets', when fish are approaching market size range.

The actual saving in diet costs associated with fish oil substitution will be noticeable, but will be dependent on oil prices, and will fluctuate over time. However, more importantly, fish oil substitution will address the pressing issue of the sustainable use of marine resources for the production of aquacultured fish. Ultimately, fish oil substitution will be necessary to ensure global market access, especially in our current environment where merchants and consumers are sensitive and aware of the sustainability issue.

Under the conditions tested in this project (Chapter 4) our research has provided insights into the digestive functioning of Yellowtail Kingfish fed diets substituting fish oil with poultry oil and canola oil. The substitution of fish oil with 100% canola oil led to a reduction in trypsin and lipase activity, particularly at the cooler water temperatures, which corroborates with our previous findings that fish growth is negatively related to the inclusion of canola oil. The lipase activity in Yellowtail Kingfish was found to be higher in diets containing fish oil compared to the 100% poultry oil and canola oil diets. It is important to note that the diets were formulated to ensure the requirements for essential fatty acids were met, despite the high substitution of fish oil with poultry and canola oil. And although the histological study did not reveal any signs of diet-induced enteritis in the digestive tract, a high number of goblet cells and low supranuclear vacuolisation were identified in fish fed all diets and in the initial fish prior to the experiment. This suggests the need to validate the histology scoring system specifically for Yellowtail Kingfish. In addition, a longer period of exposure to inappropriate nutrition may result in different, or more pronounced, structural and functional changes to the digestive system. Therefore, we recommend longer term studies to investigate the effects of fish oil substitution and suboptimal water temperatures on the digestive enzyme activities and digestive tract health and function in Yellowtail Kingfish.

Regarding the results reported in Chapters 5 and 6, the substitution of fish meal with SESBM significantly decreased the growth, feed efficiencies, nutrient retentions and apparent dietary nutrient digestibilities as SESBM inclusion increased in this project. The inclusion of SESBM did not induce any gross gastrointestinal histological problems in this project, but supplementary work within this study by Bansemer (2011) and Bansemer et al. (In press; Appendix 4), reported histological alterations to the digestive tract on a finer, but not inconsequential, scale in regard to the erosion of the mucus layer and increased goblet cell numbers in response to dietary SESBM inclusion and water temperature. Additionally, no signs of acute enteritis were observed in the foregut or hindgut of the digestive tract of Yellowtail Kingfish fed diets containing SPC at levels of up to 40%. While Bansemer et al. (In press; Appendix 4), did not observe any issues associated with SPC inclusion and the alteration of the mucus layer or goblet cell abundance in the digestive tract. Therefore, it is suggested that further research is required to first identify the normal histology and structure of the digestive tract from wild-caught Yellowtail Kingfish. Then if necessary, develop a new scoring system for identifying the signs of enteritis in cultured Yellowtail Kingfish. The inclusion of SESBM above 20% caused reductions in growth performance, and inclusions above 10% caused reductions in nutrient retentions and digestibility, while the inclusion of increasing levels of SESBM resulted in significant increases in the erosion of the mucus layer of the digestive tract. Based on this information, SESBM is not recommended for use in Yellowtail Kingfish diets. Further research is required to clarify the potential long term health effects on the digestive tract of Yellowtail Kingfish associated with feeding SESBM.

In the interim, the more refined soy protein ingredient may be used an alternative ingredient, as Bowyer et al. (2013c; Chapter 6) demonstrated that 20% SPC inclusion was a suitable substitution level for fish meal in diets for Yellowtail Kingfish. However, feed intake was numerically decreased above 20% SPC inclusion, particularly at the lower water temperature, so possible feeding stimulants may be beneficial to improve feed intake. Essential amino acid and taurine supplementation would also be required. In addition, we also recommend that a longer term studies would be beneficial to determine the effects of SESBM and SPC on growth performance, feed efficiency and health of Yellowtail Kingfish over an extended time period. The actual saving in diet costs associated with fish meal substitution may be noticeable, but will be dependent on ingredient prices, and will fluctuate over time. However, as previously mentioned, more importantly, fish meal substitution will address the pressing issue of the sustainable use of marine resources and help to ensure global market access.

Yellowtail Kingfish have a fairly narrow and parabolic temperature range over which growth and FCE are optimal. The results reported in Chapter 7 (Bowyer et al., 2013b) demonstrated that culture temperatures as little as ± 3 °C below or above 24 °C led to decreases in the growth potential and aberrations in FCE, which at the cooler temperatures was possibly linked to a reduction in trypsin activity and a reduction in protein digestibility. With regard to the culture environment, importantly, juvenile Yellowtail Kingfish appear to be somewhat tolerant of short term hypoxia, but chronic exposure to hypoxic conditions in sea cages will severely retard growth potential, worsen FCE and lead to increased mortality. This condition may be further exacerbated as fish increase in size and stocking density in the sea cage environment. The outcome of the research in this project emphasises the importance of providing normoxic conditions in sea cages for juvenile and larger Yellowtail Kingfish, and has wider implications for the water quality management and production outcomes for the sea cage farming of Yellowtail Kingfish. In addition, information on the effects of water temperature and dissolved oxygen level on the growth and digestive capacity of juvenile Yellowtail Kingfish provide further information towards improving the feed management practices for this species under changing environmental conditions.

With regards to the current status of knowledge of the nutritional requirements of Yellowtail Kingfish reported in Chapter 8, little is known and we are dependent on using information from related and unrelated species to formulate diets for this species. The information presented in this review demonstrates that there is a large knowledge gap in our understanding of the nutritional requirements for Yellowtail Kingfish. Apart from the excellent information on the temperature and size dependent protein and energy requirements for Yellowtail Kingfish provided by Booth et al. (2010), most of the other nutrient requirements specific to Yellowtail Kingfish have not been determined. Information presented here for the closely related *Seriola* spp. and other marine species may be used to formulate diets for Yellowtail Kingfish in the interim. However, this must be done with caution. There have been many instances of large unexpected differences in the nutrient requirements on inter- and intra-specific levels with fish. To add to this, many of the nutrient requirement studies examined and discussed throughout the review were carried out under near optimal conditions. Suggested dietary requirements may differ considerably when fish are exposed to stressors in the challenging environment of the sea cage (Lygren et al., 2001).

Taurine supplementation has recently received a great deal of attention with Yellowtail Kingfish and cobia. As pointed out in this review, the closely related Japanese Yellowtail cannot synthesise taurine endogenously, therefore, the dietary provision of this sulphonated organic acid is conditionally essential. Levels of 5 to 8 g kg^{-1} diet have been recommended for Japanese Yellowtail when dietary fish meal levels of at least 30% are used. More so, when fish meal taurine levels are low, or when dietary fish meal substitution reduced dietary fish meal levels below 30%. As a precautionary measure, Yellowtail Kingfish diets in the USA are now routinely supplemented with 10 g taurine kg^{-1} diet. It is recommended that diets for Australian Yellowtail Kingfish are supplemented with dietary taurine at similar levels until more is known about the conditional requirement of this nutrient for this species.

Given that Yellowtail Kingfish are extremely fast growing and have an extremely high metabolic rate, their diets will need to be formulated with very high nutrient specifications using high quality, readily digestible ingredients to meet their nutritional requirements. To add to this, without additional knowledge, and due to the current status of fish meal and fish oil supply and prices, one would assume that the diets for Yellowtail Kingfish will be more expensive than diets currently used for the culture of salmonid and other fish species that have had their nutritional requirements relatively well determined. Clearly, when replacing dietary fish meal and fish oil in the quest to manufacture sustainable diets more nutrition research is needed in the areas of essential amino acid requirements, the addition of supplemental taurine, adequate levels and ratios of DHA to EPA, fortification with vitamins

and minerals, particularly, vitamin A, D, C and E, iron, selenium, and available phosphorous levels to ensure optimum growth and reduce effluent.

The research presented in this report has screened the use of four dietary ingredients of commercial interest, for their potential inclusion or removal, in commercial diets for Yellowtail Kingfish. Information provided by this research has led to the development of more cost-effective, sustainable diets for the culture of healthy Yellowtail Kingfish, at optimal and suboptimal water temperatures. The results from these trials, and the literature review in Chapter 8, have highlighted that there is more research required on the nutritional requirements of Yellowtail Kingfish, particularly in relation to nutrient-environment interactions. Particular focus should be on ensuring that anti-nutritional factors or dietary deficiencies do not lead to the occurrences of diet-induced enteritis, green liver syndrome or other potential growth limiting factors and health problems. The culture of Yellowtail Kingfish, like most species, is complex and as outlined in this research, the industry are faced with a multitude of health problems directly associated with nutrient-environment interactions, specifically green liver syndrome and a potentially diet-induced enteritis. Although the majority of findings and conclusions arising from this research are based upon the fish tested within laboratory conditions and over a short duration of time (~5 weeks), these findings have given further insight into these health conditions. As well as a deeper understanding of the response of Yellowtail Kingfish to temperature and dietary manipulations, the insights gained from this research can be used in future investigations. The commercial production of Yellowtail Kingfish is a relatively new industry, with commercial production in Australia only coming from one location at present and almost exclusively from one company. However, based on results of this research, the feed companies in Australia have altered the formulation for their production diets for Yellowtail Kingfish and they no longer contain canola oil or SESBM, although further long term research is necessary to validate these outcomes. Therefore, the results of this research are novel and have provided baseline nutritional information that have enhanced the Australian seafood industry by providing new information to help increase the sustainable production of Yellowtail Kingfish through an increase in production efficiency and profitability. In addition, it has provided new information to produce quality and nutritious seafood for consumers.

Recommendations

1. Normoxia in sea cages for juvenile Yellowtail Kingfish is important, especially at high water temperature. Therefore, careful monitoring of dissolved oxygen levels especially during summer, particularly during, and immediately following feeding, is recommended to ensure that the levels in the sea cages are as close to normoxic as possible. Monitoring the extended weather forecast for conditions such as heat waves, storm events or dodge tides would allow feed quantities and feed duration times to be accurately calculated to reflect the daily water temperature and dissolved oxygen conditions. Yellowtail Kingfish are voracious, active feeders, so over-feeding should be avoided because fish have a high oxygen demand after feeding due to increases in post-prandial metabolism, after which can lead to sharp reductions in the surrounding dissolved oxygen levels.

2. At this stage of research, it is recommended that the fish oil in diets for Yellowtail Kingfish is not substituted with canola oil. If other alternative oils such as poultry oil are included into the diets, then it is necessary to ensure that the diets contain the minimum level of essential n-3 LC-PUFA, which is 2.0-2.4% of the dry diet for juvenile Japanese Yellowtail (Deshimaru *et al.* 1982; NRC 2011), to ensure that the essential fatty acid requirements are met.

3. Plant oils and animal fats contain either zero or low amounts of cholesterol, respectively, compared to fish oil; therefore it is recommended that if fish oil is substituted with

alternative oils, particularly plant oils which contain phytosterols, then it is necessary to ensure that the diets are supplemented with additional cholesterol to avoid the effects of green liver syndrome and hypocholesterolemia. Additionally, if canola oil is to be used at even low levels it should be screened for the presence of trypsin inhibitors and other anti-nutritive factors.

4. It is recommended that caution be used when substituting fish meal with soy products. Due to reduced growth performance, feed utilisation and health concerns, we do not recommend the use of SESBM in diets for Yellowtail Kingfish. Soy protein concentrate was the more promising soy ingredient, and dietary inclusions of 10-15% are acceptable. Feeding stimulants may be useful to improve feed intake at levels above 20% SPC. We also recommend further research into different plant and/or animal protein ingredients (depending on market preferences, i.e., Europe has land-animal protein free requirements) that could be used instead of soy ingredients in diets for Yellowtail Kingfish.

5. Taurine is a non-essential amino acid that has been found to be required to maintain or enhance growth for *Seriola* spp. and some other marine species, particularly when fish meal is replaced using plant proteins (Gaylord et al., 2006; 2007; Lunger et al., 2007). Taurine fortification is also an important consideration when substituting fish meal with alternative plant protein ingredients. Therefore, when substituting either fish meal or fish oil with alternative ingredients, it is recommended that Yellowtail Kingfish diets are supplemented with available taurine to avoid the occurrence of the green liver syndrome. Further research into the specific requirement level of taurine for Yellowtail Kingfish is essential. As are the interactive effects of taurine and cholesterol on the metabolism of Yellowtail Kingfish.

6. Culturing Yellowtail Kingfish in winter water temperatures presents particular difficulties. Fish exhibit low growth, reduced feed intakes, and suboptimal health, i.e., green liver syndrome and signs of abnormal histology and the early symptoms of diet-induced enteritis. Although not covered in this research, it would be beneficial to ensure winter diets contain immunostimulants to prevent mortalities after stressful events, either biotic or abiotic related.

7. Based on the findings of the literature review investigating the current status of knowledge of the nutritional requirements of Yellowtail Kingfish, a large amount of additional nutritional research is required to improve our understanding of the nutritional requirements and subsequent commercial diet formulations for this species.

8. The implementation and commissioning of the CST R & D sea cage farm would be extremely beneficial to the company's future development. It would enable the company to test potential production improvements in a well replicated pilot scale system in the sea cage setting prior to incorporation into production systems.

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APPENDICES

Appendix 1. List of publications, conference abstracts, reports and theses arising from this project.

Refereed Publications

1. Bansemer, M., 2011. Effects of soybean meal and water temperature on the mucus layer and the development of sub-acute enteritis in Yellowtail Kingfish (*Seriola lalandi*), Faculty of Sciences, School of Animal and Veterinary Sciences. Adelaide University, Adelaide, Australia.
2. Bansemer, M., Forder, R., Howarth, G.S., Suitor, G.M., Bowyer, J.N., Stone, D. A.J., In press. The effects of solvent extracted soybean meal, soy protein concentrate and water temperature on the intestinal mucus layer and goblet cell abundance and the development of sub-acute enteritis in Yellowtail Kingfish, *Seriola lalandi*. Aquaculture Nutrition.
3. Bellgrove, E.J., Forder, R.E.A., Howarth, G.S., Stone, D.A.J., Under Review. Inducing sub-acute enteritis in yellowtail kingfish (*Seriola lalandi*): The effect of dietary inclusion of soybean meal on hindgut morphology and myeloperoxidase activity. Aquaculture.
4. Bowyer, J.N., Qin, J.G., Adams, L.R., Thomson, M.J.S., 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, 368-369, 19-28.
5. 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.
6. Bowyer, J.N., Qin, J.G., Smullen, R.P., Ward, L.R., Stone, D.A.J., 2012. The use of plant and animal oils to improve the sustainable production of yellowtail kingfish, *Seriola lalandi*. In: Miller, M.R. (Ed.), Report from the Biennial Scientific Meeting of the Australasian Section of the American Oil Chemists Society (AAOCS), Adelaide, November 2011. Nutrients, pp. 372-398.
7. Bowyer, J.N., Qin, J.G., Stone, D.A.J., 2012. Protein, lipid and energy requirements of cultured marine fish in cold, temperate and warm water. Reviews in Aquaculture 4, 1-23.
8. Bowyer, J.N., Rout-Pitt, N., Bain, P.A., Stone, D.A.J., Schuller, K.A., 2012. Dietary fish oil replacement with canola oil up-regulates glutathione peroxidase 1 gene expression in yellowtail kingfish (*Seriola lalandi*). Comparative Biochemistry and Physiology - Part B 162: 100-106.
9. Bowyer, J.N., Booth, M.A., Qin, J.G., Stone, D.A.J., 2013. Temperature and dissolved oxygen influences growth and digestive enzyme activities of Yellowtail Kingfish (*Seriola lalandi*). Aquaculture Research 1–11 doi:10.1111/are.12146.
10. Bowyer, J.N., Qin, J.G., Smullen, R.P., Adams, L.R., Thomson, M.J.S., Stone, D.A.J., 2013. 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.
11. Bowyer, J.N., Qin, J.G., Smullen, R.P., Adams, L.R., Thomson, M.J.S., Stone, D.A.J., 2013. 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.
12. Collins, G.M., Ball, A.S., Qin, J.G., Bowyer, J., Stone, D., 2012. Effect of alternative lipids and temperature on growth factor gene expression in yellowtail kingfish (*Seriola lalandi*). Aquaculture Research. DOI: 10.1111/are.12067.

13. 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.

Other Reports

David A.J. Stone, D.A.J., Bowyer, J.N. (Editors); Final Report Sustainable Feeds and Feed Management for Yellowtail Kingfish. Project No. 2009/728.

Adams, L.R., Bowyer, J.N., Stone, D.A.J., 2012. The assessment of soybean enteritis like condition in juvenile Yellowtail Kingfish *Seriola lalandi* held under different feed and temperature regimes. University of Tasmania.

Stone, D.A., 2012. Analysed and presented data to CST management regarding the nutrient profile of commercial diets fed to the companies Yellowtail Kingfish in their sea cage facilities. (Commercial in Confidence).

Conference Presentations and Posters

1. Matthew Bansemer*, Gordon S. Howarth, Rebecca Forder, Suitor, G.M., Jenna N. Bowyer, David A.J. Stone., (Accepted). Effects of solvent extracted soybean meal and water temperature on the intestinal mucus layer and the development of sub-acute enteritis in yellowtail kingfish, *Seriola lalandi.* World Aquaculture Society Conference, Adelaide, SA, Australia, June 7 - 11, 2014. (Oral presentation).

2. Jenna N. Bowyer*, Jian G. Qin, Richard P. Smullen, Louise R. Ward, David A.J. Stone., (2012). The response of yellowtail kingfish (*Seriola lalandi*) digestive enzyme activity to the replacement of dietary fish oil. International Symposium on Fish Nutrition and Feeding conference, Molde, Norway, 4-7th June, 2012. (Poster presentation).

3. David A.J. Stone*, Richard .P. Miegel, Sarah .J. Pain and Gordon .S. Howarth, (2012). Water temperature effects gut transit time, digestive enzyme activity and nutrient digestibility in yellowtail kingfish, *Seriola lalandi.* Skretting Australasian Aquaculture Conference, Melbourne, Australia, May 1 - 4, 2012. (Oral presentation).

4. Jenna N. Bowyer*, Jian G. Qin, Richard P. Smullen, Louise R. Ward, David A.J. Stone (2012). The influence of temperature on fish meal substitution with solvent extracted soybean meal and soy protein concentrate in yellowtail kingfish, *Seriola lalandi.* Skretting Australasian Aquaculture Conference, Melbourne, Australia, May 1 - 4, 2012. (Oral presentation).

5. Matthew Bansemer*, Gordon S. Howarth, Rebecca Forder, Jenna N. Bowyer, David A.J. Stone, (2012). Effects of solvent extracted soybean meal and water temperature on the intestinal mucus layer and the development of sub-acute enteritis in yellowtail kingfish, *Seriola lalandi.* Skretting Australasian Aquaculture Conference, Melbourne, Australia, May 1 - 4, 2012. (Oral presentation).

6. Emma J. Bellgrove*, Rebecca E.A. Forder, Gordon S. Howarth, David A.J. Stone, (2012). Grape seed extract: a potential new treatment for “soybean meal-induced” enteritis in yellowtail kingfish, *Seriola lalandi.* Skretting Australasian Aquaculture Conference, Melbourne, Australia, May 1 - 4, 2012. (Oral presentation).

7. Kathryn A. Schuller*, Wan Abdul Rahim Wan Ahmad and David A.J. Stone, (2012). Fish oil replacement affects indicators of product quality in barramundi, *Lates calcarifer.* Skretting Australasian Aquaculture Conference, Melbourne, Australia, May 1 - 4, 2012. (Oral presentation).

8. Jenna N. Bowyer*, Jian G. Qin, Richard P. Smullen, Louise R. Ward, David A.J. Stone, (2011). The use of plant and animal oils to improve the sustainable production

of yellowtail kingfish *Seriola lalandi.* Australasian Section of the American Oil Chemists' Society (AAOCS), Adelaide, SA, Australia, November 9 - 11, 2011. (Oral presentation).

9. Jenna N. Bowyer*, Jian G. Qin, Richard P. Smullen, Louise R. Ward, David A.J. Stone, (2011). The influence of water temperature on fish oil substitution in yellowtail kingfish *Seriola lalandi*. World Aquaculture Society Meeting, World Aquaculture, Natal, Brazil June 6 - 10, 2011. (Oral presentation).
10. Kathryn A. Schuller*, Nathan Rout-Pitt, Peter A. Bain, Jenna Bowyer and David A.J. Stone, (2011). Impact of fish oil replacement on the expression of antioxidant genes and genes involved in the synthesis of omega-3 long chain polyunsaturated fatty acids in yellowtail kingfish (*Seriola lalandi*). World Aquaculture Society Meeting, World Aquaculture, Natal, Brazil June 6 - 10, 2011. (Oral presentation).
11. J Bowyer*, D Fisher, J Qin, DAJ Stone, (2010). Hyper-salinity affects the efficacy of AQUI-S® with cultured yellowtail kingfish, *Seriola lalandi*. Australasian Aquaculture Conference, Hobart, Australia, May 23 - May 26th, 2010. (Poster presentation).

Other Presentations

David Stone, J. Bowyer, J. Qin, R. Smullen, M. Bansemer, R. Forder, G. Howarth, H. Wang, L. Ward, M. Thomson. Assessing alternative dietary proteins and lipids for Yellowtail Kingfish. Presented at the Australian Seafood CRC Participant's Forum and Science Day. Comfort Inn Haven Marina, 6-10 Adelphi Terrace, Glenelg North, Adelaide, SA, Australia, 11 - 12 July, 2011.

David Stone. Impacts of fish meal and fish oil replacement on gut health of Yellowtail Kingfish. The Australian Seafood CRC, Yellowtail kingfish health workshop. SARDI Aquatic Sciences, West Beach, SA, 12th July, 2012.

Mark Booth. Summary of YTK results from the Aquafin CRC Project 1B5: Feed technology for temperate fish species (Volume 2: Diet development). Technology Transfer Meeting for the AS CRC "Sustainable Feeds & Feed Management for Yellowtail Kingfish" project (2009/728). Port Lincoln Marine Science Centre, Port Lincoln, Tuesday 22 March 2011 to Wednesday 23 March 2011.

Mark Booth. Summary of findings from the "Effect of temperature and stocking size on growth of juvenile Yellowtail Kingfish" activity from the "Understanding YTK" project (2008/903). Technology Transfer Meeting for the AS CRC "Sustainable Feeds & Feed Management for Yellowtail Kingfish" project (2009/728). Port Lincoln Marine Science Centre, Port Lincoln, Tuesday 22 March 2011 to Wednesday 23 March 2011.

Jenna Bowyer. Alternative lipid and protein studies with YTK at optimal (22 °C) and suboptimal (18 °C) water temperatures. Technology Transfer Meeting for the AS CRC "Sustainable Feeds & Feed Management for Yellowtail Kingfish" project (2009/728). Port Lincoln Marine Science Centre, Port Lincoln, Tuesday 22 March 2011 to Wednesday 23 March 2011.

Mathew Bansemer. Effects of alternative dietary proteins (solvent extracted soybean meal) on the intestinal mucus layer in Yellowtail Kingfish. Technology Transfer Meeting for the AS CRC "Sustainable Feeds & Feed Management for Yellowtail Kingfish" project (2009/728). Port Lincoln Marine Science Centre, Port Lincoln, Tuesday 22 March 2011 to Wednesday 23 March 2011.

Louise Ward. Histological evaluation of the digestive tract (hindgut) of Yellowtail Kingfish fed alternative lipids. Technology Transfer Meeting for the AS CRC "Sustainable Feeds &

Feed Management for Yellowtail Kingfish" project (2009/728). Port Lincoln Marine Science Centre, Port Lincoln, Tuesday 22 March 2011 to Wednesday 23 March 2011.

David Stone. The effects of temperature on gut evacuation rates in Yellowtail Kingfish. Technology Transfer Meeting for the AS CRC "Sustainable Feeds & Feed Management for Yellowtail Kingfish" project (2009/728). Port Lincoln Marine Science Centre, Port Lincoln, Tuesday 22 March 2011 to Wednesday 23 March 2011.

David Stone. Using sustainable oils in fish feed for a healthier product. Presented at the South Australian Branch of the Australian Marine Sciences Association Annual General Meeting at the Anchorage, SA Sea Rescue Squadron, West Beach, Adelaide, SA, Australia, March 19, 2010.

Students and Theses

Postdoctoral Research Fellow

Dr Maxi Canepa, Visiting Post-doctoral Research Fellow (Fellowship granted by CONICET (Argentina)). Project entitled "Effect of temperature and plant protein inclusion on the growth factor gene expression in Yellowtail Kingfish" Flinders University, School of Biology, Aquaculture. SA, Australia, 2010 - 2012. Hosted by Flinders University at the Port Lincoln Marine Science Centre to work on AS CRC projects.

Doctor of Philosophy students

Dr Jenna Bowyer; CRC Project: 2008/736 entitled "Nutritional factors influencing the performances of Yellowtail Kingfish cultured at low temperatures". Flinders University, School of Biology, Aquaculture. Graduated October 2012.

Dr Bowyer published eight peer reviewed manuscripts in international scientific journals arising from this project.

Dr Bowyer was employed as the Sustainability and Communications Officer by Skretting Australia in August 2012.

Honours students

Mathew Bansemer, AS CRC Scholarship Honours project (2009/778) entitled "The effects of feeding alternative protein sources on the intestinal mucus layer and mucosal architecture in Yellowtail Kingfish". University of Adelaide, School of Animal and Veterinary Sciences. Graduated with 1st Class Honours, July 2011. One manuscript in Aquaculture Nutrition arose from this project.

Mathew went on to do a CRC funded PhD Scholarship. Matthew Bansemer (PhD candidate with Flinders University and SARDI), AS CRC PhD Scholarship project (2011/251) entitled "Improvement of abalone nutrition with macroalgae addition". Mathew is working in association with the CRC funded project (2010/736) entitled "Development of formulated diets for cultured abalone" which involves the AS CRC, the Australian Abalone Growers Association, SARDI Aquatic Sciences, Flinders University and University of Tasmania.

Geoffrey Collins, AS CRC Scholarship Honours project (2009/780) entitled "Effects of temperature and plant protein inclusion on the growth factor gene expression in Yellowtail Kingfish". Flinders University, School of Biology, Aquaculture. Graduated

with 1st Class Honours, December 2010. One manuscript in Aquaculture arose from this project.

Geoffrey is currently a PhD candidate with James Cook University, Queensland in the field of marine sciences.

Nathan Rout-Pitt, AS CRC Scholarship Honours project (2009/782) entitled “Impact of fish oil replacement on the expression of anti-oxidant genes and genes involved in the synthesis of highly unsaturated omega-3 fatty acids in Yellowtail Kingfish liver and muscle tissue”. Flinders University, Faculty of Science and Engineering, School of Biology, Aquaculture. Graduated with 2nd Class A level Honours, December 2010. One manuscript in Comparative Biochemistry and Physiology arose from this project.

Kopano Machailo, AS CRC Scholarship Honours project entitled “Yellowtail Kingfish growth response, flesh quality and PUFA content through microalgae formulated aqua-feeds”. Flinders University, Flinders Medical Science and Technology, Medical Biotechnology. Graduated with 2nd Class A Level Honours, December 2010.

Emma Bellgrove, Honours project entitled “Grape seed extract: A potential new treatment for “soybean meal induced enteritis” in Yellowtail Kingfish (*Seriola lalandi*)”. University of Adelaide, School of Animal and Veterinary Sciences. Graduated with 1st Class Honours, December 2011.

Emma was later employed on this project to assist with the preparation of the literature review investigating the nutrient requirements for Yellowtail Kingfish in this project (Chapter 8). One manuscript under review Aquaculture arose from this project.

Richard Miegel, Honours project entitled “The determination of gut transit times and digestive tract health for Yellowtail Kingfish and mulloway”. University of Adelaide, Discipline of Agricultural and Animal Science (Graduated with 1st Class Honours).One manuscript in Aquaculture arose from this project.

Undergraduate student projects

Suitor, G.M., Summer Scholarship project entitled “Assessing the intestinal mucus layer and mucosal architecture in yellowtail kingfish”. University of Adelaide, School of Animal and Veterinary Sciences. Supervisor Dr Rebecca Forder December 2013 – February 2013.

Kate Simmons, Clinical Research project entitled “Establishing reference intervals for serum biochemistry for the Yellowtail Kingfish, *Seriola lalandi*”. University of Adelaide, School of Animal and Veterinary Sciences. August 2011 – November 2011.

Appendix 2. Technology transfer meeting agenda, list of participants and meeting outcomes.

Meeting Agenda

Technology Transfer Meeting (Objective 1, Subproject 1) for the AS CRC "Sustainable Feeds & Feed Management for Yellowtail Kingfish" project (2009/728)

Date: Tuesday 22 March 2011 to Wednesday 23 March 2011

Venue: Conference Room, Port Lincoln Marine Science Centre, Port Lincoln

Meeting Objective

Hold meeting with CST management, R & D and production staff to outline the specific aims of project, develop a framework for the identification, uptake, preliminary testing and implementation into production systems of technology developed during the project and identify areas of improvement for sea cage stock-taking.

Desired Outcome of Meeting

- CST staff members are aware of the project objectives and understand the specific project aims;
- Areas for improvement for sea cage stock-taking are identified; and
- Development of a draft framework for the identification, uptake, preliminary testing and implementation into production systems of technology developed during the project.

Invited Participants

Research and Development Providers
David Stone (Project Principal Investigator), SARDI
Mark Booth (Project Co-Investigator), NSW Department of Primary Industries
Jian Qin (Project Co-Investigator), Flinders University
Louise Ward (Project Co-Investigator), University of Tasmania
Jenna Bowyer (AS CRC PhD student), Flinders University/SARDI
Matthew Bansemer (AS CRC Honours Student), Adelaide University/SARDI

Feed Industry Project Participant
Richard Smullen, Ridley Aquafeed

CST Invitees
Clifford Ashby, Managing Director
Marcus Stehr, Executive Director, Marine Operations
Mike Thomson (Project Co-Investigator), CST Research & Development Manager
Joe Ciura (Project Co-Investigator), CST Technical Manager
Chris Brookes, Marine Operations Manager - Port Lincoln
Jason Clarke, Feed Manager – Port Lincoln
Damian Critchley, Health Manager/R & D Farm Manager
Sam Feige, Database Manager/R & D Farm Assistant – Port Lincoln
Donald MacNeil, Marine Operations Manager – Whyalla
Tony Barton, Feed/Health Manager – Whyalla

Tony Octoman, Marine Operations Manager – Arno Bay
Ben Underdown, Health Manager – Arno Bay
Shaun Roberts, Feed Manager – Arno Bay
Trent D'Antignana, Quality Assurance Manager – Port Lincoln

Program – Day 1 Tuesday March 22

Day 1 Session: 1: 9.00 am – 10.30 am - Presentations (15 minutes each)

- Introduction – David Stone – Outline project aims and objectives (20 mins)
- CST Management (To be announced): The importance of optimal diet selection and effective feed management to meeting CST objectives
- Joe Ciura – CST FCR strategy
- Mike Thomson – R & D farm
- Questions and discussion

Morning tea: 10.30 am – 10.45 am

Day 1 Session 2: 10.45 am – 12.30 pm - Presentations (20 - 30 minutes each)

- Richard Smullen – Summary of previous YTK diet research (and other relevant trials)
- Mark Booth – Summary of YTK results from the Aquafin CRC Project 1B5: Feed technology for temperate fish species (Volume 2: Diet development)
- Mark Booth – Summary of findings from the "Effect of temperature and stocking size on growth of juvenile Yellowtail Kingfish" activity from the "Understanding YTK" project (2008/903).

Lunch: 12.30 pm – 1.00 pm

Day 1 Session 3: 1.00 pm – 3.00 pm - Presentations (20 - 30 minutes each)

- Jenna Bowyer: Alternative lipid and protein studies with YTK at optimal (22 °C) and suboptimal (18 °C) water temperatures.
- Mathew Bansemer: Effects of alternative dietary proteins (solvent extracted soybean meal) on the intestinal mucus layer in Yellowtail Kingfish.
- Louise Ward: Histological evaluation of the digestive tract (hindgut) of YTK fed alternative lipids.
- David Stone – The effects of temperature on gut evacuation rates in YTK.
- Questions and discussion

Afternoon tea: 3.00 pm – 3.1 5pm

<u>Day 1 Session 4</u>: 3.15 pm to 4.15 pm

- CST procedures:
    - A review of historical growth, FCR and feed rate data (Joe) – 10 mins
    - Current CST stocking, counting and grading strategy (Joe) – 10 mins
    - Current CST work instructions for feeding including use of daily feed sheets (Joe) – 10 mins
    - Alternative non-invasive weighing methods under development, e.g. Vaki (Mike & Jason) – 10 mins
- Questions and discussion, i.e. areas for improvement – 10 mins
- *Summarise and wrap up Day 1*

<u>Day 1 Session 5</u>: 4.30 pm – 5.30 pm

Co-Investigators Meeting for the Sustainable Feeds and Feed Management for Yellowtail Kingfish Project

Dinner 7.00 pm – 9.00 pm

- Invitees only
- To be informed of venue on the day
- Informal dress

Program – Day 2 Wednesday March 23

<u>Day 2 Session 1</u>: 9.00 am – 10.30 am Planning and discussion

Specific strategies that will facilitate rapid technology transfer from the R & D experimental phase through to incorporation into on-farm routine production practices.

Compile a list of activities required during a 15 minute sessions on each of the following major topics (who will do each activity, and where each component of the research will be done into a matrix table):
Uptake of key findings from previous diet studies, e.g. Aquafin CRC

- Uptake of SARDI tank diet trials to date
- Plan for uptake of results of trials in R & D farm:
    - Diet trial
    - Feed regime / growth model trials
    - Use of revised growth model
- Uptake of alternative weighing methods
- Uptake of review of stocking, counting strategy
- Training requirements

Morning tea: 10.30 am – 10.45 am

Day 2 Session 2: 10.45 am – 1.00 pm Planning and discussion (Continued)

Specific strategies that will facilitate rapid technology transfer from the R & D experimental phase through to incorporation into on-farm routine production practices.

- *Summarise and wrap up the meeting*

Lunch: 1.00pm – 1.30pm

Day 2 Session 3: 1.30 pm – 4.00 pm

Co-Investigators Meeting for of the Sustainable Feeds and Feed Management for Yellowtail Kingfish Project

- *Meeting closes 4.00 pm*

Meeting Attendees and Outcomes

Meeting attendees

Present: David Stone, Jian Qin (day1), Jenna Bowyer (day1), Tony Barton, Shaun Roberts , Mark Booth, Matthew Bansemer, Louise Ward, Mike Thompson, Damian Critchley, Sam Feige, Joe Ciura (day 2), Marcus Stehr (part day1), Morten Deichmann (part day1)
Apologies: Clifford Ashby, Richard Smullen, Joe Ciura (day1), Marcus Stehr (day2)

Meeting outcomes

Day 1 commence: 9.00am
Personal round the table introductions
Introduction to meeting and agenda review addressed by David Stone

- Project duration March 2001 to May 2012.
- Overall budget $600K cash contribution.
- Overview of smaller projects within larger project.
- Objectives and activity Gantt chart presented.
- Aim to reduce FCR from 2.2 to 1.7 by end of Jan 2014.

Marcus Stehr joins the meeting extensively, reviewing Cleanses Tuna five pillar strategy plan and Cleanseas' challenges and concerns with mortalities across the board.

- Reduction of deformities and increase of survivability within the hatchery
- Genetics research to identify contributing brood stock and the connection with mortalities and deformities.
- Fingerling vaccinations to be researched for possible implementation to reduce early stages of mortalities.
- Predator mitigation strategies to reduce mortalities.
- Fallowing plan to reduce seafloor degradation, fluke infestations and mortalities.
- Dietary development to reduce FCR and increase profitability.

CST activities as part of the project, briefed by Mike Thomson.

- FCR Strategy Work Instruction (WICS-075) presented and read.
- CST in-house feed analysis has reduced in recent, float tests are still done by CST.
- All CST sites have the capacity to be fed by an automated feed system.
- Project FCR targets from 2008-2012 table assessed. Table shows actual FCR's are higher than target FCR's to end of a year class cycle.
- YTK have high feed response, but may not be necessarily eating as per M.Deichmann.
- Move to standardise the feeding across all sites. Variables will be temperature and size differences between sites.
- CST R & D farm activities timeline presented. Project to start in July 2011.
- Winter diets contribute considerably to FCR's.
- 2011YC fish to be used in trial at 1 kg.
- R & D farm trials will entail subprojects;
    - 3a – Test and validate growth-feed intake model in winter water temperatures.
    - 3b – Test and validate growth-feed intake model in summer water temperatures.
    - 6 – Identification of the optimal protein:energy ratios.
    - 9 – Investigation of alternative protein sources.

- Length of trials currently in discussion with Skretting, to be finalised in the coming weeks.
- Data temperature logger and DO meter to be used regularly in R & D station.
- Farm is proposed to be located nearby an existing water quality monitoring station (external to CST).
- Bathing of fish to be done in sync with production cages.
- Density less than 10kg m^3.
- 2.5 staff dedicated to R & D farm. Data accuracy essential and to be assessed weekly.
- R & D farm layout extensively explained including, but not limited to, mooring systems, cage design, net design, changing method and mesh sizing, orientation, barge layout, feed and camera systems.
- All parts should be in place by mid-April to commence construction of farms.
- Importance of start and end fish weights stressed.

Action: Start time of trial to be discussed and agreed upon between project co-ordinators.
Action: Feed analysis required in future, for the testing of vitamins, phosphorous, carbohydrate levels, to ensure manufactures' specs are accurate.
Action: Procedure and frequency of weight checks to be further discussed and decided upon by project investigates.
Action: Another meeting to be held prior to commencement of R & D farm projects to finalise procedures and actions. Communication between all parties will be vital to the results.

Research Mark Booth and Aquafin CRC on temperature, presented by Mark Booth.
Full presentation is available upon request.

- Estimation of digestible protein & energy requirements of YTK.
- Temperature and metabolic rate findings – optimum temperature for YTK = 22^o Mulloway = 28 ^{o}C.
- Model derived from finding presented graphically.
- Balance of protein/energy very important.
- Carbohydrate studies and finding presented with glucose injections.

Understanding YTK Project findings presented.

- Ridley/Skretting diet comparisons presented graphically at 12^o and 15^o with weights of 2 g and 45 g.

Preliminary results of Alternative Lipid and Protein studies presented by Jenna Bowyer.
Presentation is available upon request.

- Subproject 8a – replacement of fish meal with soy protein concentrate results presented from conclusions based on trials in suboptimal (18^o) and optimal (22^o) water temperatures.
- Subproject 8b – replacement of fish meal with solvent extracted soy bean meal results presented from conclusions based on trials in suboptimal (18^o) and optimal (22^o) water temperatures.
- Canola oil has significant impact on green liver.
- 100% fish oil and 100% poultry oil had similar results.
- Growth is compromised with 100% canola oil.

Preliminary evaluation of the gut in alternative dietary oils for YTK, presented by Louise Ward.

Presentation is available upon request.

- Non marine products may impact on hind gut hardening in accordance with trial results.
- YTK sensitive to dietary changes based on findings.
- Distal intestine histology presented.
- 100% canola increases deterioration in the hind gut.

Effects of solvent extracted soy bean meal on YTK intestine mucus layer, presented by Matthew Bansemer.
Presentation is available upon request.

- Water temperature does not result in a significant reduction of intestinal mucus.
- Reduction of mucus layer high as soybean extract is added.

Action: Letter of support from Cleanseas to be sent regarding honours student project which may result in funding from CRC.

Water temperature and its effects on gut evacuation in YTK project by Richard Miegel, presented by David Stone.
Presentation is available upon request.

- Red intestine (winter syndrome).
- Trial conducted in summer feeding twice a day results show up to 16hours for evacuation.
- Trial conducted in winter fed once each 2 days results show up to 48 hours for evacuation.
- Lower digestion time in cooler water would result in altered diets being in contact with digestion track for longer.
- Dry matter, protein, fat energy digestion at anterior and posterior sections shows more absorption at lower temperatures within the anterior, due to being stored in this area for longer.

Review of CST YTK historical growth, FCR and feed rates in compassing all year classes (2004-2011), presented by M.Thomson.

- Aim to harvest out at a near weight of 3.5kg with an 18-20month life cycle.
- Policy 26 – Reduction of fish loss strategy briefed.
- Policy 14 – CST fish containment strategy briefed.
- Gut health analysis to be included in future CST practices.
- WI-CS045 Fish Stock Control briefed.
- All 2011YC to be counted and graded by end of June 2011.
- Evaluation of two electronic weight methods and current findings.

Day 1 concluded 5.45pm

Day 2 commence 9.00am

Review of yesterday's meeting
Joe Ciura presents a number of spread sheets containing historical data.

- Growth patterns over 2004-2009 year class' using winter diets.
- 10% canola oil currently used in CST diets.
- Mortality spikes in 2011 year class in relation between poor weather/water conditions shows grumbling mortality rates in every year class between February and April.

- Length obtained in 2009 year class but limited condition.

Headline Issues/activities addressed – who is responsible for what and when.
Open discussion and input.
Action: David Stone to e-mail Tony Barton regarding project details to do Masters.
Action: Confirmation of optimal temperature for YTK possibly including salinity levels and DO levels.
Action: Fish health training to be looked into. (FRDC training).
Action: Mike to present summary of headline issues to board for consideration.
Action: Joe to look into environmental station.

Appendix 3. The assessment of soybean enteritis like condition in juvenile Yellowtail Kingfish *Seriola lalandi* held under different feed and temperature regimes.

Adams, L.R.[1], Bowyer, J.N.[2], Stone, D.A.J.[2,3]

[1]National Centre for Marine Conservation and Resource Sustainability (NCMCRS), Australian Maritime College, University of Tasmania, Locked bag 1370, Launceston, Tasmania 7250
[2]School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide, SA, Australia 5001
[3]South Australian Research and Development Institute (SARDI), PO Box 120, Henley Beach, SA, Australia, 5022

Summary of Major Experimental Outcomes

Soybean enteritis–like condition was not observed in Yellowtail Kingfish fed diets containing alternate oil sources (poultry oil or canola oil; Chapter 3), or the alternative protein sources, solvent extracted soybean meal (SESBM, Chapter 6) or soy protein concentrate (SPC, Chapter 7).

There was no evidence that dietary factors have influenced significant tissue changes in the protein and oil replacement experiments. The majority of tissues changes indicative of inflammation associated with enteritis development were minimal and very close to normal scores (according to the scoring system developed for Atlantic Salmon). There was a very high presence of goblet cells (responsible for mucus production), and reduction in absorptive enterocytes (primary site of digestion) in fish across all experiments, which included the initial fish population (before feeding experiments started) and experimental fish that were fed fish oil and fish meal control diets.

Foregut tissues had more goblet cells and less enterocyte vacuoles than hindgut tissues across all experiments, particularly in the soybean ingredient fed fish. Normal baseline Yellowtail Kingfish reference tissue will be important for future histological assessment, and if different to salmon, a Yellowtail Kingfish specific intestinal scoring system should be developed.

Introduction

Alternative protein ingredients in aquaculture feeds are assessed on the basis of growth performance of fish, apparent digestibility and nutrient utilisation from ingredients, and increasingly on the health of fish. While plant ingredients provide availability and cost benefits for use in feeds, they may contain antinutritional factors specific to the plant variety or cultivar, including oligosaccharides, non-starch polysaccharides, alkaloids, protease inhibitors, tannins, saponins, lectins, phytic acid and gossypols.

Soybean meal inclusion in salmon feeds has been well documented to cause an inflammatory response in the mucosal lining of the distal intestinal tract of Atlantic Salmon, described as soybean enteritis (Van den Ingh et al., 1991). Although the specific component responsible is still unknown (Urán et al., 2008), the alcohol soluble fractions and in particular soy saponins are thought to be responsible. Many inhibitory substances can be removed by solvent extraction, heat or pressure treatment and the more processed the soy protein fraction in salmon feeds, the less inflammatory response is observed (Baeverfjord and Krogdahl, 1996) however at additional cost. This condition has been described to a limited extent in rainbow trout (Romarheim et al., 2008), and carp, snapper and Atlantic cod (Urán et al., 2008) although often does not progress to severe levels equivalent to those considered pathological in Atlantic Salmon (Olsen et al., 2007), and studies have shown potential to reverse the effects of enteritis with return to normal feeding (Baeverfjord and Krogdahl, 1996).

The anterior distal intestine (hindgut) is the primary site in salmonids where enteritis-like changes are observed, and are thought to be associated with both the distal intestine being a site of protein absorption, and the longer digesta residence time immediately posterior to the ileal valve between the foregut and hindgut. Yellowtail Kingfish possess two ileal valves, separating the foregut, midgut and hindgut, and as differential nutrient absorptive regions have not been described for juveniles of this

species, both immediate post-valve regions were sampled as potential sites of dietary related cellular changes.

The aim of this report was to provide a histological assessment of the anterior (foregut) and distal (hindgut) intestinal tissue from the digestive tract of Yellowtail Kingfish sampled from four separate experiments which were part of the larger CRC funded “Sustainable Feeds and Feed Management for Yellowtail Kingfish (2009/728) “project. The four experiments were designed to evaluate the growth performance and digestive tract health of Yellowtail Kingfish that had been previously fed the alternative oil sources, poultry oil and canola oil (Chapter 3 of final report) and alternative protein sources, solvent extracted soybean meal (SESBM; Chapter 6 of final report) and soy protein concentrate (SPC; Chapter 7 of final report) at water temperatures of 18 and 22 °C.

Materials and Methods

Experimental conditions and fish

Histological evaluation was done on sections of the anterior (foregut) and distal (hindgut) intestine tissue of Yellowtail Kingfish. Refer to relevant Chapters in the final report for a detailed description of the experimental conditions and tissue samples collection methods used for the Yellowtail Kingfish that had been previously fed the alternative oil sources, poultry oil and canola oil (Chapter 3) and alternative protein sources, solvent extracted soybean meal (SESBM; Chapter 6) and soy protein concentrate (SPC; Chapter 7). A brief summary of experimental conditions under which the fish were exposed too are provided in Table 1.

Table A1. Experimental background of experimental conditions for Yellowtail Kingfish used in digestive tract histology assessment.

Item	Test ingredient % inclusion level for soy products or oil type	Water temperature (°C)	Replicate fish samples for histological evaluation
Chapter 7 (SPC) Soy protein concentrate Experiment 2A	0, 20, 30, 40	18 & 22	4 tanks per diet (consisting of 2 tanks/temp with 7 fish per tank)
Chapter 6 (SESBM) Solvent extracted soybean meal Experiment 2B	0, 10, 20, 30	18 & 22	4 tanks per diet (consisting of 2 tanks/temp with 7 fish per tank)
Chapter 3 Dietary oils at 18 ℃ Experiment 3A	FO, PO, CO, FO/PO, FO/CO	18	3 tanks per feed consisting of 3 fish per tank
Chapter 3 Dietary oils at 22 ℃ Experiment 3B	FO, PO, CO, FO:PO, FO:CO	22	3 tanks per feed consisting of 3 fish per tank

FO = 100% fish oil; PO = 100% poultry oil; CO = 100% canola oil. FO:PO = 1:1 blend of fish oil and poultry oil; FO:CO = 1:1 blend of fish oil and canola oil.

Intestinal histology

Fish dissection, tissue collection and tissue fixation were carried out at SARDI Aquatic Sciences Centre West Beach, SA. Intestinal tissue samples were transported to the University of Tasmania, Launceston; in cassettes in 70% ethanol saturated moist packaging. Upon receipt, cassettes were immediately immersed in 70% ethanol until processing. All tissues were dehydrated prior to embedding in paraffin wax, and were sectioned at 5 µm. Tissue sections were stained with haematoxylin and eosin (H&E) and tissue structure examined under light microscopy. Goblet cell abundance was confirmed by differentiation with H&E with alcian blue (pH 2.5) which stains acidic mucopolysaccharides blue.

The morphology of the anterior (foregut) and distal (hindgut) intestine sections were assessed according to the following criteria, which have been used to classify conditions of soybean meal (SBM)-induced enteritis in Atlantic Salmon (Baeverfjord and Krogdahl, 1996): (1) widening and shortening of the intestinal folds, (2) loss of supranuclear vacuolisation in the absorptive cells (enterocytes), (3) widening of the central lamina propria within the intestinal folds, with increased amounts of connective tissue, (4) infiltration of a mixed leucocyte population in the lamina propria and submucosa, and (5) goblet cell proliferation. Sections were randomised and blind read, and allocated semi quantitative scores on a scale of 1-5 according to Uran, (2008), where 1 represents normal tissue structure and 5 represents extensive structural changes (Appendix A). Where more than one person scored slides, cross-referenced scoring was undertaken for consistency.

Statistical analysis.

Data were assessed for normality by residual plots and homogeneity of variance by Levene's test, and where required data were square root transformed. In some cases, transformation did not meet assumptions for Levene's, therefore raw data were analysed by analysis of variance at P=0.01 across all experiments, to minimise type 2 errors. Data for hindgut and foregut results from individual fish were statistically analysed separately. Therefore experiments E3A and E3B were analysed by one-way ANOVA (P=0.01), and experiments E2A and E2B were analysed by two factor ANOVA (factors: temperature and diet). Initial fish were included in one-way analysis of variance for E3A and E3B, but not E2A and E2B. Where a significant interaction by 2-way ANOVA between temperature and diet was observed, data were analysed by one-way ANOVA for each factor separately. Significant variation in means was assessed by Tukey's HSD. Means and standard errors are presented.

Results

Experiment 2A (E2A): Dietary soy protein concentrate at 18 and 22 °C

Hindgut histology

Mucosal fold height remained unchanged from the initial fish condition at the start of the experiment (Figure A1). Lamina propria width was narrowest in 0% SPC fed fish (normal), and statistically increased to a slight change in 40% SPC fed fish at both temperatures. Subepithelial mucosa tissues were not significantly affected by temperature or diet. Goblet cell abundance was moderate across all fish, and significantly higher in 0% SPC than 40% SPC fed fish at both temperatures. Goblet cells were more abundant at 22 ºC than 18 ºC. Supranuclear vacuoles were not significantly affected by diet, but were significantly reduced at 18 ºC compared with 22 ºC.

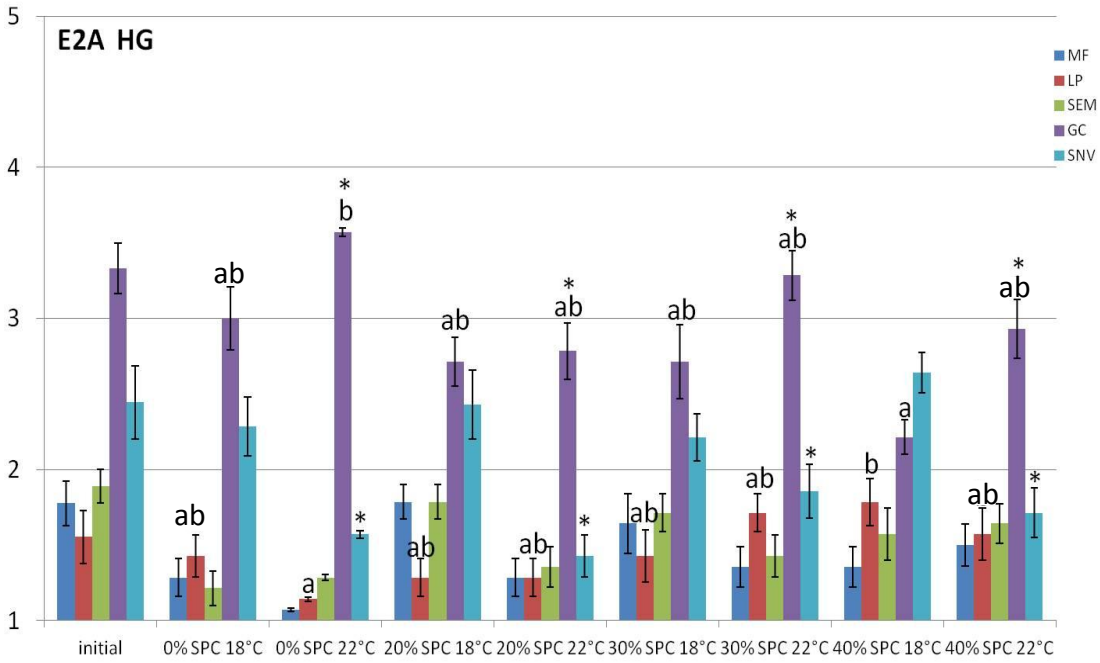

Figure A1. Hindgut distal intestinal scores for Yellowtail Kingfish *Seriola lalandi* fed feeds containing soy protein concentrate at 18 ºC and 22 ºC, indicating changes to mucosal fold height (MF), lamina propria width (LP), cellular infiltration of the subepithelial mucosa (SEM), goblet cell abundance (GC) and reduction in supranuclear vacuolisation (SNV). Different letters within each criterion indicate statistically significant differences. Asterisks indicate significant differences by temperature at $P<0.01$.

Foregut histology

Initial population fish had a slightly decreased mucosal fold height, increased lamina propria width and increased cellular infiltration of subepithelial mucosa than experimental fish (Figure A2). However the initial fish had lower goblet cell abundance and increased vacuolisation of the enterocytes supranuclear vacuolisation than fish sampled after the experimental feeding period. Scores for mucosal fold heights, subepithelial mucosa and lamina propria were generally unchanged among experimental fish ranging between 1 (normal) and 2 (only slight changes). Among experimental fish, 30% SPC at 18 ºC had greater mucosal fold heights than fish fed 0% SPC at 18 ºC, and there were no differences in fold height among other feeds or temperature combinations. Lamina propria widths were narrowest in 0% SPC fish at 18 ºC compared to fish fed 30% SPC at 18 ºC, and the other fish were statistically similar. There were no changes to cellular infiltration of subepithelial mucosal tissues with diet or temperature combinations. Goblet cell abundance was elevated in fish from all experimental treatments, with no significant differences with diet or temperature combinations. Supranuclear vacuole abundance improved (decreased) with increasing SPC inclusion.

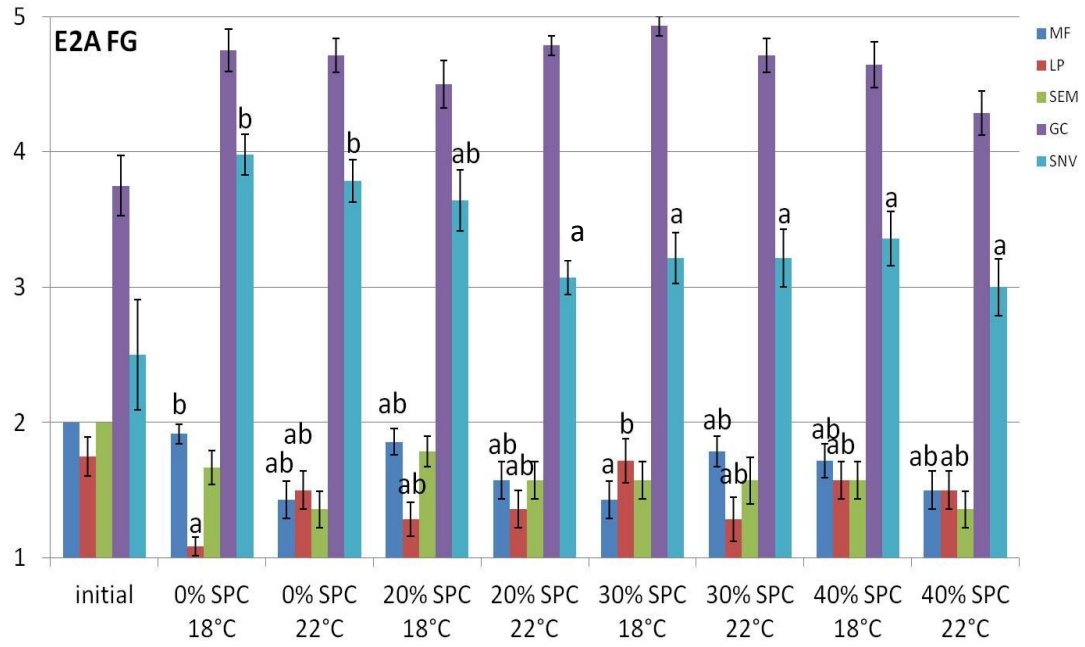

Figure A2. Foregut intestinal scores for Yellowtail Kingfish *Seriola lalandi* fed feeds containing soy protein concentrate at 18 ºC and 22 ºC, indicating changes to mucosal fold height (MF), lamina propria width (LP), cellular infiltration of the subepithelial mucosa (SEM), goblet cell abundance (GC) and reduction in supranuclear vacuolisation (SNV). Different letters within each criteria indicate statistically significant differences at $P<0.01$.

Table A2. Statistical results of hindgut and foregut histological changes in Yellowtail Kingfish *Seriola lalandi* after feeding varying levels of soy protein concentrate (SPC) at 18 and 22 °C.

	Temperature	Diet	Interaction (2-Way)
SPC Experiment 2A Hindgut			
Mucosal folds	NS	NS	NS
Lamina propria	NS	*	NS
Sub epithelial mucosa	NS	NS	NS
Goblet cells	**	*	NS
Supranuclear vacuoles	**	NS	NS
SPC Experiment 2A Foregut			
Mucosal folds	NS	NS	*
Lamina propria	NS	NS	*
Sub epithelial mucosa	NS	NS	NS
Goblet cells	NS	NS	NS
Supranuclear vacuoles	NS	**	NS

Statistical significance: NS $P>0.01$, * $P<0.01$, ** $P<0.001$.

Experiment 2B (E2B): Dietary solvent extracted soybean meal at 18 and 22 °C

Hindgut histology

Mucosal fold height and lamina propria width remained unchanged from the initial fish condition at the start of the experiment, and was not different among experimental fish (Figure A3). Cellular infiltration of the subepithelial mucosa tissues was significantly more prevalent in fish fed 0% and 10% SESBM at 18 ºC than 20% SESBM at 18 ºC; however all other fish were similar. Goblet cell abundance was moderate with no differences across all fish. Supranuclear vacuoles were moderately reduced in fish fed 20% SESBM at 18 ºC and 30% SESBM at 22 ºC, and significantly more supranuclear vacuoles were present in fish fed 10% SESBM at 22 ºC, however no clear trend associated with diet was present.

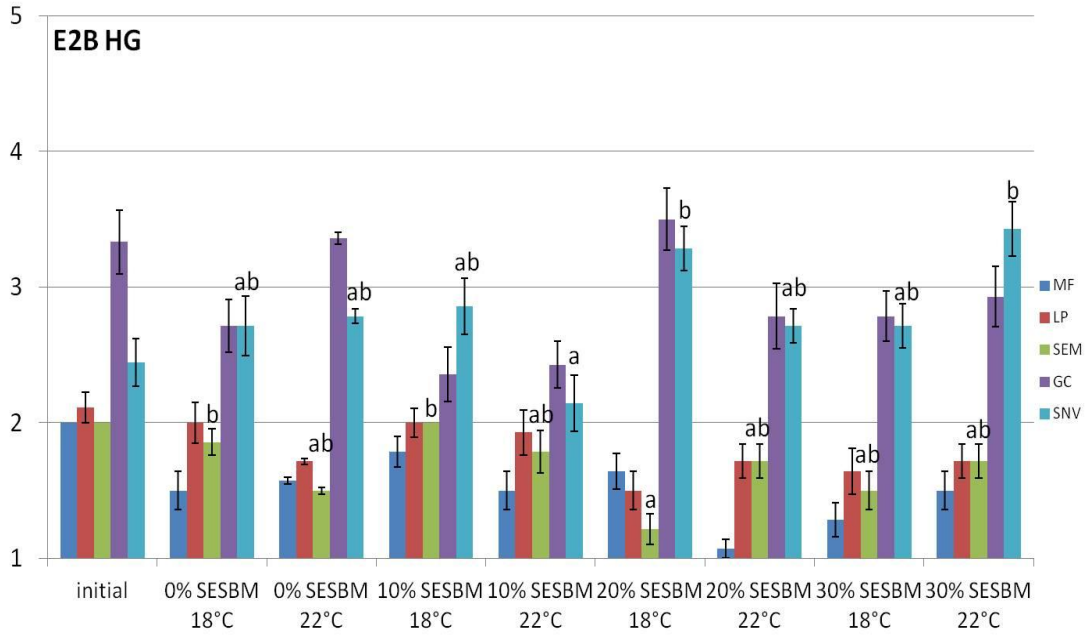

Figure A3. Hindgut distal intestinal scores for Yellowtail Kingfish *Seriola lalandi* fed feeds containing solvent extracted soy bean meal at 18 ºC and 22 ºC, indicating changes to mucosal fold height (MF), lamina propria width (LP), cellular infiltration of the subepithelial mucosa (SEM), goblet cell abundance (GC) and reduction in supranuclear vacuolisation (SNV). Different letters within each criterion indicate statistically significant differences. *P*<0.01.

Foregut histology

Initial fish had slightly higher mucosal fold height and more subepithelial mucosal infiltration than experimental fish (Figure A4). Lamina propria width was similar to experimental fish, and goblet cell and supranuclear vacuolisation was much better in initial fish than experimental fish. Lamina propria and mucosal fold heights were similar between all experimental fish. Subepithelial mucosal infiltration was significantly less in 0% SESBM fish at 22 ºC than 10% SESBM fish at 18 ºC, but otherwise there were no differences between temperature and feed combinations. Goblet cell abundance was elevated in all fish, and significantly less in 30% SESBM at 18 ºC than other temperature and feed combinations. Supranuclear vacuolisation was most reduced in 0% SESBM, and was improved but not significantly different among feeds contains SESBM.

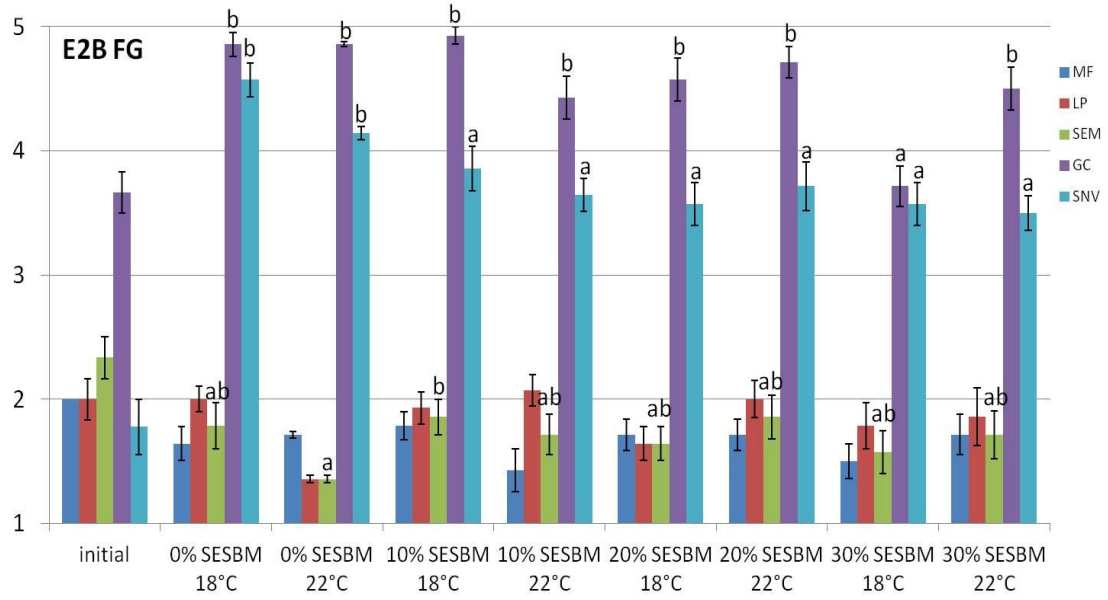

Figure A4. Foregut intestinal scores for Yellowtail Kingfish *Seriola lalandi* fed feeds containing solvent extracted soy bean meal at 18 ºC and 22 ºC, indicating changes to mucosal fold height (MF), lamina propria width (LP), cellular infiltration of the subepithelial mucosa (SEM), goblet cell abundance (GC) and reduction in supranuclear vacuolisation (SNV). Different letters within each criterion indicate statistically significant differences at $P<0.01$.

Table A3. Statistical results of hindgut and foregut histological changes in Yellowtail Kingfish *Seriola lalandi* after feeding varying levels of solvent extracted soybean meal (SESBM) at 18 and 22 °C.

	Temperature	Diet	Interaction (2-Way)
SESBM Experiment 2B Hindgut			
Mucosal folds	NS	NS	NS
Lamina propria	NS	NS	NS
Sub epithelial mucosa	NS	*	*
Goblet cells	NS	NS	NS
Supranuclear vacuoles	NS	NS	**
SESBM Experiment 2B Foregut			
Mucosal folds	NS	NS	*
Lamina propria	NS	NS	*
Sub epithelial mucosa	NS	NS	NS
Goblet cells	**	NS	NS
Supranuclear vacuoles	NS	**	NS

Statistical significance: NS $P>0.01$, *$P<0.01$, **$P<0.001$.

Experiment 3A (E3A): Dietary oil sources and blends at 22 °C

Hindgut histology

At 22 °C, responses of fish in the experiment were similar between those fed experimental diets and the initial fish across all parameters measured (Figure A5). Generally mucosal fold height, lamina propria width, cellular infiltration of the subepithelial mucosa scores ranged between normal and slight changes. Supranuclear vacuolisation was moderately reduced across all fish. Goblet cells were abundant in fish across all feed treatments.

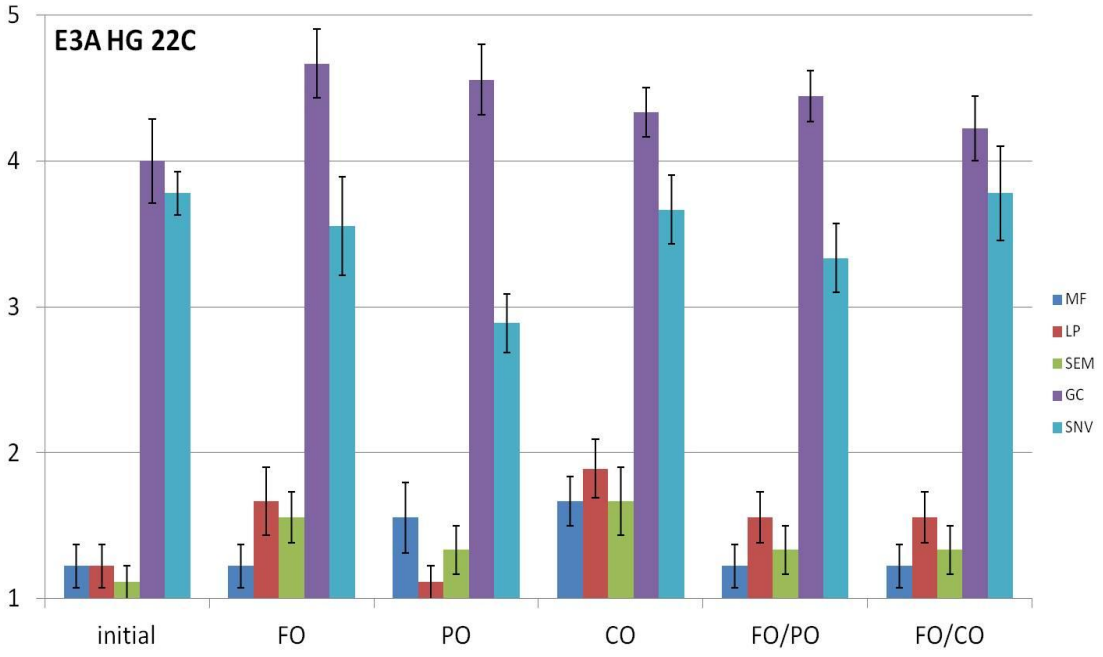

Figure A5. Foregut intestinal scores for Yellowtail Kingfish *Seriola lalandi* fed feeds containing oil sources; fish oil (FO), poultry oil (PO), canola oil (CO), fish and poultry oil blend (FO/PO) and fish and canola oil blend (FO/CO) at 22 ºC, indicating changes to mucosal fold height (MF), lamina propria width (LP), cellular infiltration of the subepithelial mucosa (SEM), goblet cell abundance (GC) and reduction in supranuclear vacuolisation (SNV). No statistical differences were observed at $P<0.01$, by one-way ANOVA.

Foregut histology

At 22 °C, there were no differences in mucosal fold heights or lamina propria across fish and all showed only slight tissue changes after feeding with different oils (Figure A6). Subepithelial mucosal infiltration was low across all feed treatments, and significantly less in initial fish compared to canola oil fed fish, and similar between all other fish. Goblet cells abundance was very high in fish across all treatments, and not statistically different between oils fed. Supranuclear vacuolisation of absorptive enterocytes was moderately to severely reduced in all fish (including initial fish), and significantly reduced in the fish oil (FO) and the fish poultry oil blend (FO/PO) compared to the best enterocyte vacuolisation in 100% poultry oil.

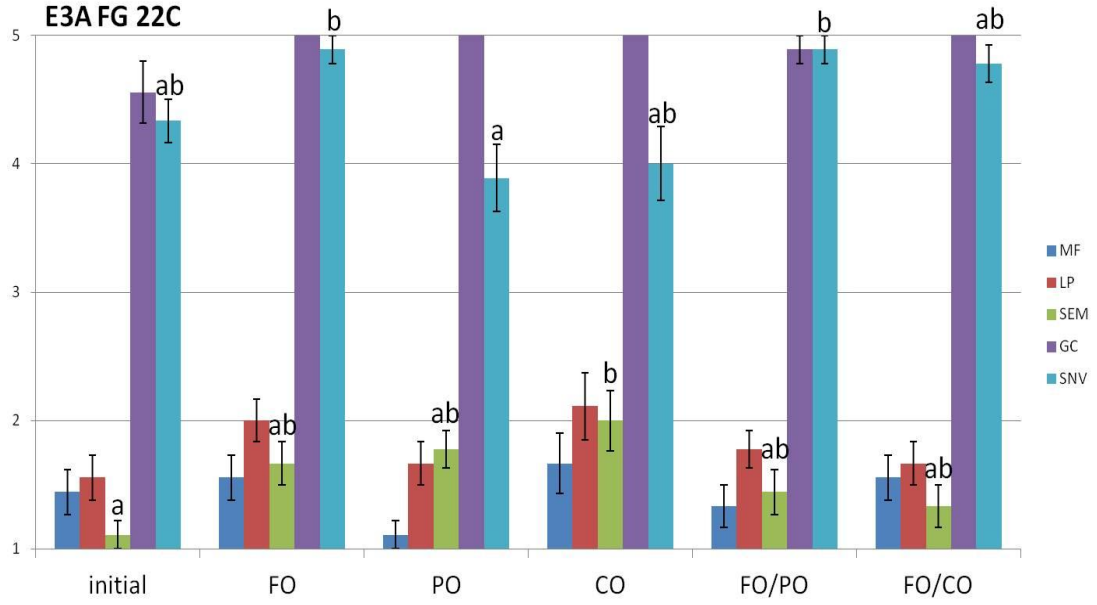

Figure A6. Hindgut distal intestinal scores for Yellowtail Kingfish *Seriola lalandi* fed feeds containing oil sources; fish oil (FO), poultry oil (PO), canola oil (CO), fish and poultry oil blend (FO/PO) and fish and canola oil blend (FO/CO) at 22 ºC, indicating changes to mucosal fold height (MF), lamina propria width (LP), cellular infiltration of the subepithelial mucosa (SEM), goblet cell abundance (GC) and reduction in supranuclear vacuolisation (SNV). Different letters within each criterion indicate statistically significant differences at $P<0.01$, by one-way ANOVA.

Experiment 3B: Dietary oil sources and blends at 18 °C

Hindgut histology

At 18 °C, initial fish had better scores than fish fed the fish oil/poultry oil blends Mucosal fold height was similar in initial fish and those fed experimental feed treatments (Figure A7). Lamina propria width was narrowest in the initial fish and fish fed canola oil, and significantly wider in fish fed fish oil/poultry oil blend. Subepithelial mucosa infiltration was least in initial fish and low-moderate in fish fed fish oil/poultry oil blend. Goblet cells were abundant in fish across all feed treatments and significantly higher in poultry oil, fish oil/poultry oil and fish oil/canola oil than initial fish. Supranuclear vacuolisation was moderately reduced in initial fish and fish oil fed fish, and significantly reduced in fish oil/poultry oil fish.

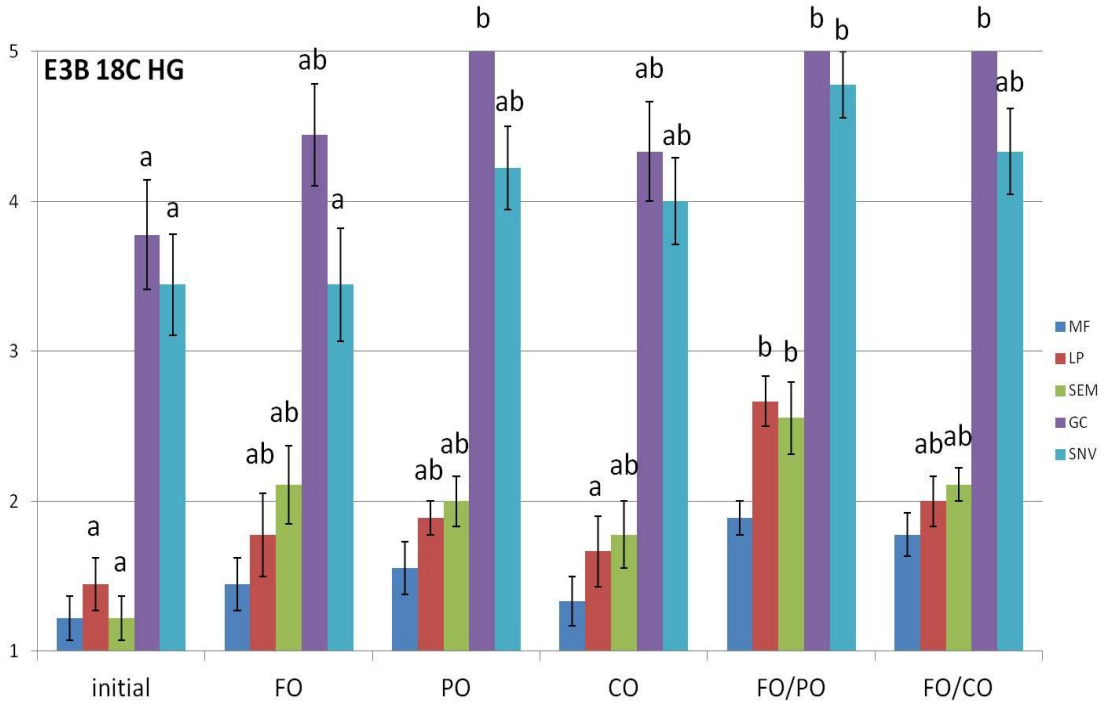

Figure A7. Hindgut intestinal scores for Yellowtail Kingfish *Seriola lalandi* fed feeds containing oil sources; fish oil (FO), poultry oil (PO), canola oil (CO), fish and poultry oil blend (FO/PO) and fish and canola oil blend (FO/CO) at 18 ºC, indicating changes to mucosal fold height (MF), lamina propria width (LP), cellular infiltration of the subepithelial mucosa (SEM), goblet cell abundance (GC) and reduction in supranuclear vacuolisation (SNV). Different letters within each criterion indicate statistically significant differences at *P*<0.01, by one-way ANOVA.

Foregut histology

At 18 °C, responses of fish in the experiment were similar between those fed experimental diets and the initial fish across all parameters measured (Figure A8). Mucosal fold height, lamina propria width, cellular infiltration of the subepithelial mucosa scores ranged between normal and slight changes and were not different between initial and experimental fish. Goblet cells were very abundant in fish across all feed treatments and significantly higher in fish oil, poultry oil, fish oil/poultry oil and fish oil/canola oil fed fish compared to canola oil fed fish which had moderately abundant goblet cells. Supranuclear vacuolisation was moderately to severely reduced across all fish, where canola oil fed fish had moderate reductions to SNV compared with absence of SNV in fish oil and fish oil/poultry oil fed fish.

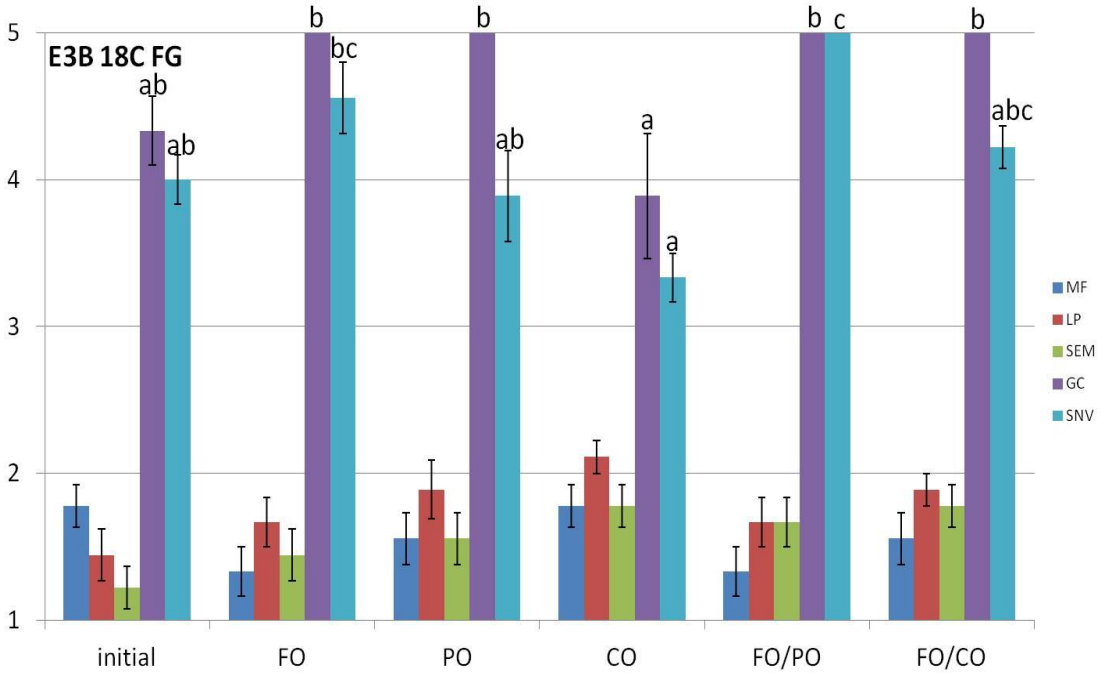

Figure A8. Foregut intestinal scores for Yellowtail Kingfish *Seriola lalandi* fed feeds containing oil sources; fish oil (FO), poultry oil (PO), canola oil (CO), fish and poultry oil blend (FO/PO) and fish and canola oil blend (FO/CO) at 18 ºC, indicating changes to mucosal fold height (MF), lamina propria width (LP), cellular infiltration of the subepithelial mucosa (SEM), goblet cell abundance (GC) and reduction in supranuclear vacuolisation (SNV). Different letters within each criterion indicate statistically significant differences at $P<0.01$, by one-way ANOVA.

General intestinal histology

In all tissues sectioned, intestinal structure was consistent with that previously described for teleost fish and consisted of a submucosal layer, subepithelial connective tissues and epithelial mucosa with abundant villi (mucosal folds, both simple and complex). Mucosal folds (villi) were comprised of epithelial mucosal cells, with a central distinct lamina propria containing a mixed leucocyte population (Figure A9).

Mucosal folds heights generally extended to or beyond the height of the ileal valve, and each villus was of uniform width from the apex to the epithelial junction (Figure A9, MF 1). A slight reduction in villi height was observed in some treatments (Figure A9, MF 2) where there was slight thickening of the villi width, however, tissue disruption and hyperplasia of villi observed in moderate to severe enteritis cases in Atlantic Salmon, were not observed in any Yellowtail Kingfish samples.

The lamina propria running centrally within each villus was generally narrow and uniform in size (Figure A9, LP 1). Slight changes were observed in some fish, generally not extending beyond the widening and cellular infiltration shown in Figure A9 (LP 2).

Normal subepithelial mucosa was indicated by a uniform and narrow connective tissue layer between the epithelial mucosa and submucosa with minimal cellular infiltration (Figure A9, SEM 1). Only slight changes to the cellular infiltration of the subepithelial mucosa were observed with increased cellularity of the connective tissues and increased leucocyte abundance (Figure A9, SEM 2).

Goblet cell abundance varied dramatically across the experimental treatments, ranging from sparsely distributed goblet cells between supranuclear vacuoles at the apical margin of enterocytes (Figure A9, GC 1) to extremely tightly packed and prolific goblet cells lining the entire margin of all mucosal folds (GC 5).
Normal supranuclear vacuoles were arranged at the apical margin of absorptive enterocytes, with basal nuclei uniformly arranged adjacent to the lamina propria (Figure A9, SNV 1). Responses in tissues ranged significantly from minor reduction in enterocytes to onset of extinction and complete extinction of supranuclear vacuolisation, where no vacuolisation was present along the apical margin, nuclei were disorganised and extended across enterocytes to the apical margin (SNV 5).

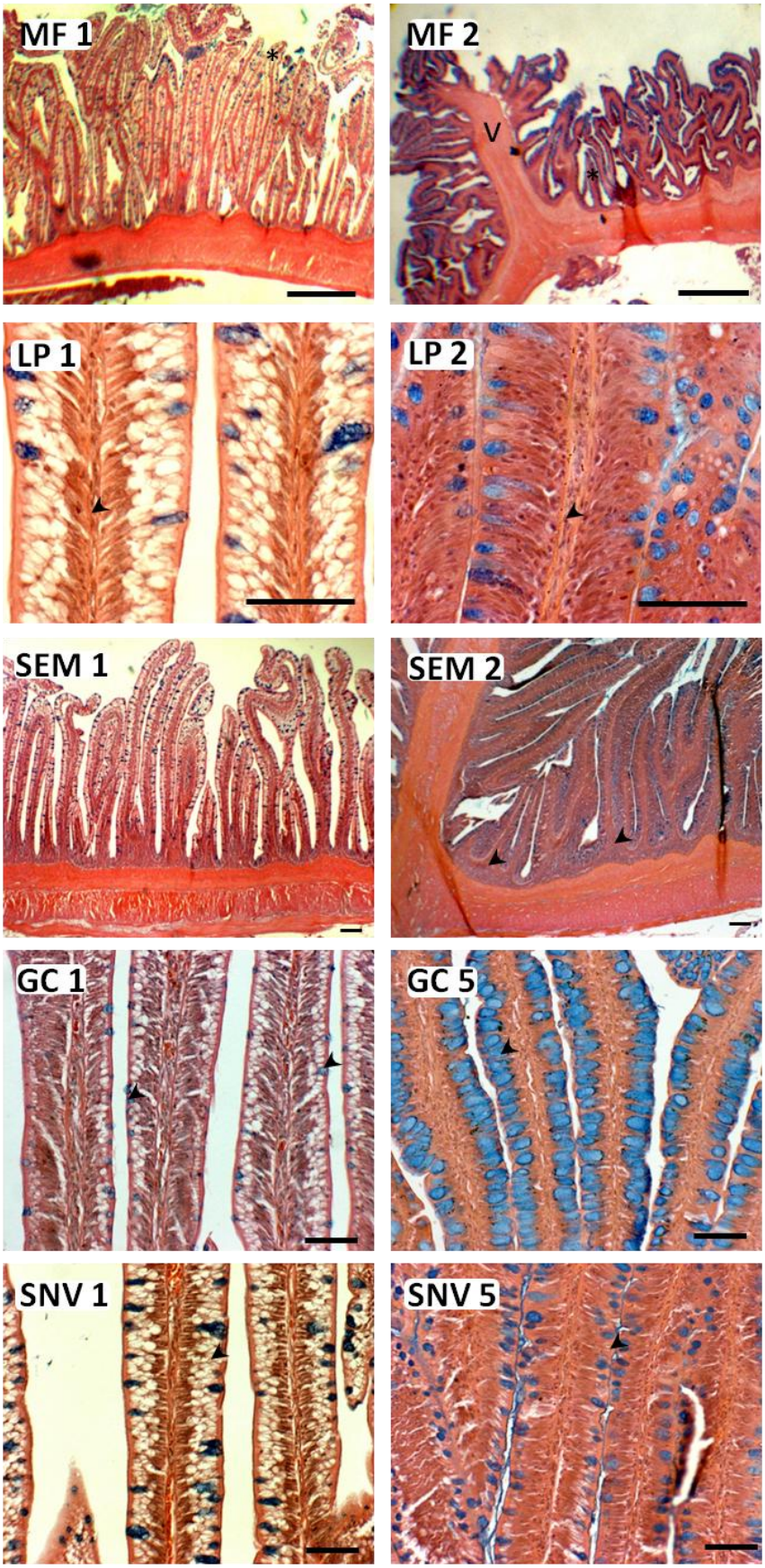

Figure 9. Intestine histology from juvenile Yellowtail Kingfish *Seriola lalandi*, 5 µm sections stained with H&E alcian blue (pH 2.5). Scores (numerals) indicate the lower and upper extremes observed in intestinal tissues across all experiments, on a

semiquantitative scale of 1-5, described for soybean enteritis in Atlantic Salmon, (Uran, 2008). MF1 shows the maximum mucosal fold (MF) height and individual mucosal folds are indicated by an asterisk (score 1 = normal). MF2 shows thickened mucosal folds of reduced height (asterisk) lining the basal mucosa and ileal valve (v) (score 2 = slight change). LP 1 shows a normal villi with narrow lamina propria (LP) (arrow) containing few leucocytes, while LP2 shows a score 2 where some cellular infiltration has caused a slight widening of the lamina propria (arrow). SEM 1; normal subepithelial mucosa appearance, minimal cellular infiltration below the intestinal epithelia, SEM 2; mild cellular infiltration below the intestinal mucosa (arrow). GC1; occasional goblet cells (GC) distributed at the proximal edge of the intestinal epithelium (stained blue with H&E alcian blue, pH 2.5), GC5 prolific abundance of goblet cells along the entire margin of the intestinal epithelium. SNV 1; wide margin of supranuclear vacuoles (SNV) within enterocytes of the epithelium and uniformly arranged nuclei adjacent to the lamina propria, SNV 5 extinction of all SNV along the epithelial margin and disorganisation of nuclei. Scale bar = 100 µm (except MF1 &2 where scale bar = 500 µm).

Summary of Histology Results

Please refer to the relevant Chapter in the final report for an in-depth discussion of results.

Experiment 2A (E2A) (SPC; Chapter 7) and 2B (E2B) (SESBM; Chapter 6) – fish meal replacement with soybean proteins at 18 and 22 °C

Foregut tissues consistently had increased goblet cell abundance and markedly reduced supranuclear vacuole presence across both experiments. Fish fed experimental feeds containing soybean protein concentrate and solvent extracted soybean meal showed responses mid-range between the initial fish and fish fed no soybean products.

Hindgut intestinal tissues were similar to the initial fish appearance and fish fed no soybean products at both temperatures, and showed normal tissue appearance with some signs of moderately increased goblet cell and reduced enterocyte vacuolisation consistent with a mild inflammatory response. Hindgut goblet cell abundance was increased at 22 ºC compared to 18 ºC in fish fed varying levels of soy protein concentrate, but temperature had no effect in fish fed solvent extracted soybean meal. It is possible that either all fish were compromised, or that the scores reflect normal Yellowtail Kingfish tissue appearance. Normal tissue appearance requires confirmation to establish if salmonid scoring systems are applicable to Yellowtail Kingfish intestinal tissues.

Experiment 3A (22 ℃) and 3B (18 ℃) (Chapter 3) – fish oil replacement with poultry and canola oil and oil blends

Foregut tissues again showed consistently increased goblet cell abundance and markedly reduced supranuclear vacuole presence across both experiments. Fish fed experimental feeds containing fish oil, poultry oil and the fish oil/canola oil blend, had the highest goblet cell abundance and the most severe reduction in vacuolisation of absorptive enterocytes (SNV). Fish fed poultry oil and canola oil had a higher presence of absorptive vacuoles (SNV) and goblet cell abundance was moderate. Hindgut tissue responses were quite similar across both oil replacement studies at 18 and 22ºC. At 22ºC there were no significant differences between initial fish or experimental fish fed any of the oil combinations. Across all fish, SNV were

moderately reduced, and goblet cell abundance was high. At 18ºC, initial fish were significantly better across most intestinal scores than fish fed fish oil/poultry oil, however there were no differences between any of the experimental feeds. Overall scores were similar to the 22ºC held fish fed the same feeds (not statistically assessed).

Discussion

Please refer to the relevant Chapter in the final report for an in-depth discussion of results.

The key criteria used to classify soybean enteritis in Atlantic Salmon were measured, but substantial changes in all parameters consistent with soybean enteritis were not observed in Yellowtail Kingfish fed varying protein and oil sources at 18ºC and 22ºC. No major inflammatory responses typical of extensive leucocyte inflammation of connective tissues or drastic reduction in villi height indicative of soybean enteritis were seen.

Generally the scores for LP, MF and SEM were very low and may indicate normal tissue structure for Yellowtail Kingfish. Initial and fish oil, and fish meal, fed fish were intended to provide the baseline reference in this experiment, however comparison to healthy Yellowtail Kingfish of naive or wild origin will be of value for reference tissue for future histological assessments to confirm baseline tissue appearance. Confirmation of normal Yellowtail Kingfish tissue appearance may provide a basis for developing a scoring system specific for Yellowtail Kingfish, rather than assuming equivalence to Atlantic Salmon.

At the same experimental inclusions of soybean ingredients, extensive changes to Atlantic Salmon intestinal structure have been well documented. Studies investigating the possible onset of soybean enteritis in species other than Atlantic Salmon, have often demonstrated only moderate dietary-related changes. Arctic char fed soybean products, showed only moderate changes to mid gut and distal gut cellular structure after 28 weeks feeding, and the moderate changes seen (similar to the present study in Yellowtail Kingfish) were not considered pathological (Olsen et al., 2007). Rainbow trout (Romarheim et al., 2008) and carp (Urán et al., 2008) demonstrated moderate and reversible changes to soybean ingredients in feeds.

More marked changes were observed, particularly related to goblet cell abundance and reduced supranuclear vacuolisation of enterocytes. SNV reduction was evident across most experiments, and is likely associated with the proliferation of goblet cells in the same region of the epithelia (although SNV reduction can be observed without GC proliferation). Reduced enterocyte vacuolisation and has been associated with reduced enterocyte absorptive function and subsequent reduced nutrient digestibility from feeds (Bakke-McKellep et al., 2006).

Goblet cell proliferation and subsequent increased mucus production have been observed in may fish species in response to exposure to several stressors and antigens including; parasite presence (Bucke, 1971), environmental contamination (Fanta et al., 2003), bacterial infection, feed borne antigens and also with starvation (Baeverfjord and Krogdahl, 1996; Chen et al., 2007). Baeverfjord and Krogdahl (1996) suggested that goblet cell abundance could be used to differentiate between starved fish (with higher goblet cell abundance) and fish affected by soybean enteritis (lower abundance), and that studies in weaner piglets fed soybean ingredients failed to change goblet cell abundance. The extent of goblet cell proliferation and associated reduction in absorptive enterocytes, particularly in the experiments

assessing oil replacement was unexpected. As the initial fish were sampled prior to exposure to feed treatments, and fish fed fish oil as a commercial reference both showed similar elevation in goblet cell abundance, suggest that non-dietary factors may be influencing the intestinal responses observed.

The observed increase in goblet cell proliferation (GC), and the reduction in supranuclear vacuolisation (SNV), observed in initial fish prior to feeding experimental diets was more severe in the larger fish used in the oil studies compared to the smaller fish used in the soybean studies. The fish used in the oil studies were held on hand at SARDI West Beach for longer periods than the smaller fish used for the soybean studies. The history of these fish differs and is as follows: All of the fish used in the oil studies were sourced from the CST Arno Bay Hatchery on the 19th November 2008 (5-10 g/fish) and were held in 5000-L tanks at SARDI West Beach and supplied with flow through ambient temperature seawater. These fish were fed a Ridley diet (50% protein, 15% lipid, 2 mm pellet provided by CST) *ad libitum* followed by a Skretting Nova 3mm diet (45% protein / 20% lipid diet) prior to the commencement of the studies. The 22 °C oil study commenced on the 23rd March 2009 (~96 g/fish initial stocking weight), and the 18 °C oil study commenced on the 22nd of July 2009 (101 g/fish initial stocking weight). The fingerlings (5-10 g /fish) used in the solvent extracted soybean (SESBM) trial were collected from the CST Port Augusta Hatchery on October 29th 2010. These fish were held in the same culture system as previously described for fish used for the oil studies, and were fed a Skretting Nova diet (50% protein, 15% lipid, 1.8 mm) *ad libitum.* The fingerlings were stocked (~22g/fish initial stocking weight) into the experimental system on November 17th 2010. The fingerlings used in the soy protein concentrate (SPC) trial were collected from the CST Arno Bay Hatchery on the January the 7th 2011 (5-10 g fish), held in the same system, and were fed *ad libitum* with the Skretting 1.8 mm diet prior to stocking (~22g/fish initial stocking weight) into the experimental system on January 24th 2010. Differences in parents (genetics), pre-feeding history and culture conditions may have influenced the differences seen in goblet cell proliferation (GC) and the reduction in supranuclear vacuolisation (SNV) in fish prior to the studies.

Conclusion

In conclusion, it appears unlikely that dietary factors are responsible for the intestinal tissue changes observed. The tissue changes associated with inflammation (of lamina propria, epithelial submucosa) and reduced mucosal fold height commonly observed in Atlantic Salmon at severe levels in response to soybean enteritis, in the current study were minimal and close to normal consistent with salmon scoring systems. Goblet cell abundance was high across all experiments, and was consistent across initial fish that weren't exposed to dietary manipulation, and fish fed control feeds containing 100% fish oil and fish meal with no alternative ingredients. The cause of high goblet cell abundance warrants further investigation and the combined tissue structural changes associated with very high numbers of goblet cells and reduced vacuolisation of absorptive enterocytes, may have potential to reduced nutrient digestion. However, fish growth performance (Bowyer et al. 2012a, Chapter 3; Bowyer et al. 2013a, Chapter 6; 2013c, Chapter 7) in these experiments did not correspond to the changes in intestinal structure which suggests that despite goblet cell and enterocyte appearance, dietary nutrient utilisation has not been compromised. Differences in parents (genetics), pre-feeding history and culture conditions may have influenced the differences seen in goblet cell proliferation (GC) and the reduction in supranuclear vacuolisation (SNV) in fish prior to the studies. However, more research is needed to clarify this. Normal baseline Yellowtail Kingfish reference tissue will be important for future histological assessment, and if different to salmon, a Yellowtail Kingfish specific intestinal scoring system should be developed.

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Appendix A. Scoring System to Assess the Development of Enteritis.

Table 2 Semi-quantitative scoring system for the different parameters used to assess the degree of enteritis developed by Atlantic salmon fed a soybean meal-based diet. From Urán *et al*. 2008.

	Score	Description
Mucosal Folds (MF)	1	Basal length
	2	Some shrinkage and bloating
	3	Diffused shrinkage and onset of tissue disruption
	4	Diffused tissue disruption
	5	Total tissue disruption
Supranuclear Vacuoles (SNV)	1	Basal SNV size
	2	Some size reduction
	3	Diffused size reduction
	4	Onset of extinction
	5	No SNV
Goblet Cells (GC)	1	Scattered cells
	2	Increased number and sparsely distributed
	3	Diffused number widely spread
	4	Densely grouped cells
	5	Highly abundant and tightly-packed cells
Eosinophilic Granulocytes (EG)	1	Few in SM basal small quantity
	2	Increased number in SM and some migration into LP
	3	Increased migration into LP
	4	Diffused number in LP and SM
	5	Dense EG in LP and SM
Lamina Propria (LP)	1	Normal size LP
	2	Increased size of LP
	3	Medium size LP
	4	Large LP
	5	Largest LP
Sub-epithelial Mucosa (SM)	1	Normal SM
	2	Increased size SM
	3	Medium size SM
	4	Large SM
	5	Largest SM

Note: The thesis and figures representing each of these stages are available online at http://edepot.wur.nl/121985

Appendix 4. The effects soy products and water temperature on the intestinal mucus layer and goblet cell abundance in yellowtail kingfish.

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The effect of dietary soybean meal and soy protein concentrate on the intestinal mucus layer and development of sub-acute enteritis in Yellowtail Kingfish (*Seriola lalandi*) at suboptimal water temperature

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Short running title: Soy effects on the yellowtail kingfish mucus layer

KEY WORDS**:** yellowtail kingfish, soy products, water temperature, mucus layer, sub-acute enteritis

Abstract

Solvent extracted soybean meal (SESBM) has been reported to cause sub-acute enteritis in certain fish species. Two 34 day experiments investigated the effects of SESBM and soy protein concentrate (SPC) on the intestinal mucus layer and development of sub-acute enteritis in the hindgut of yellowtail kingfish (*Seriola lalandi*) at 22 °C and 18 °C. Fish were fed increasing levels of SESBM (Exp. 1: 0, 10, 20, 30%) and SPC (Exp. 2: 0, 20, 30, 40%). No visual signs of inflammation in the hindgut were observed in either experiment. However, increasing dietary SESBM significantly reduced mucus layer thickness. Neutral and acidic goblet cell mucin composition increased at 18 °C and 22 °C, respectively. A significant positive linear relationship was evident between goblet cell number and SESBM inclusion at 18 °C. SPC inclusion and water temperature had no significant effect on mucus layer thickness or mucin composition. However, at 18 °C, goblet cell numbers decreased with SPC inclusion. Results suggest the early stages of sub-acute enteritis may have been manifesting in SESBM fed fish. In the long term, mucus layer alterations associated with feeding SESBM may compromise fish health. Longer terms studies should investigate the effects of feeding SESBM to yellowtail kingfish, particularly at suboptimal water temperatures.

Introduction

Soybean meal is of interest as an alternative protein in aquaculture due to its availability, high crude protein content and relatively low cost (Gatlin *et al.* 2007). However, dietary inclusions of soybean meal for many aquaculture species are often limited by high concentrations of antinutritional factors. Soybean meal has been

reported to contain oligosaccharides, β-conglycinin, glycinin, lectins, phytic acid-P, saponins and trypsin inhibitors (Francis *et al*. 2001; Gatlin *et al*. 2007). The concentration of antinutrients is greatly reduced by alcohol extraction resulting in a product referred to as SPC.

Soybean meal has been found to induce a condition first described in Atlantic salmon (*Salmo salar*) as sub-acute enteritis of the distal epithelial mucosa (van den Ingh *et al*. 1991; Baeverfjord and Krogdahl 1996). This condition is characterised by an increased proportion of inflammatory cells, decreased height and density of villi and microvilli (Merrifield *et al*. 2009), a loss of normal supranuclear vacuolization in the epithelial cells, a widening of the lamina propria and a shortening of the primary and secondary mucosal folds (Baeverfjord and Krogdahl 1996).

Enteritis has been reported in cultured yellowtail kingfish (Sheppard 2004). However, the mechanisms are still debated. Factors suggested to play a role include high fat feeds, invasion of opportunistic bacteria, cool water temperatures and dietary inclusions of plant proteins such as SESBM (Sheppard 2004). Sub-acute enteritis has a low morbidity rate with fish exhibiting very few external symptoms. However, the high mortality rate associated with this condition is a major concern to the yellowtail kingfish industry (Sheppard 2004).

Farmed yellowtail kingfish in South Australia are exposed to water temperatures that fluctuates seasonally, reaching 24 ºC in summer and falling below 12 ºC in winter. Gut transit time is prolonged in yellowtail kingfish at cooler water temperatures, becoming three times greater at 13 °C than 21 °C. This may increase the duration that the intestinal mucosa is exposed to the antinutrients present in SESBM, which may further increase the risk of inflammation (Miegel *et al*. 2010).

The mucus layer is the first line of defence from inflammation for the gastrointestinal epithelium and separates the gastrointestinal epithelial cells from the luminal environment providing protection from pathogens, destructive enzymes and corrosive chemicals (Johansson *et al.* 2013). Mucins, a major component of the mucus layer, are high molecular weight glycoproteins that are synthesised and secreted by goblet cells (Johansson *et al.* 2013). Mucins comprise a protein core with branching highly heterogonous oligosaccharide chains attached by glycoside bonds (Lievin-Le Moal and Servin 2006). Their highly heterogeneous nature provides a vast array of potential binding sites for intestinal bacteria, thereby retarding their access to the mucosal surface, reducing colonisation and favouring their removal.

Alterations to the thickness or composition of the mucus layer impair its effectiveness as a barrier, increasing the susceptibility of the gastrointestinal tract to inflammation and disease, and interfering with nutrient uptake. The role the mucus layer plays in protecting the underlying mucosa has been identified in a number of gastrointestinal inflammatory disorders including ulcerative colitis in humans (Pullan *et al*. 1994), Crohn's disease in rodent models (McGuckin *et al*. 2009; Shirazi *et al*. 2000) and necrotic enteritis in poultry (Collier *et al*. 2008; Van Immerseel *et al*. 2009).

At present, there is no information on mucus layer structure, composition or the role the mucus layer plays in protection against the development of sub-acute enteritis in yellowtail kingfish. Samples taken for this histological study were from two experiments published as Bowyer *et al.* (2013a) and Bowyer *et al.* (2013b). These were factorial experiments of soy ingredient inclusion level by water temperature. Yellowtail kingfish were fed either SESBM or SPC at optimal (22 °C) and sub-optimal (18 °C) water temperatures. These water temperatures were used as they reflect the optimal and

sub-optimal growth water temperatures for cultured yellowtail kingfish in South Australian waters. The primary aim of this study was to investigate changes in mucus layer thickness and goblet cell mucin composition and other morphological alterations in the hindgut of yellowtail kingfish and to determine the dietary level of SESBM or SPC that results in the development of sub-acute enteritis.

Materials and Methods

Experimental work was conducted in the Nutrition Laboratory at the South Australian Research and Development Institute (SARDI) Aquatic Science Centre, West Beach, South Australia. Samples were collected from two interrelated Australian Seafood CRC projects 2009/728 and JQ001. Experimental protocols followed the ethical standard approved by the Animal Welfare Committee of Flinders University (E286). Prior to being used in these experiments, fish were housed in 5,000L tanks at SARDI Aquatic Science Centre, West Beach. Juvenile yellowtail kingfish were used in each experiment and were sourced from the Arno Bay hatchery, Cleanseas Tuna Ltd, South Australia.

Fish were randomly selected from a larger population and transferred by net into 80L of seawater containing 20 mg L^{-1} AQUI-S (AQUI-S New Zealand Ltd., Lower Hutt, New Zealand) and allocated by systematic interspersion to one of sixteen, 700L tanks. Twenty four yellowtail kingfish were stocked in each tank that was supplied with recirculating seawater. Fish with an average starting weight of 22.61±0.02 g and 22.36±0.05g (mean weight ± standard deviation) were used for Experiments one and two, respectively. A fixed photoperiod of 14 h light: 10 h dark was held throughout the experiments.

Animal Housing and Feeding

In Experiment one, fish were fed one of four experimental diets that contained 0, 10, 20 and 30% inclusions of SESBM. The diet ingredient formulations and the proximate composition and calculated amino acid compositions of the four experimental diets are displayed in Table 1 and 2, respectively. In Experiment two, fish were fed one of four experimental diets that contained 0, 20, 30 and 40% inclusions of SPC. The diet ingredient formulations and the proximate composition and calculated amino acid compositions of the four experimental diets are displayed in Table 3 and 4, respectively. All feeds were prepared using a 3mm die with steam cooking extrusion at the SARDI Australasian Experimental Stockfeed Extruded Centre (AESEC) at Roseworthy, South Australia. Each diet was fed to fish held at sub-optimal (18 °C) and optimal (22 ºC) water temperatures. This resulted in a total of eight treatments for each experiment, with two replicate tanks for each treatment. Throughout both experiments, fish were fed to apparent satiation twice daily at 09:00 and 15:30. Both experiments were run for 34 days.

In Experiment one, the following water quality parameters were monitored daily (mean ± SD) for both the 22 °C (optimal, 21.8 ± 0.3 °C) and 18 °C (suboptimal, 17.9 ± 0.2 °C) treatments: dissolved oxygen (6.4 – 7.2 mg L^{-1}), pH (7.6 – 7.7), and ammonia levels (0.1 – 0.3 mg L^{-1}) and weekly measurements for salinity (38 ± 0.5 ppt) (Bowyer et al. 2013a). In Experiment two, the same following water quality parameters were monitored daily (mean ± SD) for both the 22 °C (optimal, 21.9 ± 0.5 °C) and 18 °C (suboptimal, 18.0 ± 1.2 °C) water temperature treatments: dissolved oxygen (6.3 – 7.2

mg L^{-1}), pH (7.8 – 8.0), and ammonia (0.20 – 0.21 mg L^{-1}) and weekly measurements of salinity (38 ± 0.5 ppt) (Bowyer *et al.* 2013b).

Table 1

Table 2

Table 3

Table 4

Sample Collection

At the conclusion of each experiment, fish were fasted for 24 hours before being euthanised with an overdose of AQUI-S (AQUI-S New Zealand Ltd., Lower Hutt, New Zealand). Fish were then dissected and the gastrointestinal tract removed. The distal half of the hindgut was collected from three fish per tank (n = 6 fish/treatment) and opened longitudinally onto biopsy paper. The hindgut sample was then cut in half, one half was fixed in Carnoy's solution and the second half was fixed in 10% buffered formalin. Samples fixed in Carnoy's solution were transferred to 100% ethanol after 2 hours before being transported to the University of Adelaide, Roseworthy Campus and immediately subjected to a standard histological dehydration process before being embedded in paraffin wax. Buffered formalin samples were fixed for 24 hours, processed and embedded in paraffin wax. Tissue samples were cut at 5μm on a Thermo Scientific Microm HM340E microtome (Microm International GmbH, Waldorf, Hessen, Germany), floated onto Starfrost® glass slides and allowed to stand for at least 24 hours before being stained.

Mucus Layer Thickness

Carnoys fixed samples were used to assess the mucus layer thickness. Sections were stained with periodic acid-schiffs/alcian blue pH 2.5 (PAS/AB pH 2.5) and mounted in DPX mounting medium (Ajax Finechem Pty Ltd, Taron Point, NSW, Australia). Mucus layer thickness was assessed using a similar method to that described

by Matsuo *et al*. (1997). Five photomicrographs were taken at randomly selected regions throughout the sample at 100X magnification using an Olympus WH B10X\20 microscope (Olympus, Tokyo, Japan) and Colorview Soft imaging System CX41 camera (2048 x 1538 pixel resolution) (Soft Imaging System, Brook-Anco Corp, Rochester, NY). Five random measurements of mucus layer thickness were taken between villi for each photo using the image analysis program, Video Pro® (version 6.2.1.0, Leading Edge Pty. Ltd., Adelaide, SA, Australia), resulting in 25 measurements for mucus layer thickness for each fish.

Intestinal Morphology

In Experiment one, slides were stained with PAS/AB pH 2.5 and in Experiment two slides were stained with PAS and mounted in DPX mounting medium. Photomicrographs were taken at 40X magnification using the Olympus WH B10X\20 microscope and Colorview Soft imaging System CX41 camera, described previously. Villus height, villus width and lamina propria area were measured for approximately 20 villi per section depending on the quality of the section using the image analysis program, Video Pro®. Villus height (VH; μm) was measured from the tip to the base of the villus. The apical (AW; μm) and basal (BW; μm) width of the villus was measured in order to calculate approximate villus area (VA; μm2). Villus area was calculated using the formula VA = [((AW+BW)/2)]x(VH). The lamina propria area was measured from the base of the villus to the tip of the lamina propria and expressed as a percentage of total villus area.

Goblet Cell Number and Mucin Composition

Goblet cell number and mucin composition were measured using buffered formalin fixed sections. Image J® was used to calculate the number of goblet cells per millimetre villus height. Total goblet cell numbers were calculated in Experiment one using all goblet cells observed in sections stained with PAS/AB pH 2.5. Total goblet cell numbers in Experiment two were calculated by the sum of all neutral goblet cells observed when stained with PAS and acidic goblet cells observed when stained with HID/AB pH 2.5. Total goblet cell number was expressed as total goblet cell number per millimetre of villus height.

In Experiment one, neutral goblet cell number was calculated by the sum of neutral and intermediate (goblet cells observed to contain a mixture of both neutral and acidic mucins) in sections stained with PAS/AB pH 2.5. Due to the high number of intermediate goblet cells observed in sections stained with PAS/AB pH 2.5 this method was modified for Experiment two. In Experiment two, neutral goblet cell number was calculated by the total number of goblet cells observed in sections stained with PAS. For both experiments, total acidic goblet cell number was calculated from the total goblet cell number observed in sections stained with high iron diamine/alcain blue pH 2.5 (HID/AB pH 2.5). Neutral and acidic goblet cell number is expressed as the number of neutral or acidic goblet cells observed per millimetre of villus height.

Statistics

IBM SPSS, Version 20 for Windows (IBM SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Homogeneity of variances and normality among mean values was assessed using Levene's test for equality of variance errors and the standardized residuals against the predicted mean plot. Data were analysed using a two-

factor ANOVA with dietary inclusion level of SESBM or SPC as the first factor and water temperature as the second factor. When significant interactions were observed, the data were analysed using one-factor ANOVA. Student Newman-Keuls (SNK) test was used to identify significant differences between multiple treatment means. The relationship between mucus layer thickness or goblet cell numbers and SESBM or SPC was analysed using Pearson linear regression analysis. A significance level of $P < 0.05$ was used. All data are expressed as means ± standard error of the mean, unless otherwise specified.

Results

Growth Performance and Feed Intake

For growth performance and feed intake of yellowtail kingfish from Experiment one and two refer to Bowyer *et al.* (2013a) and Bowyer *et al.* (2013b), respectively. In short, the growth and feed efficiency yellowtail kingfish in Experiment one was significantly reduced at 18 °C compared to 22 °C. In addition, second-order polynomial regression showed a negative effect on growth performance and feed efficiency in fish fed increasing dietary inclusions of SESBM at both water temperatures. This resulted in a significantly positive polynomial relationship between FCR and increasing SESBM inclusion at both water temperatures (Bowyer *et al.* 2013a).

In Experiment two, the growth and feed efficiency was also significantly reduced in Yellowtail kingfish at 18 °C compared to fish at 22 °C. In addition, second-order polynomial regression showed a negative impact on the growth of yellowtail kingfish fed increasing dietary inclusion levels of SPC. Feed intake was not affected by

dietary inclusion of SPC; however, the apparent feed conversion ratio was significantly higher above 30% SPC inclusion (Bowyer *et al.* 2013b).

Mucus Layer Thickness

Table 5
Table 6

The mucus layer in the hindgut of yellowtail kingfish predominantly occurred between the villi and was observed to be thin and discontinuous, with the tips of villi not always covered. In Experiment one, dietary SESBM inclusion level had a significant effect on mucus layer thickness (Table 5, $P = 0.003$). Mucus layer thickness was significantly thinner in fish fed 20 and 30% dietary inclusion of SESBM (Table 5). Pearson's linear regression analysis also revealed a significant negative relationship between increasing SESBM level and mucus layer thickness ($R^2 = 0.5735$, $P = 0.001$, Fig 1a). Water temperature had no significant effect on mucus layer thickness ($P = 0.399$), and there was no significant interactions between dietary SESBM inclusion and water temperature for mucus layer thickness ($P = 0.572$) (Table 5).

Figure 1

In Experiment two, there was no significant interaction between dietary SPC inclusion and water temperature for mucus layer thickness ($P = 0.885$). The dietary inclusion of SPC had no significant effect on mucus layer thickness ($P = 0.114$) (Table 6). There was also no significant linear relationship between SPC dietary inclusion and mucus layer thickness (Fig. 1b, $R^2 = 0.1598$, $P = 0.125$). There was, however, a significant effect of water temperature on mucus layer thickness ($P = 0.003$). In contrast to fish fed the SESBM in Experiment one; fish fed the SPC in Experiment two had a significantly thinner mucus layer at 18 °C compared to 22 °C (Table 6).

Intestinal Morphology

Villi in the hindgut of yellowtail kingfish were long, slender and branched and appeared similar across all treatments. In Experiment one, dietary inclusion of SESBM had no significant effect on villus height (P = 0.562), villus area (P = 0.610) and lamina propria area (P = 0.688) (Table 5). Similarly, water temperature had no significant effect on villus height (P = 0.682), villus area (P = 0.346) and lamina propria area (P = 0.084). There were no significant interactions between water temperature and dietary inclusion of SESBM for villus height (P = 0.477), villus area (P = 0.428) or lamina propria area (P = 0.064).

In Experiment two, dietary inclusion of SPC had no significant effect on villus height (P = 0.597), villus area (P = 0.217) or lamina propria area (P = 0.439) (Table 6). Similarly, water temperature had no significant effect on villus height (P = 0.818), villus area (P = 0.701) or lamina propria area (P = 0.503). There were no significant interactions between water temperature and dietary inclusion of SPC for villus height (P = 0.174), villus area (P = 0.599) or lamina propria area (P = 0.574).

Goblet Cell Number

In Experiment one, there was a significant effect of SESBM inclusion level (P = 0.044) and water temperature (P = 0.004) on total goblet cell numbers in the hind gut (Table 5). However, there was also a significant interaction between the two factors (P = 0.026) for goblet cell number. The interaction could be explained by the significant positive linear relationship between SESBM inclusion and goblet cell number at 18 °C,

whereas no significant relationship was evident between SESBM inclusion and goblet cell number at the higher temperature of 22 °C (Table 5).

In Experiment two, there were no significant interaction between dietary inclusion level of SPC and water temperature on total goblet cell number (Table 6, P = 0.394). Both SPC dietary inclusion level (P = 0.802) and water temperature (P = 0.232) had no significant effects on total goblet cell number (Table 6).

Mucin Composition

In Experiment one, there were no significant interaction between SESBM dietary inclusion and water temperature on the number of goblet cells containing either neutral (P = 0.215) or acidic mucins (P = 0.392). Dietary inclusion of SESBM had no significant effect on the number of goblet cells containing neutral (P = 0.104) or acidic mucins (P = 0.435). However, there was a significant effect of water temperature on the number of goblet cells containing neutral mucins (18>22 °C, P = 0.003, Table 5). Water temperature also had a significant effect on the number of goblet cells containing acidic mucins (18<22 °C, P = 0.009, Table 5).

In Experiment two, there were no significant interactions between SPC dietary inclusion and water temperature for the number of goblets cells containing either neutral (P = 0.628) or acidic (P = 0.085) mucins (Table 6). Water temperature also had no significant effect on the number of goblet cells containing either neutral (P = 0.558) or acidic (P = 0.102) mucins. There were also no significant effect of dietary SPC inclusion on the number of goblet cells containing neutral (P = 0.643) or acidic (P = 0.743) mucins (Table 6).

Discussion

In the current study, alterations to the mucus layer were measured through changes in mucus layer thickness and goblet cell mucin compositions. The synthesis and degradation of the mucus layer are natural processes. A balance between these processes is required to protect the epithelium against harmful organisms or detrimental compounds present in the diet (Corfield *et al*. 2001). In Experiment one, the erosion of the mucus layer in fish fed SESBM may have acted as a stimulus to increase goblet cell number in order to increase the synthesis of mucins. However, the mucus layer appears to have eroded quicker than mucins could be synthesised and secreted. In contrast, no significant erosion of the mucus layer occurred in fish fed SPC, as a result, goblet cell numbers did not significantly increase. Regional variation in the mucus layer thickness of rodent models and humans, from the stomach to the colon indicate that each region has an optimal thickness to protect the underlying mucosa (Atuma *et al*. 2001). The optimal mucus layer thickness in the hindgut appears to be significantly reduced in fish fed SESBM.

SESBM comprises a number of antinutrients including oligosaccharides, β-conglycinin, glycinin, lectins, phytic acid-P, saponins and trypsin inhibitors (Francis *et al*. 2001; Gatlin *et al*. 2007). Fish fed higher dietary inclusions of SESBM consequently consumed a higher concentration of antinutrients. The increased erosion of the mucus layer in yellowtail kingfish fed SESBM in Experiment one may have been caused directly by the higher intake of antinutrients when fish were fed higher dietary inclusion levels of this constitute. The concentration of antinutrients in SPC is considerably less than SESBM. For example, SESBM contains ~0.6% saponin which is reduced to ~0% in SPC (Brown *et al*. 2008). Dietary inclusion of SPC had no significant effect on mucus

layer thickness, further supporting the hypothesis of antinutrients exerting a direct effect on mucus layer thickness. The mechanism behind the increased erosion of the mucus layer requires further investigation.

The functioning of the mucus layer and microbiota composition, and the survival of pathogens is determined by the non-absorbed nutrients from the diet (Van der Meer and Bovee-Oudenhoven 1998). Despite viable counts of bacteria in the intestine of Rainbow trout (*Oncorhynchus mykiss*) when fed either a control fish meal diet or a diet with 50% fish meal replaced with SESBM, alterations to the bacterial population did occur (Merrifield *et al*. 2009). A higher number of *Psychrobater* spp. and yeast and a reduction in *Aeromonas* spp. were observed in fish fed a diet with 50% fish meal replaced with SESBM compared to fish fed a control fish meal diet (Merrifield *et al*. 2009). Dietary inclusion of soy products, when fed to yellowtail kingfish, may alter the luminal environment in the intestinal tract, which may disrupt intestinal-immune homeostasis, potentially changing the microbial profile leading to an overgrowth of undesirable bacterial species.

Bacteria have been hypothesised to play crucial roles in a number of inflammatory diseases. These diseases include Crohn's disease in humans (Schultsz *et al*. 1999) and necrotic enteritis in poultry (Collier *et al*. 2008). In poultry, *Clostridium perfringens* is not detrimental until the mucosa is compromised by predisposing factors such as *Eimeria* parasites. Inflammation caused by *Eimeria* parasites is believed to allow *C. perfringens* to multiply rapidly (Van Immerseel *et al*. 2009). An increased secretion of mucin may occur due to the initial exposure to *Eimeria*, which has been speculated to allow *C. perfringens* to utilize mucin as a nutritional substrate, providing this species with a growth advantage (Collier *et al*. 2008). Further investigations are

required into the role that bacteria play in the erosion of the mucus layer when yellowtail kingfish are fed SESBM.

In Experiment one, the lower SGR and final weight gain of yellowtail kingfish fed high dietary SESBM inclusion levels (Bowyer *et al*. 2013a), may have been affected by the utilisation of amino acids for mucin synthesis. Glycine, serine and proline are prominent in mucin released in the intestine, and sourcing these non-essential amino acids has been suggested to dominate mucosal maintenance (Moran 2011). Reduced performance occurred in poultry fed low crude protein diets, despite dietary inclusions of free essential amino acids (Moran 2011). Improved performance also occurred in chickens fed increased levels of non-essential amino acids, particularly glycine, serine and proline (Moran 2011).

In Experiment two, a significantly thinner mucus layer was observed in fish at 18 °C compared to fish at 22 °C. In Experiment one, the mucus layer was also numerically thinner in fish at 18 °C compared to fish at 22 °C, however, this was not significant. As the mucus layer in the hindgut of yellowtail kingfish becomes compromised at water temperatures slightly lower than their optimal, it is reasonable to hypothesise that the protective properties of the mucus layer may be further impaired in fish at the extremely low winter water temperature of 12 °C. If yellowtail kingfish held at such low water temperatures are fed diets containing SESBM, sub-acute enteritis may subsequently develop. Additionally, at the cooler water temperature of 13 °C, the digesta of yellowtail kingfish takes approximately three times longer to be voided compared to 21 ºC (Miegel *et al*. 2010). A reduction in gut transit time at low water temperatures may have increased the duration that the intestinal mucosa was exposed to the antinutrients present in SESBM. This, in conjunction with a compromised mucus

layer may further increase the vulnerability of underlying epithelial cells to antinutrients present in SESBM. This hypothesis requires further investigation before yellowtail kingfish are fed dietary SESBM during periods of suboptimal winter water temperatures.

These experiments were not designed to directly compare between fish fed SESBM and SPC primarily due to space limitations. Caution should be exercised when comparing between experiments. However, the mucus layer thickness of fish fed the 0% control fish meal diet in Experiment two was approximately 8μm thicker at both water temperatures than fish in Experiment one. The 0% control fish meal diet used in each experiment were identical. The observed difference may have been due to a different cohort of yellowtail kingfish used for each experiment or a difference in sampling techniques between individuals. It may be advantages in future experiments to perform a factorial within the same experiment to compare dietary inclusions of SESBM and SPC for yellowtail kingfish.

In Experiment one, goblet cell mucin composition was significantly affected by water temperature and a significant increase in the number of goblet cells containing acidic mucins was measured in yellowtail kingfish at 22 ºC compared to 18 °C. Similarly, an increased number of goblet cells containing acidic mucins was observed at 22 °C in Experiment two, although statistical significance was not attained ($P = 0.102$). Bacteria have evolved to produce a number of specific mucin-degrading compounds such as proteases, glycosidases, sialidases and sulfatases (Derrien *et al.* 2010). Compared to neutral mucins, the strong negative charge exhibited by acidic mucins increases resistance to degradation by bacterial enzymes, as well as offering superior binding sites to facilitate the sloughing of detrimental biotic and abiotic factors (Derrien

et al. 2010). The decreased synthesis of acidic mucins in fish at 18 °C may result in an increased susceptibility of yellowtail kingfish to infection.

In the current study, there was a limited inflammatory response to both dietary SESBM and SPC in the hindgut of yellowtail kingfish. Typical characteristics of sub-acute enteritis were measured using the scoring system developed for Atlantic salmon by Urán (2008). However, villus height, villus area and lamina propria area in the current study were not significantly affected by dietary SESBM or SPC inclusion at either water temperature. Villus morphology appeared similar to that reported for Atlantic salmon fed a control fish meal diet (Urán 2008).

However, in Experiment one, there was a positive relationship between SESBM inclusion and increasing goblet cell numbers at 18 °C. Goblet cell number has been observed to be more responsive than other variables measured in Atlantic salmon fed 10% dietary SESBM over 57 days (Urán *et al*. 2009). Results from Urán *et al*. (2009) and from the current study suggest that an increase in goblet cell number may indicate the first stage of sub-acute enteritis. Saponins have also been suggested to be the primary cause of sub-acute enteritis in Atlantic salmon (van den Ingh *et al*. 1996). This is consistent with the hypothesis of alcohol soluble antinutrients being the primary cause of sub-acute enteritis in fish, as yellowtail kingfish showed no signs of sub-acute enteritis, including increases in goblet cell number, when fed SPC.

Both experiments were run for 34 days. Fish in all treatments increased in size by greater than 300% and significant differences in growth performance indices between treatments were observed (Bowyer *et al.* 2013a; Bowyer *et al.* 2013b). Atlantic salmon have been shown to develop sub-acute enteritis within 20 days of being fed a diet containing an inclusion of 20% SEBSM (Urán *et al*. 2008). In the short-term,

yellowtail kingfish appear to be more tolerant to dietary inclusion of SESBM than Atlantic salmon (Urán 2008), but less tolerant than species adapted to plant proteins, such as Channel catfish (*Ictalurus punctatus*) (Evans *et al*. 2005). However, it is unknown as to whether any damage to digestive tract ultrastructure, such as microvilli, occurred in the current study. Further investigations are required. If fish are fed diets containing high dietary concentrations of SE SBM for a longer duration, the characteristics of sub-acute enteritis may be exacerbated.

In conclusion, this study demonstrated that feeding SESBM to yellowtail kingfish resulted in a reduction in mucus layer thickness. This, in addition to the shift from acidic to neutral goblet cell mucin composition at suboptimal water temperature, presumably led to a reduction in the protective ability of the mucus layer. Increased goblet cells numbers in relation to feeding SESBM indicated that the mucosa was likely compromised and that this may have been the first stage of sub-acute enteritis in yellowtail kingfish. If yellowtail kingfish are fed a diet containing SESBM and are exposed to further stress, such as colder water temperatures indicative of those experienced during winter in Spencer Gulf, sub-acute enteritis may subsequently develop. In contrast to fish fed SESBM, in Experiment two, dietary SPC caused no significant alterations to the mucus layer and morphology of the digestive tract.

Based on the growth performance published in Bowyer *et al*. (2013a) and Bowyer *et al*. (2013b) and observations made in this study, we recommend that SESBM be excluded from yellowtail kingfish diets until further research is undertaken to assess the long term effects of feeding this ingredient under production conditions, particularly at low sub-optimal winter water temperatures. We would also recommend a dietary inclusion level of 20% SPC for yellowtail kingfish diets.

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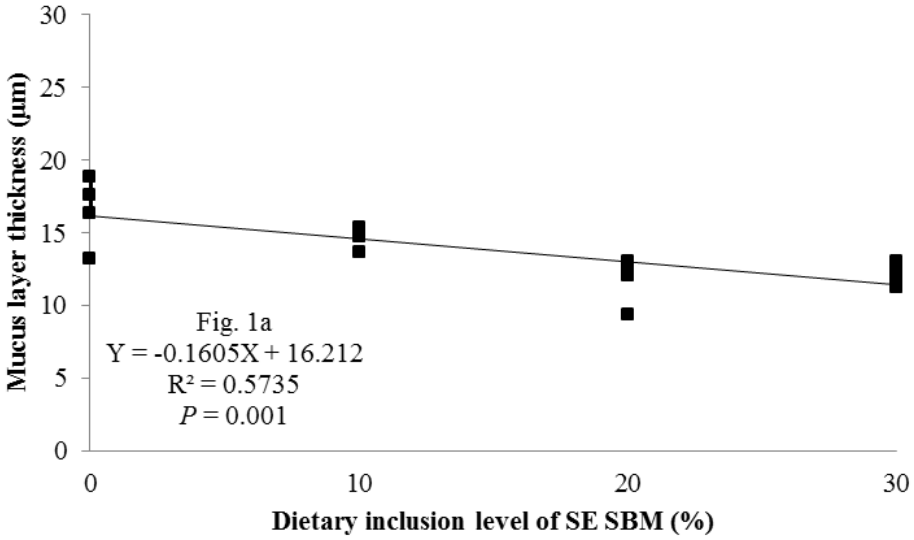

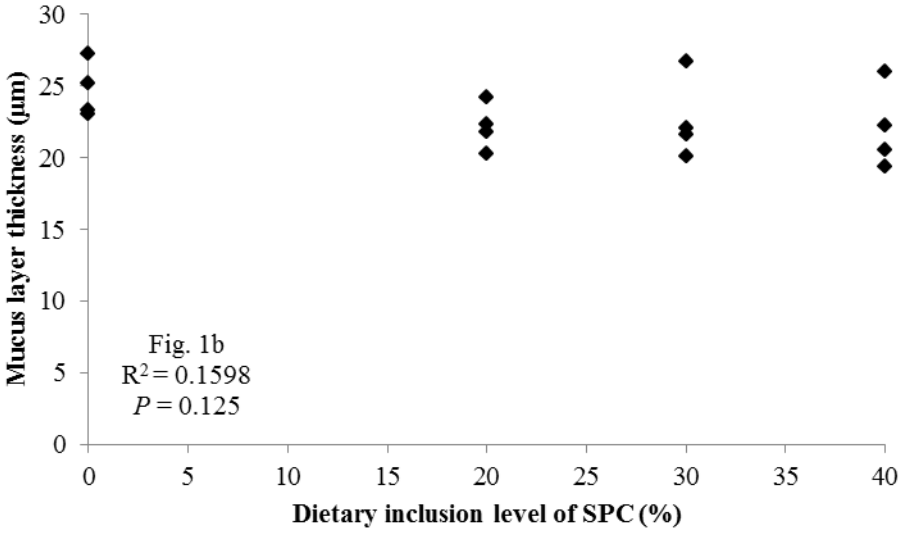

Figure 1a & b. Mucus layer thickness between villi in the hindgut of yellowtail kingfish fed increasing dietary inclusion of SE SBM (Fig 1a) or SPC (Fig 1b). Pearson linear regression analysis (n = 4).

Table 1. Diet formulations (g kg^{-1} dry basis) of the four experimental diets (formulated on a digestible protein and lipid basis) fed to yellowtail kingfish in Experiment one.

	Diet (%)			
Ingredients [a]	0	10	20	30
Herring meal	460.0	360.0	260.0	160.0
Solvent extracted soybean meal	0.0	100.0	200.0	300.0
Wheat 14	90.0	90.0	90.0	90.0
Wheat gluten meal	73.9	77.7	77.7	95.0
Fish oil	93.4	99.5	105.6	111.9
Soy lecithin	5.0	5.0	5.0	5.0
Wheat starch	81.9	46.6	11.8	0.0
Poultry by-product meal	60.6	60.7	60.7	60.7
Blood meal	23.6	45.3	70.8	97.2
Choline chloride	3.0	3.0	3.0	3.0
Corn gluten meal	90.0	90.0	90.0	47.8
Vitamin/mineral premix[b]	2.0	2.0	2.0	2.0
Vitamin C (Stay C)[c]	3.0	3.0	3.0	3.0
Vitamin E	0.4	0.4	0.4	0.4
Betaine	5.0	5.0	5.0	5.0
Monosodium phosphate	4.6	5.5	6.5	7.6
Taurine	3.6	4.5	5.3	6.1
Lysine	0.0	0.7	1.0	1.6
Methionine	0.0	1.1	2.2	3.7
Total	*1000.0*	*1000.0*	*1000.0*	*1000.0*

Yttrium oxide was added to the diets at a rate of 200 mg kg−1.
[a] Supplied by Ridley Aquafeeds, QLD, Australia.
[b] A proprietary product supplied by Lienert Australia Pty Ltd., Australia.
[c] Rovimix® Stay-C® 35, DSM Nutritional Products, Basel, Switzerland.

Table 2. Proximate composition and calculated amino acid composition (dry basis) of the SESBM ingredient and the four experimental diets (formulated on a digestible protein and lipid basis) fed to yellowtail kingfish in Experiment one.

		Diet (%)			
Ingredients [a]	SESBM	0	10	20	30
Analysed proximate composition					
Dry matter (g kg^{-1})	881.0	920.2	919.1	918.0	917.2
Crude protein (g kg^{-1})	524.4	496.2	508.0	504.4	498.8
Crude lipid (g kg^{-1})	20.0	208.8	197.0	186.4	178.8
Ash (g kg^{-1})	70.0	82.1	75.7	66.1	61.0
NFE (g kg^{-1})[a]	385.6	212.9	228.8	255.9	270.0
Starch (g kg^{-1})	27.0[b]	151.7	117.1	83.0	68.1
NSP (g kg^{-1})[c]	358.6	61.2	111.7	172.9	201.9
Phosphorous (g kg^{-1})	n/a	14.8	13.2	12.2	10.8
Gross energy (MJ kg^{-1})	198.6	230.5	239.2	249.0	239.5
Calculated amino acids (g kg^{-1})					
Arginine	40.4	29.0	29.7	30.4	30.9
Histidine	13.6	15.8	15.8	15.8	15.5
Isoleucine	23.4	24.3	23.5	22.6	21.4
Leucine	39.3	48.0	49.1	50.5	49.0
Lysine	32.9	33.5	33.4	33.3	33.3
Methionine	6.16	14.0	14.0	13.9	13.8
Phenylalanine	25.9	24.6	25.8	27.1	27.4
Threonine	20.7	21.9	21.9	21.9	21.5
Tryptophan	6.76	5.5	5.7	6.0	6.2
Valine	24.6	29.3	29.9	30.6	30.9
Σ IAA[d]	233.7	246.0	248.8	252.0	250.0
Taurine	n/a	8.0	8.0	8.0	8.0

NFE, nitrogen-free extract; NSP, non-starch polysaccharides; IAA, indispensable amino acids.
[a] By difference: NFE=(100−crude protein−total fat−ash).
[b] Reported SESBM ingredient starch value from NRC (2011).
[c] By difference: NSP=(NFE−starch).
[d] Σ IAA: total indispensable amino acid.

Table 3. Ingredient formulation (g kg^{-1} dry basis) of the four experimental diets (formulated on a digestible protein and lipid basis) fed to yellowtail kingfish in Experiment two.

	Diet (%)			
Ingredients [a]	0	20	30	40
Herring meal	460.0	260.0	160.0	60.0
Soy protein concentrate	0.0	200.0	300.0	400.0
Wheat 14	90.0	90.0	90.0	90.0
Wheat gluten meal	73.9	71.2	71.2	71.2
Fish oil	93.4	107.4	114.5	121.5
Soy lecithin	5.0	5.0	5.0	5.0
Wheat starch	81.9	60.3	49.1	38.1
Poultry by-product meal	60.6	60.6	60.6	60.7
Blood meal	23.6	25.6	25.5	25.1
Choline chloride	3.0	3.0	3.0	3.0
Corn gluten meal	90.0	90.0	90.0	90.0
Vitamin/ mineral premix[2]	2.0	2.0	2.0	2.0
Vitamin C (Stay C)[3]	3.0	3.0	3.0	3.0
Vitamin E	0.4	0.4	0.4	0.4
Betaine	5.0	5.0	5.0	5.0
Monosodium phosphate	4.6	6.4	7.4	8.3
Taurine	3.6	5.4	6.2	7.1
Lysine	0.0	2.6	4.0	5.5
Methionine	0.0	2.1	3.1	4.1
Total	*1000.0*	*1000.0*	*1000.0*	*1000.0*

Yttrium oxide was added to the diets at a rate of 200 mg kg^{-1}.
[1] Supplied by Ridley Aquafeeds, QLD, Australia.
[2] A proprietary product supplied by Lienert Australia Pty Ltd, Australia.
[3] Rovimix® Stay-C® 35, DSM Nutritional Products, Basel, Switzerland.

Table 4. Proximate composition and calculated amino acid composition (dry basis) of the SPC ingredient and the four experimental diets (formulated on a digestible protein and lipid basis) fed to yellowtail kingfish in Experiment two.

		Diet (%)			
Ingredients	SESBM	0	20	30	40
Analysed proximate composition					
Dry matter (g kg^{-1})	879.0	938.2	938.5	949.9	935.0
Crude protein (g kg^{-1})	720.6	496.2	498.5	491.6	490.2
Crude lipid (g kg^{-1})	1.0	208.8	177.4	184.0	178.1
Ash (g kg^{-1})	63.0	82.1	66.9	60.2	50.9
NFE (g kg^{-1})[1]	215.4	199.8	241.3	250.9	262.6
Starch (g kg^{-1})	0.0	151.7	127.1	114.6	102.4
NSP (g kg^{-1})[2]	*215.4	48.1	114.2	136.3	160.2
Phosphorous (g kg^{-1})	n/a	14.8	12.7	11.6	10.0
Gross energy (MJ kg^{-1})	20.6	23.1	23.3	23.2	23.0
Calculated amino acids (g kg^{-1})					
Arginine	57.2	29.0	30.1	30.6	31.2
Histidine	19.2	15.8	14.7	14.1	13.5
Isoleucine	33.5	24.3	23.5	23.2	22.8
Leucine	56.4	48.0	47.0	46.5	45.9
Lysine	46.7	33.5	32.4	31.9	31.4
Methionine	8.9	14.0	13.7	13.5	13.3
Phenylalanine	36.4	24.6	25.4	25.8	26.1
Threonine	29.6	21.9	21.0	20.6	20.1
Tryptophan	8.9	5.5	5.5	5.5	5.5
Valine	35.0	29.3	28.2	27.6	27.0
Σ IAA[3]	*331.8*	*245.9*	*241.5*	*239.1*	*236.7*
Taurine	n/a	8.0	8.0	8.0	8.0

NFE, nitrogen-free extract; NSP, non-starch polysaccharides; IAA, indispensable amino acids.
[1] By difference: NFE = (100 – crude protein – total fat – ash).
[2] By difference: NSP = (NFE – starch).
[3] Σ IAA: total indispensable amino acid.
*NSP of SPC ingredient based on the assumption that SPC contains no starch.

Table 5. Mucosal architecture, goblet cell mucin composition and mucus layer thickness in the hindgut of yellowtail kingfish fed solvent extracted soybean meal (mean ± SE; n=2)[1].

Water temperature (°C)	18				22				ANOVA					
									Water temperature (A)	SE SBM level (%) (B)				AxB
SE SBM level (%)	0	10	20	30	0	10	20	30		0	10	20	30	
Villus height (µm)	752±8	799±50	859±139	783±1	906±70	764±41	843±31	759±77	ns	ns	ns	ns	ns	ns
Villus area (µm^2)	79498±1751	86733±1501	92168±10852	90399±156	87845±6283	77956±7627	89078±10896	75925±5245	ns	ns	ns	ns	ns	ns
Lamina propria area[2]	18.48±1.62	19.48±0.71	21.63±0.03	19.28±0.27	23.31±0.06	20.49±2.43	19.17±0.15	24.89±2.46	ns	ns	ns	ns	ns	ns
Total goblet cell number[3,4]	87.94±0.68^b	97.49±4.42^b	128.58±15.51^a	121.51±0.04^{ab}	94.37±2.50^b	89.76±1.86^b	91.25±6.13^b	90.25±2.40^b	*	*	*	*	*	*
Neutral goblet cell number[5]	95.06±0.25	97.62±1.88	105.21±3.52	102.05±2.20	92.80±2.17	91.41±3.14	92.93±0.32	95.39±1.06	18>22	ns	ns	ns	ns	ns
Acidic goblet cell number[6]	43.15±5.61	45.77±3.52	57.85±0.34	45.52±6.18	66.99±1.37	53.05±9.11	62.07±9.04	63.12±6.54	18<22	ns	ns	ns	ns	ns
Mucus layer thickness (µm)	15.43±2.18	14.52±0.82	12.32±0.26	11.57±0.30	17.62±1.28	15.00±0.24	11.21±1.87	12.77±0.25	ns	Y	Y	Z	Z	ns

[1] ns: denotes non significance ($P > 0.05$); For variables with a significant effect of SE SBM and no interaction, values without a common upper case letter are different (Y indicates the highest value; $P < 0.05$); For variables with a significant effect of water temperature and no interaction, differences are indicated with a greater than (>) or less than (<) symbol ($P < 0.05$); A x B = Two-factor ANOVA interactions, * denotes significant interaction ($P < 0.05$), for variables with a significant interaction, differences are compared using a one-factor ANOVA, SNK test, values without a common superscript are significantly different (^{a} indicates the highest value; $P < 0.05$).

[2] Lamina propria area is expressed as a percentage of total villus area.

[3] Expressed as total number of gobet cells per millimetre villus height observed in samples stained with PAS/AB pH 2.5.

[4] There is a strong significant linear positive relationship between SE SBM inclusion and total goblet cell number at 18 °C ($y = 1.318x + 89.108$, $R^2 = 0.631$, $P = 0.019$). There was no significant relationship between SE SBM inclusion and total goblet cell number at 22 °C ($R^2 = 0.09$, $P = 0.471$) (Pearson linear regression analysis, n = 2).

[5] Expressed as total number of neutral and intermediate (both neutral and acidic) gobet cells per millimetre height observed in samples stained with PAS/AB pH 2.5.

[6] Expressed as total number of acidic gobet cells per millimetre villus height observed when stained with HID/AB pH 2.5.

Table 6. Mucosal architecture, goblet cell mucin composition and mucus layer thickness in the hindgut of yellowtail kingfish fed soy protein concentrate (mean ± SE; n=2)[1].

Water temperature (°C)	18				22				ANOVA					
									Water Temperature (A)	SPC level (%) (B)				AxB
SPC level (%)	0	20	30	40	0	20	30	40		0	20	30	40	
Villus height (µm)	1021±45	878±39	874±141	980±9	971±33	981±42	1114±216	759±12	ns	ns	ns	ns	ns	ns
Villus area (µm^2)	198203±51808	132309±7783	133314±25941	149160±7506	172082±53266	144218±6052	162214±29254	102517±5426	ns	ns	ns	ns	ns	ns
Lamina propria area[2]	12.35±0.66	15.20±1.85	12.53±1.88	12.51±0.65	12.49±1.96	13.79±0.44	13.98±1.21	14.80±0.65	ns	ns	ns	ns	ns	ns
Total goblet cell number[3,4]	133.68±15.58	125.05±12.00	101.41±4.64	92.15±7.54	125.91±38.76	123.06±3.69	119.12±30.79	150.05±14.08	ns	ns	ns	ns	ns	ns
Neutral goblet cell number[5]	87.30±19.35	77.27±13.74	55.55±1.42	58.43±2.79	81.06±31.84	65.62±6.48	75.78±18.66	80.33±5.87	ns	ns	ns	ns	ns	ns
Acidic goblet cell number[6]	46.38±3.77	47.78±1.74	45.86±3.22	33.72±10.33	44.86±6.91	57.44±2.79	43.34±12.13	69.72±8.21	ns	ns	ns	ns	ns	ns
Mucus layer thickness (µm)	23.19±0.13	21.05±0.77	20.90±0.76	19.99±0.59	26.24±1.00	23.31±0.96	24.42±2.33	24.17±1.88	18<22	ns	ns	ns	ns	ns

[1] ns: denotes non significance ($P > 0.05$); For variables with a significant effect of water temperature and no interaction, differences are indicated with a greater than (>) or less than (<) symbol ($P < 0.05$).

[2] Expressed as a percentage of lamina propria area to total villus area.

[3] Expressed as the sum of gobet cells observed in samples stained with PAS/AB pH 2.5 and HID/AB pH 2.5 per millimetre villus height.

[4] There is a strong significant negative linear relationship between SE SBM inclusion and total goblet cell number at 18 °C: y = -1.082x + 137.428, R^2=0.638, P=0.017; and 22 °C R^2=0.05, P=0.593 (Pearson linear regression analysis, n = 2).

[5] Expressed as total number of neutral gobet cells per millimetre height observed in samples stained with PAS.

[6] Expressed as total number of acidic gobet cells per millimetre villus height observed when stained with HID/AB pH 2.5.