Yellowtail Kingfish health and feed analysis

Dr. Trent D'Antignana

Project No. 2012/737

30/7/2012

Title: Yellowtail Kingfish health workshop and feed analysis

Authors: Trent D'Antignana[1,2],

Author Affiliations:

[1]Lincoln Marine Science Centre, Flinders University, Port Lincoln SA

[2]Cleanseas Tuna Pty Ltd, Port Lincoln, SA

ISBN: [978-0-9805789-3-5]

The Australian Seafood CRC is established and supported under the Australian Government's Cooperative Research Centres Program. Other investors in the CRC are the Fisheries Research and Development Corporation, Seafood CRC company members, and supporting participants.

Office Mark Oliphant Building, Laffer Drive, Bedford Park SA 5042
Postal Box 26, Mark Oliphant Building, Laffer Drive, Bedford Park SA 5042
Tollfree 1300 732 213 Phone 08 8201 7650 Facsimile 08 8201 7659
Website www.seafoodcrc.com ABN 51 126 074 048

Australian Government
Fisheries Research and Development Corporation

An Australian Government Initiative
CRC AUSTRALIA

Table of Contents

Non-Technical Summary

2012/737 –Yellowtail Kingfish health workshop and feed analysis

PRINCIPAL INVESTIGATOR: Dr Trent D'Antignana

ADDRESS:

Cleanseas Tuna Pty Ltd

Lincoln Marine Science Centre

PO Box 2023, Port Lincoln SA 5606

PROJECT OBJECTIVES:

1. To conduct detailed nutritional analysis and review commercial diets to identify whether dietary formulations are sufficient for optimal fish performance.
2. To review the existing knowledge and attain advice from experts in the field of aquatic animal health with an emphasis on nutrition and enteritis.
3. Development of a comprehensive disease investigation plan which will provide the basis of all future CRC projects concerned with YTK production.
4. Identify remedial strategies which can be immediately implemented to reduce mortality and improve growth in the existing stock.

OUTCOMES ACHIEVED

This project reviewed and examined the health status of Yellowtail Kingfish (YTK) produced in 2011 and 2012 and investigated the nutritional content of their diet with the aim to develop and validate CST's R&D direction and remedial strategies. Nutritional analysis of diets ranging in size from 200um to 9mm, suggested that taurine (Tau) and LCPUFA's were the only nutrients that could be limiting. In fact, all diets appeared to be deficient in Tau, based on the 0.5-1% dietary inclusion level recommended by Japanese researchers. The clinical observations were also consistent with a dietary Tau deficiency. The case history, dietary analysis, clinical observations and CST hypothesis that the fish were suffering from a Tau induced immuno-disregulation was then presented at the workshop for peer review. The panel of 30 experts in the field of aquatic animal health, microbiology, intestinal health, nutrition, virology and physiology were unable to provide an alternative theory to explain the unacceptable and uneconomical rates of mortality and growth. It was

agreed that mitigation strategies and future R&D should focus on elucidating the relationship between Tau and the health status of YTK. As a result CST is now reviewing the nutritional formulation of its diets, top coating existing diets with 1% crystalline Tau and systematically addressing a number of action items to further elucidate the role of Tau on YTK health.

OUTPUTS

- Comprehensive nutritional analysis completed on nursery, juvenile, growout and broodstock diets, with a focus on dietary taurine and LCPUFA's.
- Dietary analysis results were reviewed by an independent fish nutritionist to determine the nutritional adequacy of the diets in respect to the published literature on recommended dietary inclusion levels for particular nutrients.
- YTK health workshop successfully executed and attended by experts in the field of aquatic animal health, microbiology, intestinal health, nutrition, virology and fish physiology.
- A short to medium term action plan has been developed to further CST's understanding of the disease picture and the role nutritional factors namely Tau, essential fatty acids and cholesterol may be playing.
- The workshop has provided CST with the necessary information to allow informed, strategic decisions to be made with regard to maximising its return from its available Australian Seafood CRC research funds.

SUMMARY

This project addressed the need to further CST's understanding of the disease issues severely impacting YTK production. It also provided an environment to share ideas and peer review CST's approach to dealing with the outlined case history and its proposed remedial strategies and R&D activities. As a consequence of this project, CST is significantly more confident that it is dealing with a nutritional deficiency that has resulted in immuno-disregulation. The main findings from this project are below:

- The requirement for Taurine (Tau) by marine carnivores such as YTK is high and the level in the diet should be between 0.5-1.0%. Considering this recommendation it would appear that all diets are deficient in Tau, particularly those for larvae and +1kg fish.

- Hematological parameters and gross hepatic observations are consistent with a Tau deficiency.
- Addition of crystalline Tau should be immediately adopted to mitigate losses.
- Development of a routine feed sampling and analysis protocol is required as diets often do not meet the nutrient specification.
- Viral pathogens are unlikely to be responsible for the observed mortality and reduced growth. Likewise bacterial and protozoan parasites would appear to be secondary pathogens. These may or may not kill the fish and may or may not induce enteritis.
- The diagnostic approach taken by CST and Future Fisheries Veterinary Services in developing the presented case history would appear to be appropriate. As there was no alternative hypothesis to explain the observed case history the workshop furthered CST's belief that the primary case for the disease picture was immuno-disregulation as a result of inadequate dietary Tau, and potentially EPA/DHA and cholesterol. However there is still a lack of evidence linking Tau to the enteritis observed in the 2011 year class.
- A short to medium term action plan was formulated with CST's R&D manager accountable for its delivery. These actions will provide the basis for ongoing remedial strategies, improved farm husbandry and future R&D in YTK health and nutrition.

KEYWORDS: Yellowtail Kingfish, taurine, nutrition, health, enteritis

Acknowledgements

The author would like to thank:

- Mike Thomson, Damian Critchley, Jason Clark, Phil Nielsen and Clean Seas Tuna Ltd farm operations staff for assistance with sample collection.
- Dr. Mark Shepard and Prof. Masashi Maita for their insight, guidance and continued assistance with the diagnostic pathology on YTK.
- David Stone of the South Australian Research and Development Institute for his review of the nutritional profile of the diets.
- Dr. Christine Huynh for her assistance with coordinating field pathology and preparing the diagnostic reports on the case history summary.

- Dr Fran Stephens of the WA Aquatic animal health lab for her presentation on YTK histopathology in SA and WA.
- All workshop participants for their time and support in developing remedial strategies and a focused R&D plan.
- The Australian Seafood CRC and the Fisheries Research and Development Corporation for their continued financial support and assistance in helping to establish a financially viable YTK business in Australia.

1. Introduction

Cleanseas Tuna (CST) is the largest producer of Yellowtail Kingfish (YTK) in Australia. Production forecasts for 2013-14 were expected to be ≈ 2500T, however since December 2011 the company has experienced unprecedented mortalities in both its 2011 and 2012 year classes. In addition to the mortalities, fish performance has been severely compromised, with a 30% -50% reduction in growth recorded in the 2011 and 2012 year classes respectively. Though a considerable amount of information has been gathered on the disease picture in both year classes, the primary cause of the disease still remains to be elucidated. As a consequence, CST has decided to downsize its YTK production to ≈300T, a decisions which has resulted in a significant number of people being made redundant. For YTK production to increase in South Australia the primary cause of the disease picture needs to be identified and an effective treatment needs to be determined. To this end, CST proposed the following:

- Conduct a workshop attended by experts in the field of aquatic animal health.
- Undertake extensive dietary analysis to determine whether there are dietary factors contributing to the disease picture.

Through the workshop and dietary analysis it was anticipated that CST would be able to develop a disease investigation plan and remedial strategy for immediate implementation. If successful, this project will provide the first step towards rebuilding a sustainable YTK industry in Australia.

1.1 Need

The long term viability of YTK production is in question given the significant and unprecedented health issues Cleanseas has experienced in recent times. This is demonstrated by the fact that the cumulative mortality is presently 39.2% in the 2011 year class and 37.71% in the younger 2012 year class. Though the primary cause for poor fish performance remains unclear, disease investigations conducted by Future Fisheries Veterinary Services (FFVS) have provided insight. It is known that the disease picture is different between the year classes and investigations have found a mixture of disease agents, a high occurrence of intestinal disease and abnormalities, lesions and inflammation of major organs. Given the complex and potentially multifactorial nature of the disease picture CST seeks the advice from experts in the field of fish nutrition, pathology, aquatic animal health, intestinal health, virology, and protozoan parasitology. To this end, CST proposed and implemented a workshop to review the information gathered thus far in order

to develop remedial strategies for immediate implementation. The workshop should develop a disease investigation plan to elucidate the cause of its disease issues. In addition to the workshop, CST there is a need to conduct nutritional analysis of its diets as dietary factors maybe a significant contributor to the disease profile being observed.

1.2 Objectives

1. Conduct detailed nutritional analysis and review commercial diets to determine whether dietary formulations are sufficient for optimal fish performance.
2. To review the existing knowledge and attain advice from experts in the field of aquatic animal health with an emphasis on nutrition and enteritis.
3. Develop a comprehensive disease investigation plan to provide the basis of all future CRC projects concerned with YTK production.
4. Identify remedial strategies which can be immediately implemented to reduce mortality and improve growth in the existing stock.

2. Methods

2.1 Feed analysis

Dietary analysis was conducted to identify whether the nutritional formulation of the diets could be contributing to the disease picture being observed in the 2011 and 2012 year classes. As differences in survival of juvenile fish reared on Skretting Australia and Ridleys Aquafeed diets were evident, the first round of testing focused on 3-4 mm diets (see table 1). The feed sampling protocol, simply entailed removing 2kg of feed from, 1 x 25kg bag and sending 500g off to a fee-for-service laboratory for analysis. The remaining sample was retained for future analysis. These diets were analyzed for proximate (protein, energy, lipid, ash, moisture, carbohydrate) and mineral composition, amino acid and fatty acid analysis and Taurine (Tau) by AsureQuality, NZ. On examination of the results it was evident that further testing was required across all diets types and sizes. Consequently all diets in stock ranging in size from 200um to 9mm were examined in the second round of testing (see table 2). A more robust feed sampling protocol was utilized in the second round of testing and though dependent on the amount of feed in stock, it basically entailed taking a composite sample from 3 bags (25kg, 0.5T and 1T bags depending on the type used) when there was < 30T in stock and then 1 bag/10T of feed when there was >30T of feed in stock. Where possible, samples were taken from the start, middle and end of a particular run/batch. The total weight of the composite sample was 1.5kg of which each individual simple contributed

equally. The composite samples were then agitated and a 250g subsample dispatched to AsureQuality for fatty acid, cholesterol and Tau analysis.

The results of the dietary analysis were reviewed internally by CST staff, Future Fisheries Veterinary Services, Dr. Mark Shepard and Prof. Masashi Maita. In addition, the results were reviewed externally by Dr. David Stone, who compared the nutritional profile of the diets to that recommended by the National Research Council 2011(NRC 2011).

Table 1: Feed samples taken from the Port Lincoln and Arno Bay hatchery feed stores during the first round of nutritional testing. The (R) or (S) indicates whether the manufacture was Ridley's Aquafeed or Skretting respectively.

Feed sample analysis							
Sample ID	**Product Name**	**Local or imported**	**Pellet size**	**Bag size**	**Batch no**	**Date or Manufacture**	**No. of bags sampled**
PORT LINCOLN Feed Store							
A	Marine CST (R)	Local	4	25	244607	26/04/2012	**1**
B	Optima YT 100 (S)	Local	4	25	9019366	17/04/2012	**1**
C	Protec (S)	Local	3	25	9018984	6/02/2012	**1**
D	Protec ME (S)	Local	4	20	9018060	21/11/2011	**1**
E	Marine CST (R)	Local	3	25	227300	1/03/2012	**1**
F	Marine CST (R)	Local	4	25	237737	4/04/2012	**1**
G	Protec (S)	Local	2.3	25	9017559	29/09/2011	**1**
Hatchery Feed Store							
H	Gemma Diamond (S)	Imported	1.5 mm	20kg	7121550	29/06/2010	**1**

Table 2: Feed samples taken from the Arno Bay and Port Lincoln feed stores during the expanded second round of nutritional testing. The (I), (R) or (S) indicates whether the manufacture was INVE, Ridley's Aquafeed or Skretting respectively.

Feed sample analysis							
Sample ID	**Product Name**	**Origin**	**Size**	**Bag size**	**Batch no**	**Date or Manufacture**	**No. of bags sampled**
1	NRD 2/4 (I)	Imported	200-400um	1kg	120-003539-A00	14/10/2011	1
2	O.range Wean S (I)	Imported	200-400um	3kg	120-002850-A00	n/a	1
3	Marine CST (R)	local	3mm	25kg	183176	3/10/2011	3
4	Protec ME (S)	local	4mm	1T	9018057	21/11/2011	3
5	Protec ME (S)	local	4mm	1T	9018247	9/12/2011	2
6	Protec ME (S)	local	4mm	1T	9018117	29/11/2011	3
7	Protec ME (S)	local	4mm	1T	9019793	31/05/2012	1
8	Protec ME (S)	local	4mm	1T	9018194	7/12/2011	5
9	Optima YT100 (S)	local	4mm	1T	9019560	5/05/2012	3
10	Optima YT100 (S)	local	4mm	1T	9019367		1
11	Triumph (R)	local	4mm	25kg	182429	30/09/2011	3x 25kg bag
12	Protec ME (S)	local	6mm	1T	9019795	31/05/2012	3
13	Optima YT500 (S)	local	6mm	1T	9019370	17&24/4/12	3
14	Marine winter (R)	local	6mm	1T	256261	2/06/2012	3
15	Marine CST (R)	local	6mm	25kg	237739	4/04/2012	3
16	Marine CST (R)	local	6mm	1T	195538	11/11/2011	3
17	Optima YT1000	local	9mm	1T	9019342	18/04/2012	7
18	Optima YT1000 - medicated (S)	local	9mm	1T	9019755	31/05/2012	3
19	Optima YT1000 (S)	local	9mm	1T	9019098	27/03/2012	4
20	Optima YT1000 (S)	local	9mm	1T	9018887	23/03/2012	4
21	Optima YT1000 (S)	local	9mm	1T	9019013	16/03/2012	4
22	Optima YT1000 (S)	local	9mm	1T	9019438	27-28/4/12	6
23	Optima YT1000 (S)	local	9mm	1T	9019226	12/04/2012	3
24	Marine CST (R)	local	9mm	0.5T	235090	24/03/2012	4
25	Marine CST (R)	local	9mm	1T	216314	30/01/2012	4
26	Marine CST (R)	local	9mm	1T	243504	21/04/2012	5
27	Vitalis SA (S)	Imported	9mm	25kg	9010458	26/04/2012	1

2.2 Diagnostic pathology

Diagnostic pathology was conducted on production fish from both year classes and included normal, moribund, emaciated and well-conditioned fish. Gross pathology and tissue samples (liver, heart, spleen, intestine, kidney, muscle pyloric caeca) were taken by either Future Fisheries Veterinary Services (FFVS) or CST health technicians. Tissue pathology was determined by either the Western Australian Fish Health Laboratory or QML Pathology whilst the Australian Animal Health Laboratory was utilized to examine for the presence of viral pathogens. FFVS was contracted by CST to coordinate, collate and assimilate all diagnostic pathology and develop a case history document on the disease picture in both year classes.

2.3 Site visit/Workshop

Visiting experts Dr. Mark Shepard (Sakana Veterinary Services Ltd, Canada) and Prof. Masashi Maita (Tokyo University of Fisheries and Technology) arrived in Port Lincoln on the 8/7/12 for a 3 day site visit/fish examination, prior to the health workshop on the 12/7/12. Dr. Matthew Landos (FFVS) and Dr. Trent D'Antignana accompanied the two visiting experts and coordinated the sampling of cages WK11-11GS and WK12-11GL on 9/7/12 and cages ABK11-12, ABK12-12 and PAK20-12GL on 10/7/12. All cages examined were based in Port Lincoln and represented cages from different year classes, fed different diets and suffering varying degrees of background mortality. From each of the cages, moribund, emaciated/small and normal/well-conditioned fish were sampled. Blood samples were immediately taken from the cordial vein by needle and syringe and stored in a heparinized vial on ice. All fish were subjected to gross pathology and selected fish were sampled for histology. Blood samples were analyzed for hematocrit and osmotic fragility on return to the Lincoln Marine Science Centre whilst plasma cholesterol and urea nitrogen were analyzed the following day from plasma which had been frozen at -20^{o}C. Hematological methods are outlined in section 2.3.1.

The YTK health workshop was held at the SARDI Aquatic Sciences, West Beach on the 12/7/12 and chaired by CST, Chief Executive Officer, Dr. Craig Foster. The workshop agenda and the titles of the presentations are provided in Appendix 9.3, whilst the names of the attendees, their area of expertise and their affiliations is provided in Appendix 9.4. The minutes of the workshop were recorded by Dr. Erin Bubner. The workshop was concluded by developing remedial strategies for CST and exploring potential areas requiring further investigation.

2.3.1 Hematological methods

Demonstration of the haematological methods, including haematocrit, osmotic fragility, plasma cholesterol and urea nitrogen were provided by Masashi Maita to Trent D'Antignana and LMSC staff during his site visit and examination of CST fish for Tau deficiency. The methods provided by Masashi Maita are outlined briefly below.

2.3.1.1 Haematocrit

The haematocrit value was determined using a modified version of the method used by Vazquez and Guerrero (2007). In brief, the haematocrit value was determined by the standard micro-haematocrit method, and expressed in percentage. Duplicate heparinised

blood samples were loaded into standard heparinised capillary tubes (100µl), spun in a micro-haematocrit centrifuge at 12,000 rpm for 5 min and measured using a ruler.

2.3.1.2 Osmotic Fragility

NaCl solutions (0.0% - 0.8% NaCl, 0.05% increments) were prepared using distilled water. Eppindorf tubes were then filled with 1ml of each of the different saline solutions. 1µl of heparinised blood was subsequently pipetted into each of the saline solutions and mixed by gentle inversion (to prevent physical homolysis), before being placed on ice. After 30 min on ice the eppindorfs were centrifuged for 7 min @ 1000g. 300µl of the supernatant from each of the different saline solutions was then dispensed in to a 90 well micro-plate and read @ 540nm.

2.3.1.3 Plasma Cholesterol

Heparinized blood samples were centrifuged at 1500g for 10 min. The plasma was then transferred to 1.5ml Eppendorf tubes and stored at -20 ^{o}C prior to analysis. On analysis plasma samples were defrosted at room temperature and inverted several time to mix. Plasma cholesterol was then measured using an enzymic colourmetiric test kit method (Wako Pure Chemicals, Cholesterol E test kit: 439-17501, Novachem, Australia).

2.3.1.4 Plasma Urea Nitrogen

Plasma was prepared as per the method in 2.3.1.3. The quantitative determination of urea nitrogen in plasma was determined using an enzymic colourimetric (urease-indophenol) test kit (Wako Pure Chemicals, Urea N B: 279-36201, Novachem, Australia)

3. Results/Discussion

3.1 Feed analysis

The nutritional profiles of the diets were examined to identify whether dietary factors were contributing to the disease picture being observed in the 2011 and 2012 YTK year classes. The first round of testing entailed a comprehensive examination of 3-4mm diets and one larval rearing diet as these were the predominate diets fed to the 2012 year class prior to and during their increased mortality events. On review of the results against recommended dietary inclusions, it was evident that two key nutrients were potentially limiting (Tables 3-6). Based on the levels recommended by NRC 2011 and Maita (pers. comm.) it was evident that all diets were deficient in Tau. The suggested minimum requirement for free Tau was

500mg/100g, however the levels in CST's commercial diets ranged between 127mg – 331mg, approximately half the recommended dietary inclusion level. In addition, three of the diets contained less than the NRC 2011 recommendation for 2% dietary inclusion of LC PUFA's. The proposed impact and mechanism that a low dietary Tau content and insufficient LC PUFA's could have on the health status of CST's YTK is outlined in the case history report by Landos and Huynh (2012) provided in Appendix 9.5 of this document. Given these results and the admission that one of the diets was incorrectly formulated a second round of testing was conducted, focusing on, Tau, cholesterol and EPA/DHA.

The results from this second round of testing clearly demonstrated that all diets were well below the recommended free Tau level with the dietary levels in all but one of the diets ranging between 111-397mg/100g (Table 7). Though one diet (diet no. 14; Marine Winter) had a free Tau level of 412mg/100g this diet was specified to contain 0.7% added crystalline Tau thus the levels measured were substantially lower. It was evident that the diets with the lowest tau were the 9mm diets, in particular those from Skretting. This was probably a reflection of the percentage fish meal (FM) used in the diet. Matsunari *et al* (2003, 2005, 2006) demonstrated that the requirement for Tau was likely to be greater in broodstock and larvae and suggested that the min Tau level in the diet should be 1%. As the broodstock diets (diet no. 27) and the larval rearing diets (diet no. 1 and 2) contained only 1/3 to 1/5 that recommended by Matsunari *et al* (2003, 2005, 2006) it is highly probable that YTK have been deficient in Tau throughout their life.

Examination of the dietary EPA, DHA and DPA levels suggested that ≈ 30% of the diets were on the cusp of the recommended dietary inclusion level of LCPUFA's for YTK, however of most concern was the level of LCPUFA's in the larval rearing diets (diet no. 1 and 2, see Table 7), particularly diet 1 which contained ≈ ½ that recommended by the NRC (2011). In light of these results a review of the diets used in the hatchery is recommended.
It is well known that cholesterol is catabolized to bile salt, and bile salt conjugates with Tau and is excreted as bile. Consequently the cholesterol content in the feed was also measured as a deficiency in cholesterol and or Tau could affect lipid emulsification and ultimately absorption. It is well documented that plant oils and plant meals are almost devoid of cholesterol, whilst fish oil (FO) and FM have high content (USDA data base 2012). Subsequently, fish oil and fish meal substitution can have a significant impact on the level of dietary cholesterol (pers comm. David Stone). Though there would appear to be no dietary cholesterol specification for YTK, Maita *et al* (2006) observed increased mortality in yellowtail (*Seriola quinqueradiata*) when fed a diet low in cholesterol (180mg/100g) and Tau (71mg/100g), when compared to a commercial FM/FO based diet containing 285g/100g of

cholesterol and 322mg/100g of Tau. Yun *et al*, (2012) observed improved growth in turbot when fed a diet substituted with 1% additional Tau and cholesterol compared to a diet which contained 300mg/100g of cholesterol and 0.11% Tau. Examining the cholesterol content in CST's YTK diets showed that they contained between 280-418mg/100g with the cholesterol content being slightly higher in the larger pellets sizes an artefact of the increased dietary lipid inclusion level. Considering there is no recommended dietary inclusion level for cholesterol in YTK, and few studies have examined it, it is difficult to say whether this nutrient is limiting. Though the impact of FM and FO substitution on the cholesterol content of a particular diet remains to be elucidated it clearly should be considered when formulating diets.

3.2 Diagnostic pathology

A case history report based on the diagnostic pathology reports from QML and the Western Australian Fish Health Laboratory was compiled by Future Fisheries Veterinary Services. In this document the authors Matthew Landos and Christine Huynh summarized the key lesions observed in both the 2011 and 2012 year class YTK and provided theories as to how nutritional deficiencies could be contributing to the clinical pathology being observed. This report titled "Yellowtail Kingfish health workshop" is provided in Appendix 9.5.

3.3 Workshop

The YTK health workshop delivered 6 presentations on YTK health, nutrition, pathology and recent R&D trials to attendees. The slides from these presentations are provided in Appendix 9.6a-f. The workshop was concluded with general discussion to review and develop a coherent and comprehensive R&D and remedial strategy to elucidate the cause for poor fish performance and high mortality and alleviate it immediately on farm. As a consequence of this discussion, a list of suggested activities, research priorities and remedial strategies for CST to pursue were tabled and are summarized below. They are organised under the subheadings, Strategic recommendations, Investigative recommendations and R&D recommendations.

Strategic recommendations:

- Encourage complete disclosure of the nutritional composition and specification of the diets from feed manufactures to veterinarians, pathologists and physiologists to better understand the role that nutrition may be playing in the health status of YTK.

- Agree to conduct an independent audit of the feed suppliers to determine the adequacy of their QA system in regard to testing the quality of their raw materials and identifying nutrient losses post milling.
- Obtain historical data on the nutritional formulation and raw material origins for both Skretting and Ridleys diets between 2006 to 2012 and compare the nutritional profile of those diets to winter and summer diets commercially used in Japan.
- Initiate discussions with the porcine veterinarians at the Adelaide Veterinary School to explore the similarities between coccidian induced hose pipe syndrome in pigs and the intestinal thickening/enteritis present in the 2011 year class YTK.

Investigative recommendations:

- Monitor the uptake of nutrients in fish (tissue and blood) fed diets containing different levels of Tau, cholesterol and fatty acids. Examine their impact on packed cell volume, mean corpuscular volume, bilirubin, plasma urea nitrogen and albumin.
- Considering viral examination research requires diseased tissue with known lesions, paraffin blocks previously prepared for histology analysis should be re-processed for electron microscopy.
- CST should begin discussion with the Aquaculture Protein Centre in Norway as they conducting fundamental research around bile salt nutrition, amino acid metabolism and intestinal physiology.
- Pursue an examination of the historical data on the fatty acid and amino acid content of the raw materials used by both manufactures in 2006 and 2012, years where acceptable and unacceptable fish performance were observed respectively.
- Assess the nutritional composition of CST's YTK nursery/larval rearing diets, to determine whether they contain adequate levels of essential nutrients. Review the diet/feeding history in the hatchery to ascertain whether there has been a significant shift in the brand of diets used in the hatchery.
- Create an archival tissue/blood bank for future reference. This may be particularly relevant in the event that the fish improve. The inventory of samples could be used to compare the osmotic condition in the fish over time.
- Undertake antimicrobial action/inhibition tests on the diets to eliminate the presence of antimicrobials in the feed as a factor contributing to the observed lack of intestinal biodiversity.

- Review hatchery practices in 2012 compared to previous years to identify whether there were significant changes in husbandry practices.

R&D recommendations:

- Instigate longer feed trials and validate tank trials with sea based R&D trial before commercial scale nutritional changes are made to the diets on a.
- Conduct a wild fish sampling episode to determine what is “normal” is regard to gut and liver physiology, haematology and tissue amino acid and fatty acid profile.
- If the current CST Tau supplementation trial demonstrates a positive response to Tau a CRC project should be developed encompassing a tank and sea cage Tau dose response trial on naive fish.
- Undertake a commercial trial with a “deluxe” diet (100% FM/FO; >0.5% tau; elevated LCPUFA’s, vitamin E, vitamin C, choline chloride, and cholesterol) and compare fish performance to YTK conditioned on a standard commercial diet. The purpose of this trial would be to identify whether nutritional factors are the primary cause for poor fish performance and elevated mortalities. Such a trial would also allow CST to understand what are the baseline parameters are (haematology, tissue composition and morphology) in fish cultured under commercial cage conditions.
- Conduct a gut biota culture study or pyro sequencing to identify/speciate and enumerate the intestinal fauna. The purpose being to determine whether a bacterial agent is a primary or secondary agent associated with the enteritis.
- Conduct a tank based, diagnostic trial on sick fish to determine whether they respond to Tau supplementation.

Table 3: Proximate composition of predominately 3 - 4 mm commercial diets for Yellowtail Kingfish produced by either Ridley's Aquafeed or Skretting Australia. Recommended dietary inclusion levels are also provided.

	Type	Units	A	B	C	D	E	F	G	H	Requirement (dry basis)	Reference
Proximate	Ash	%m/m	9.5	11.8	13.1	12.1	8.3	8.1	10.4	10.1	<15	
	Protein		54.3	52.6	49.4	50.3	54.5	53.2	51.7	57.6	45-50	
	Moisture		7.0	9.1	8.6	8.8	5.0	6.1	7.1	9.1	<12	NRC 2011
	Fat		18.7	18.0	19.7	18.1	19.2	22.1	24.0	17.0	15-20	
	Carbohydrate		10.5	8.5	9.2	10.7	13.0	10.5	6.8	6.2	<20	NRC 2011
	Energy		1790	1710	1730	1710	1860	1900	1880	1710	1700-1900	

Table 4: Mineral composition of predominantly 3 - 4 mm commercial diets for Yellowtail Kingfish produced by either Ridley's Aquafeed or Skretting Australia. Recommended dietary inclusion levels are also provided.

	Type	Units	A	B	C	D	E	F	G	H	Requirement (dry basis)	Reference
Minerals	Aluminium	mg/kg	62	43	110	86	46	20	49	86	Trace	NRC 2011
	Antimony		<0.01	<0.01	0.48	0.11	<0.01	<0.01	0.030	<0.01	Trace	NRC 2011
	Arsenic		1.2	2.0	1.4	1.2	1.1	1.3	1.3	2.7	< 3	NRC 2011 (APVMA 2011)
	Barium		3.5	2.8	13	8.4	2.7	3.8	4.1	2.0	Trace (Not essential)	NRC 2011
	Beryllium		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	NR	
	Bismuth		<0.01	<0.01	0.043	<0.01	<0.01	<0.01	<0.01	<0.01	NR	
	Boron		5.0	3.6	2.5	1.1	2.8	3.1	0.6	1.9	Trace	
	Cadmium		0.38	0.68	0.49	0.56	0.23	0.52	0.50	0.28	Trace	
	Caesium		0.023	0.019	0.022	0.015	0.025	0.024	0.019	0.028	NR	
	Calcium		27000	35000	43000	38000	17000	17000	27000	23000	NR get from seawater	NRC 2011
	Choline	mg/100g	178	164	133	164	181	192	165	186	100	NRC 2011
	Chromium	mg/kg	0.96	0.69	0.85	1.3	0.55	0.43	0.57	1.9	Trace	NRC 2011
	Cobalt		1.3	0.14	0.19	0.17	1.2	1.2	0.13	0.22	Trace	NRC 2011
	Copper		7.9	9.4	12	11	12	12	13	11	5	NRC 2011 for Atlantic salmon and Grouper
	Iodide(Potassium Iodide)	ug/100g	290	300	340	390	200	160	470	760	110	NRC 2011 for Rainbow Trout
	Iron	mg/kg	240	660	530	570	490	420	660	320	160	Webster and Lim 2002
	Lead		0.11	0.22	0.43	0.32	0.068	0.068	0.26	0.16	<10	APVMA 2001
	Lithium		0.38	0.19	0.19	0.15	0.21	0.18	0.12	0.33	Trace	
	Magnesium		1800	1600	1500	1400	1500	1800	1300	1700	400	NRC 2011 for Atlantic salmon

Table 4 (cont):

Minerals	Manganese	mg/kg	59	150	51	41	30	38	37	35	10	NRC 2011 for Atlantic salmon
	Mercury		0.088	0.074	0.044	0.046	0.052	0.016	0.032	0.040	<40	APVMA 2001
	Molybdenum		0.58	0.57	0.38	0.43	1.0	0.62	0.41	0.42	Trace	NRC 2011
	Nickel		1.2	0.64	0.46	0.92	1.3	1.5	0.52	1.3	Trace	NRC 2011
	Phosphorus		18000	20000	23000	20000	17000	14000	17000	18000	8000	NRC 2011 for Red Drum and Atlantic Salmon
	Potassium		5200	6100	4900	5000	4900	6900	5800	9700	NR get from seawater	Webster and Lim 2002
	Rubidium		2.9	2.0	2.2	2.3	3.3	2.9	5.0	1.7	Trace (Not essential)	NRC 2011
	Selenium		1.7	1.9	1.6	1.9	1.5	1.3	1.6	1.5	0.15	NRC 2011 for Rainbow Trout
	Silver		<0.05	<0.05	<0.05	<0.05	0.065	0.055	<0.05	<0.05	Trace (Not essential)	NRC 2011
	Sodium		4900	6300	6900	5700	6700	7200	6900	7700	NR get from seawater	Webster and Lim 2002
	Strontium		84	84	85	83	66	46	49	46	Trace (Not essential)	NRC 2011
	Thallium		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.011	<0.01	NR	
	Tin		0.047	<0.03	0.040	<0.03	<0.03	<0.03	<0.03	<0.03	Trace	NRC 2011
	Uranium		0.026	0.025	0.023	0.019	0.032	0.019	0.073	0.79	NR	
	Vanadium		1.6	0.24	0.26	0.26	0.80	0.25	0.26	2.3	Trace	NRC 2011
	Zinc		140	86	110	110	120	120	170	170	30	NRC 2011

Table 5: Amino acid composition of predominantly 3 - 4 mm commercial diets for Yellowtail Kingfish produced, by either Ridley's Aquafeed or Skretting Australia. Recommended dietary inclusion levels are also provided.

	Type	Units	A	B	C	D	E	F	G	H	Requirement (dry basis)	Reference
Amino Acids	Alanine	mg/100g	3426	3194	3432	3188	3231	2985	3575	3186	Not essential	NRC 2011
	Arginine		3720	3596	3639	3885	4156	3399	3921	3614	1600 (1.6%)	NRC 2011
	Aspartic acid		4832	4251	4826	4690	5844	5230	4585	5561	Not essential	NRC 2011
	Glutamic acid		7460	7038	5038	5136	6491	6621	5389	8436	Not essential	NRC 2011
	Glycine		3198	3666	4225	4102	3187	3185	4106	3617	Not essential	NRC 2011
	Histidine		2371	1632	1284	1201	2148	1884	1731	1547	650 to 850 (0.65-0.85%)	Webster and Lim 2002
	Isoleucine		2241	1957	1943	2076	2177	1901	1894	2535	1030	NRC 2011
	L cystine		1049	1056	1339	1423	1285	1019	1177	892	1200 (1.2% - met + Cys)	NRC 2011
	Leucine		3880	3429	4018	3851	4493	4118	3821	4161	1330 (1.33%)	NRC 2012
	Lysine		3940	3165	2832	2850	4050	4065	3915	4719	1900 (1.9%)	NRC 2011
	Methionine		1106	912	920	877	982	1005	934	1354	800 (0.8%)	NRC 2011
	Phenylalanine		2210	2057	2357	2240	2584	2488	2225	2434	1050 (1.05%)	NRC 2012
	Proline		2224	2049	3354	3414	2258	1742	2364	2205	Not essential	
	Serine		2091	2187	2515	2600	2514	2291	2156	2149	Not essential	
	Taurine		191	223	245	158	127	234	218	331	500 (0.5%)	NRC 2011
	Threonine		2114	1944	2047	2043	2387	2143	2094	2263	1060 (1.06%)	NRC 2011
	Tryptophan		649	519	510	517	665	618	575	647	270 (0.27%)	NRC 2011
	Tyrosine		2046	2025	1480	1457	1698	1495	2240	1771	1710 (1.71% - Tyr + Phe)	NRC 2011
	Valine		3082	2836	3694	3616	3846	3528	3283	3472	1160 (1.16%)	NRC 2011

Table 6: Fatty acid composition of predominantly 3 - 4 mm commercial diets for Yellowtail Kingfish, produced by either Ridley's Aquafeed or Skretting Australia. Recommended dietary inclusion levels are also provided.

	Type	Units	A	B	C	D	E	F	G	H	Requirement (dry basis)	Reference
Fatty Acids	Butyric C4:0	mg/100g	<10	<10	<10	<10	<10	<10	<10	<10		
	Caproic C6:0		<10	<10	<10	<10	<10	<10	<10	<10		
	Caprylic C8:0		<10	<10	<10	<10	<10	<10	<10	<10		
	Capric C10:0		<10	<10	<10	<10	<10	<10	<10	<10		
	Lauric C12:0		11	18	18	18	14	22	21	17		
	Trisdecanoic C13:0		<10	<10	<10	<10	<10	<10	<10	<10		
	Myristic C14:0		430	900	540	560	800	1020	1070	1100		
	Pentadecanoic C15:0		55	87	66	63	70	83	78	89		
	Palmitic C16:0		3890	3040	4110	3750	3660	4290	4680	2800		
	Margaric C17:0		62	82	94	86	80	82	84	76		
	Stearic C18:0		1030	720	1400	1220	960	1100	1160	560		
	Arachidic C20:0		36	52	33	34	42	45	54	37		
	Docosanoic C22:0		24	47	16	16	28	18	19	19		
	Tetracosanoic C24:0		<10	19	<10	<10	16	<10	11	11		
	Total SFA		**5538**	**4946**	**6277**	**5747**	**5670**	**6660**	**7177**	**4709**		

Table 6 (cont):

Fatty Acids	Decenoic C10:1	mg/100g	<10	<10	<10	<10	<10	<10	<10	<10		
	Myristoleic C14:1		41	46	60	50	46	62	55	37		
	Pentadecenoic C15:1		<10	16	13	11	<10	16	12	13		
	Palmitoleic C16:1		1050	1230	1130	1170	1380	1640	1730	1160		
	Heptadecenoic C17:1		<10	22	<10	10	<10	19	<10	17		
	Octadecenoic C18:1n-6		12	19	16	15	<10	18	15	19		
	Octadecenoic C18:1n-7		460	490	470	440	530	610	600	420		
	Oleic C18:1n-9		6040	3010	6300	5260	4220	5170	5820	1560		
	Eicosenoic C20:1n-9		120	310	120	110	140	150	170	410		
	Eicosenoic C20:1n-11,13		16	19	17	17	12	17	22	37		
	Docosenoic C22:1n-9		14	46	10	10	18	16	15	41		
	Docosenoic C22:1n-11,13		<10	<10	<10	<10	<10	<10	<10	<10		
	Tetracosenoic C24:1		24	66	24	24	36	38	38	82		
	Total MUFA		**7777**	**5274**	**8147**	**7096**	**6382**	**7756**	**8465**	**3796**		

Table (6 cont)

Fatty Acids	Linoleic C18:2n-6	mg/100g	1930	870	1440	1210	1280	1440	1430	640	2000 - 3900 (2% fish >50g; 3.9% for larvae)	NRC 2011
	Gamma Linolenic C18:3n-6 (GLA)		27	41	28	29	42	51	48	37		
	Steridonic C18:4n-3		94	290	110	130	220	270	230	380		
	Eicosadienoic C20:2n-6		24	35	23	20	19	29	24	31		
	Dihomo-gamma-linoleic C20:3n-6		18	22	16	17	20	24	29	21		
	Arachidonic C20:4n-6		97	140	72	94	150	120	130	160		
	Docosatetraenoic C22:4n-6		30	59	37	34	58	33	38	67		
	Docosapentaenoic C22:5n-6		25	76	29	34	62	69	94	98		
	Total n-6 PUFA		**2151**	**1243**	**1645**	**1438**	**1631**	**1766**	**1793**	**1054**		
	Alpha Linolenic C18:3n-3		330	210	270	240	220	250	280	190		
	Eicosatrienoic C20:3n-3		<10	17	<10	<10	<10	11	10	17		
	Eicosatetraenoic C20:4n-3		57	200	47	58	88	110	160	280		
	Eicosapentanaeoic C20:5n-3 (**EPA**)		560	1730	640	790	1420	1630	2130	2190		
	Heneicosapentaenoic acid C21:5n-3		<10	<10	<10	<10	<10	<10	<10	<10		
	Docosapentaenoic C22:5n-3		120	240	98	110	170	210	260	270		
	Docosahexaenoic C22:6n-3 (**DHA**)		700	1800	680	830	1540	1170	960	1680		
	Total n-3 PUFA		**1767**	**4180**	**1735**	**2028**	**3438**	**3370**	**3790**	**4610**		
	EPA+DHA		**1260**	**3530**	**1320**	**1620**	**2960**	**2800**	**3090**	**3870**		
	Total LC PUFA		**1380**	**3770**	**1418**	**1730**	**3130**	**3010**	**3350**	**4140**		
	n-3:n-6		**0.8**	**3.4**	**1.1**	**1.4**	**2.1**	**1.9**	**2.1**	**4.4**		

Table 7: Concentration of selected nutrients found in commercial diets for Yellowtail Kingfish that were produced by either Ridley's Aquafeed or Skretting Australia. Taurine values marked in yellow were provided by the feed suppliers.

Feed sample analysis							
Product Name	Fat (%m/m)	Taurine (mg/100g)	DHA (mg/100g)	EPA (mg/100g)	DPA (mg/100g)	LCPUFA (mg/100g)	Cholesterol (mg/100g)
1	12.9	397	1550	650	120	2320	379
2	15.7	317	2180	920	160	3260	362
3	20.3	177	1300	1370	190	2860	350
4	19.8	186	1050	1020	130	2200	305
5	19.9	183	1350	1270	150	2770	302
6	20.2	203	1200	1070	130	2400	281
7	18.9	283	1580	1180	200	2960	290
8	19.3	191	940	930	120	1990	280
9	20.2	260	2620	2190	210	5020	296
10	17.9	279	1980	1740	230	3950	280
11	23.2	209	1970	2920	370	5260	418
12	20.7	222	2150	1580	260	3990	295
13	20.8	257	2010	2080	280	4370	297
14	25.6	412	2170	3680	410	6260	340
15	22.0	268	840	960	130	1930	286
16	19.3	301	1000	900	130	2030	295
17	24.2	193	2810	2680	330	5820	301
18	24.5	240	2770	2750	350	5870	309
19	25.4	131	1250	1350	180	2780	295
20	26.2	120	940	880	120	1940	335
21	25.7	112	1140	1240	170	2550	313
22	24.4	242	3430	3090	350	6870	308
23	24.6	111	1160	830	160	2150	368
24	24.3	190	920	840	130	1890	326
25	24.2	163	940	930	130	2000	316
26	21.6	161	1970	2660	320	4950	289
27	25.4	211	3390	3100	330	6820	307

4. Benefits and Adoption

On refection of the recommendations provided at the workshop CST has developed a short to medium term action plan to further develop its understanding of the disease picture and the role nutritional factors namely Tau, essential fatty acids and cholesterol may be playing. The current list of activities being pursued by CST and predominantly coordinated through its R&D Manager, Dr. Trent D'Antignana are below. They are organised under 3 sub-headings in order of priority:

Short term (1-2 months post workshop):

- Top coat existing commercial diets (4 – 9 mm) with 1% crystalline Tau and administer to all production fish.
- Provide 1% Tau to all broodstock diets (tuna and YTK).
- Investigate/develop techniques to enrich live feeds and larval rearing diets with Tau for both YTK and tuna.
- Take liver samples from 2011 and 2012 year class production fish to check for a Tau supplementation dose response.
- Confirm difference between "free Tau" and "total Tau" and identify which methods are used by Masterlab, Eurofins and AsureQuality
- Review the diet/feeding history in the hatchery to ascertain whether there has been a significant shift in the brand of diets used in the hatchery.
- Utilize Skretting to determine whether the Japanese yellowtail industry utilizes bile powders.
- Quantify remaining funds with CRC and acquit any outstanding projects.
- Dispatch histology slides/ tissue mounted in paraffin (only those known to contain lesions) from QML to the Aquatic Animal Health Lab for electron microscopy examination.

Medium term (<6 month post workshop):

- Attain historical data on the nutritional profile of both Skretting and Ridleys diets from 2006 and 2012.
- Compare the nutritional profile of Australian YTK diets to those used in Japan.
- Enlist David Stone and engage a student to conduct a literature review on the nutritional requirements of YTK so as to develop a set of guidelines on the ideal winter and summer diets for YTK.

- Complete the feed trial being undertaken in the SBT hatchery examining the impact of 2% Tau supplementation on YTK performance, survival and haematology.
- Begin making arrangements for a wild fish sampling episode to attain samples for haematology, tissue amino acid and fatty acid profile, intestinal and organ physiology.

Long term (>6 months post workshop):

- Develop a relationship with the Aquaculture Protein Centre in Norway considering they are undertaking fundamental research on amino acid metabolism and its impact on fish physiology and performance.
- Liaise with David Stone, Skretting and Ridley's to develop a new CRC project examining the impact of Tau on YTK health and performance.
- Utilize Grant Richards (Parasite Diagnostic Services) and Bec Forder (Adelaide Uni, Veterinary School) to help develop techniques to commercially enumerate coccidian/myxidia in the gut of YTK.
- Engage Darren Trott (Adelaide Uni, Veterinary School) to determine the similarities between the hose pipe syndrome observed in pigs to that observed in YTK.
- Determine the most suitable techniques to identify and enumerate protozoan gut fauna and flora.

In summary, it is hoped that these activities can elucidate the root cause for the existing disease profile observed in YTK. If successful, this action plan should provide a platform for increased investor confidence ultimately reversing the current demise of the YTK industry. This will not only benefit CST, but the aquaculture industry in general providing flow on benefits in terms of employment and revenue for regional Australia.

5. Further Development

To address some of the planned activities, it is proposed that Australian Seafood CRC projects; 2008/711 "Addressing key aquatic animal health issues limiting production of Australian Yellowtail Kingfish (*Seriola lalandi*)" and 2009/728 "Sustainable feeds and feed management of Yellowtail Kingfish" are terminated and the remaining funds returned to the research fund pool. A new ASCRC proposal will then be developed to investigate the role of

fish nutrition on the health status of YTK. It is anticipated that a full proposal will be submitted in November so as to utilise fingerlings produced by CST in the spring/summer of 2012.

6. Planned Outcomes

Using the outputs for the project as a guide it would appear that the majority of the outcomes for this project have been accomplished. As a result of this project CST, has a much greater understanding of the nutritional requirements of YTK, the composition of their diets and their nutrient specifications. Dietary analysis and review clearly implied that certain nutrients may be limiting and that routine sampling/testing and auditing of the feed suppliers QA systems should be adopted. The workshop provided an avenue for improved understanding of the factors contributing to the disease picture and essentially validated that CST has taken the appropriate steps to reduce mortality and identify the route cause. Considering no other hypothesis was presented by the panel of experts, CST is confident the primary cause for the increased mortality and reduced production is due to inadequate nutrition. CST now has a clear, peer reviewed direction as to what action is required to mitigate losses in growout and what R&D should be undertaken to better understand the role of nutrition in YTK health. A Seafood CRC research proposal examining YTK health and nutrition will be submitted in the immediate future.

It is anticipated that if the proposed short to medium term measures identified above are effectively implemented, the root cause of the failure to thrive in the current year classes of YTK will be addressed and the mortality rates and growth rates will improve significantly and be at least on par with those observed in 2008/9 and 2009/10 production seasons.

7. Conclusion

This project addressed the need to further CST understanding of the disease issues impacting YTK production. It also provided an environment to share ideas and peer review CST approach to dealing with the outlined case history and its proposed remedial strategies and R&D activities. As a consequence of this project, CST is far more confident that it is dealing with a nutritional deficiency induced immuno- incompetence. The main conclusions included:

- The requirement for Tau by marine carnivores such as YTK is high and the level in the diet should be between 0.5-1.0%. Considering this recommendation it was

apparent that all diets were deficient in Tau, particularly those for larvae and +1kg fish.

- Hematological parameters and gross hepatic observations were consistent with a Tau deficiency.
- Addition of crystalline Tau should be immediately adopted to mitigate losses.
- Development of a routine feed sampling and analysis protocol is required as diets often do not contain the level of nutrients specified.
- It is unlikely that viral pathogens are responsible for the observed mortality and reduced growth. Likewise bacterial and protozoan parasites would appear to be secondary pathogens. These may or may not kill the fish and may or may not induce an enteritis.
- On peer review it would appear that the diagnostic approach taken by CST and Future Fisheries Veterinary Services in developing the presented case history was appropriate and considering there was no alternative hypothesis to explain the observed case history the workshop furthered CST's belief that the primary cause for the disease picture was immuno-disregulation as a result of inadequate dietary Tau, and potentially EPA/DHA and cholesterol. However there is still a lack of evidence linking Tau to the enteritis observed in the 2011 year class.
- A short to medium term action plan has been formulated and CST's R&D manager is accountable for its delivery. These actions will provide the basis for ongoing remedial strategies, improved farm husbandry and future R&D in YTK health and nutrition.

8. References

Maita, M., Maekawa, J., Satoh, K., Futami, K. and Satoh, S.(2006). Disease resistance and hypocholesterolemia in yellowtail *Seriola quinqueradiata* fed a non-fishmeal diet. Fisheries Science, 72: 513-519.

Matsunari, H., Takeuchi, T. Murata, Y., Takahashi, M., Ishibashi, N., Chuda, H. and Arakawa, T. (2003). Changes in the Tau content during the early growth stages of artificially produced yellowtail compared with wild fish. Nippon Suisan Gakkaishi, 69 (5); 757-762.

Matsunari, H., Takeuchi, T., Takahashi, M. and Mushiake, K.(2005). Effect of dietary taurine supplementation on growth performance of yellowtail juveniles *Seriola quinqueradiata.* Fisheries Science, 71: 1131-1135.

Matsunari, H., Hamada, K., Mushiake, K. and Takeuchi, T. (2006). Effects of Tau levels in broodstock diet on reproductive performance of yellowtail, *Seriola quinqueradiata*. Fisheries Science, 72: 955-960.

NRC (2011). Nutrient requirements of fish and shrimp. Animal nutrition series, National Academic Press, Washington, D.C.

USDA (2012). National Nutrient Database for Standard Reference – release 24. #15236 Fish, salmon, Atlantic, farmed, raw. http://ndb.nal.usda.gov/ndb/foods/list

Vazquez, R.C and Guerrero, G.A. (2007). Characterization of blood cells and hematological parameters in *Cichlasoma dimerus* (Teleostei, Perciformes). Tissue and Cell, 39: 151-160.

Yun, B., Ai, Q., Mai, K., Xu, W., Qi, G. and Luo, Y. (2012). Synergistic effects of dietary cholesterol and Tau on growth performance and cholesterol metabolism in juvenile turbot (Scophthalmus maximus L.) fed high plant protein diets. Aquaculture, 324-325: 85-91.

9. Appendices

Appendix 9.1: Intellectual property

No intellectual property has been generated by this project.

Appendix 9.2: Staff

Please refer to Appendix 9.4 for all people who attended the workshop. People outside of this list who were involved in this project are outlined below.

Clean Seas Tuna
Philip Nielsen
Jason Clark

Flinders University
Maximiliano Canepa

Future Fisheries Veterinary Services
Dr. Christine Huynh

Appendix 9.3: Workshop agenda

CLEANSEAS | sustainable seafood

Australian Seafood CRC –Yellowtail Kingfish health workshop

Date	Thursday 12th July, 2012
Time	9:00 am – 5:00pm (Refreshments/lunch will be provided)
Location	SARDI Aquatic Sciences, 2 Hamra Ave, West Beach, SA 5024
Chair	Craig Foster

9:00am	Welcome – Craig Foster (CEO, Cleanseas tuna)
9:05am	Health workshop program overview - Trent D'Antignana (R&D manager)
9:10am	Consideration and comparative clinical pathology – Mark Shepard
10:00am	Clean Seas Tuna Pty Ltd:Yellowtail Kingfish health workshop - Matt Landos
10:50am	**MORNING TEA**
11:30am	Yellowtail kingfish health workshop (cont) - Matt Landos
12:10am	A Japanese perspective on yellowtail health: The problem of Tau deficiency – Masashi Maita
12.30am	**LUNCH**
1:10pm	Yellowtail kingfish lesions seen in sea caged fish from SA as well as WA - Fran Stephens
1:30pm	Assessment of soybean enteritis like condition in juvenileYellowtail Kingfish *Seriola lalandi* held under different feed and temperature regimes
2:10pm	Dietary investigations and determining their impact on fish performance and survival – Trent D'Antignana
2:30pm	General discussion – Craig Foster
3:30pm	**AFTERNOON TEA**
4:00pm	General discussion (cont) - Review and development of a coherent and comprehensive R&D strategy to elucidate the cause for poor fish performance and high mortality – Trent D'Antignana
5:00pm	**CLOSE**

Cleanseas Tuna Ltd.
PO Box 159
7 North Quay Boulevard
Port Lincoln 5606 South Australia
Telephone +61 (0)8 8621 2900
Facsimile +61 (0)8 8621 2990
www.cleanseas.com.au

ABN 61 094 380 435

Appendix 9.4: List of attendees at the YTK health workshop

Table 8: YTK health workshop attendees, held at SARDI Aquatic Sciences on the 12th of July 2012.

Participants	Business/Institution	Role/Field of Expertise
Craig Foster	CST	Chief executive officer
Mike Thomson	CST	Technical manager
Trent D'Antignana	CST	R&D manager
Damian Critchly	CST	Health manager
Matt Landos	Future Fisheries Veterinary Services	Aquatic veterinarian for CST
Erin Bubner	Flinders University	Biologist
John Carragher	Logifish Consulting	Fish physiologist
Michelle Dennis	QML Pathology	Pathologist
Fran Stephen	WA Fisheries, Aquatic Animal Health Lab	Pathologist
Richard Smullen	Ridleys Aquafeed	Nutritionist
Kristian Just	Ridleys Aquafeed	CST account manager
Mark Crane	Australian Animal Health Laboratory	Virologist
Graham Mair	Australian Seafood CRC	Aquaculture/genetics
Shane Robberts	PIRSA Fisheries and Aquaculture	Aquatic animal health
James Munro	SARDI	Virologist/Veterinarian
Rebecca Forder	Adelaide University	Digestive tract specialist
Mark Booth	NSW Fisheries and Aquaculture	Nutritionist
David Stone	SARDI	Nutritionist
Matthew Bransden	Skretting	CST account manager
Rhys Hauler	Skretting	Nutritionist
Marcus Stehr	CST	Board member
Mehdi Doroudi	PIRSA Fisheries and Aquaculture	Veterinarian
Erica Starling	Indian Ocean Aquaculture	YTK aquaculture
Masashi Maita	Tokyo University of Fisheries and Technology	Fish physiology and nutrition
Marty Deveney	MISA Marine Biosecurity	Epidemiologist
Mark Shepard	Sakana Veterinary Services Ltd	Aquatic veterinarian
Gavin Begg	SARDI	Chief Scientist
Lens Stephens	Australian Seafood CRC	Veterinarian
Andrew Barns	University of Queensland	Microbiologist
Charles Caragell	Adelaide University	Veterinarian
Grant Richards	ParaSite Diagnostic Services	Coccidian/myxidian expert

Appendix 9.5: Case history report prepared by Dr. Matt Landos and Christine Hynh

Future Fisheries
VETERINARY SERVICE Pty Ltd
ABN: 520 830 961 17
PO Box 7142 East Ballina NSW 2478
Phone 0437 492 863
matty.landos@gmail.com

Australian Seafood CRC & Clean Seas Tuna Pty Ltd Yellowtail kingfish health workshop

Dr Matt Landos BVSc(HonsI)MANZCVS(Aquatic Animal Health Chapter

Christine Huynh BVSc

July 12, 2012

Future Fisheries
VETERINARY SERVICE Pty Ltd
ABN: 520 830 961 17
PO Box 7142 East Ballina NSW 2478
Phone **0437 492 863**
matty.landos@gmail.com

Figure 1: Clean Seas Tuna Sites forYellowtail Kingfish Production

SUMMARY OF KEY LESIONS IN CLEAN SEAS TUNA KINGFISH POPULATIONS

PORT LINCOLN 2012 YEAR CLASS (see document CST PL 12 histo-gross path)

Histological lesions

1) Enteritis with occasional bacterial or protozoal overgrowth (dysbiota)
2) Renal myxosporidiosis & renal tubular proteinosis
3) Focal hepatocyte necrosis and cellular atrophy
4) Epicarditis associated with myxozoan plasmodia (Kudoa-like)
5) Bacterial and parasitic branchitis
6) Skeletal muscle myxosporean-associated myopathy
7) Increased melanomacrophage centres in spleen

Clinical features

Poor growth and food conversion

Elevated mortality rates

No missed feeds, and held in calm protected site

Reduced mortality after changes to diet, and 10 day course of oral oxytetracycline, however still not back to historically low levels which would be expected. Caudal fin erosion – elevated prevalence, observed in May 2012.

ARNO BAY 2012 YEAR CLASS (see doc FFVS CST YC 2012 AB histo-gross path)

Histological lesions

1) Enteritis with occasional bacterial or protozoal overgrowth
2) Epicarditis associated with myxozoal stages
3) Epitheliocystis and gill fluke infestation and associated branchitis and fusion of lamellae
4) Focal hepatic necrosis and cellular atrophy, bile duct proliferation
5) Renal myxosporidiosis and tubular proteinosis, tubular cell degeneration
6) Skeletal muscle granulomatous foci of inflammation
7) Spleen high numbers of melano-macrophage centres and erythrophagia
8) Pancreatic fibrosis

Clinical features

Poor growth and food conversion

Elevated mortality rates

Exposed to rough weather and underfeeding

Negligible response in mortality rate to 10 day oxytetracycline course

Non-responsive to 30 days course of Amprolium (24mg/kg) and Salinomycin (0.6mg/kg)

Vaccinated (*P.damselae ssp damselae*) fish (Cage 10-12) perform better early on, but subsequently succumbed.

WHYALLA-ARNO BAY 2011 YEAR CLASS

Histological lesions

1) Chronic severe gill and skin fluke infestations

2) Epitheliocystis gill infections

3) Moderate to severe intestinal coccidiosis and myxidiosis and associated lymphocytic proliferative enteritis

4) Disseminated protozoan infection (possibly coccidian/myxidia)

5) Mycobacteriosis (in Whyalla cages, which has spontaneously resolved after move to Arno Bay)

6) Blood fluke (in Whyalla cages, which has not appeared to progress after move to Arno Bay)

7) Spleen lymphoid depletion

8) *Photobacterium damselae* ssp damselae systemic infection as fingerlings only

9) Renal myxosporidiosis, tubular proteinosis, occasional fish with haemopoietic necrosis

10) Fibrosing pancreatitis

Clinical features

Elevated mortality rates

High susceptibility to multiple synchronious diseases

Poor growth and food conversion

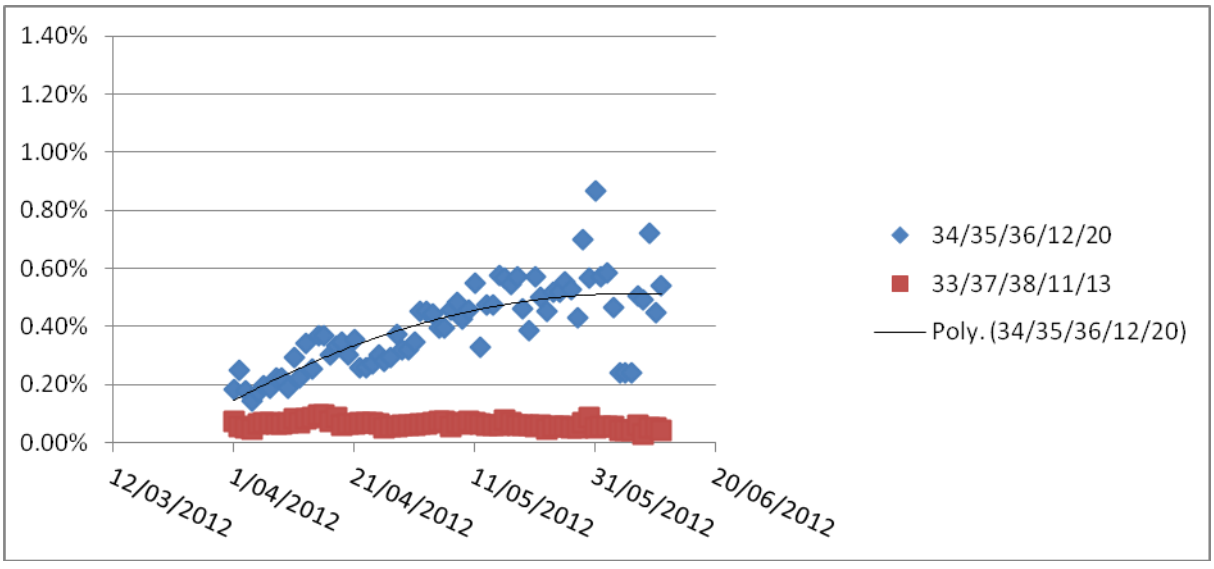
Poor response to 10 day trimethoprim sulfonimide treatment for enteric coccidia

Poor response to oxytetracycline treatment for epitheliocystis

Poor response to amprolium treatment to try and control renal myxosporean

Poor response to combined amprolium/salinomycin treatment to try and control enteric protozoa (coccidian/myxidia)

Diet trial between companies since December 2011 on Arno Bay grid site, reveals significantly lower mortality on Ridley 9mm feed (red) compared to fish on Skretting 9mm Optima YT(blue). The proximate analysis of the two feeds is detailed in table 1 below.



Graph 1: daily percentage mortalities from year class 2011 cages. Brick(Ridley), Blue(Skretting)

The Ridley 9mm diet is closer to the diet recommendations of (Booth, Allan, & Pirozzi, 2010) with higher DP levels. The Ridley diet also is reported to contain higher levels of fishmeal. The Tau levels of these diets are not known at this stage. Given the close proximity of cages

on each diet, similarity of water conditions, feeding methods and cage management, it appears less likely that the difference in mortality is due primarily to infectious disease. And more likely that feed is playing a key role in causing the elevated mortality. The slow/chronic increase in mortality rate, is also more consistent with a non-infectious disease curve, than that observed during infectious disease outbreaks. The pathology results indicate that some infectious diseases are active in the fish (intestinal protozoa/bacterial infection, gill epitheliocystis, gill and skin flukes), and it is my hypothesis that the severity of these diseases is being exacerbated by dietary factors which are leading to immunocompromise of the fish. Detailed dietary analysis of the two diets is underway.

Table 1: Diets – Proximate analysis

	50-100	50-100	500	1000		2000
pellet size (mm)	3, 4 Optima YT Skretting	3,4 Marine CST Ridley	6 Optima YT Skretting	9 Optima YT Skretting	9 Marine CST Ridley	11 Optima YT Skretting
fish size (g)	50-300		300-1000	1000-2000		2000+
Crude protein	50%	54%	45%	40%	47%	35%
Crude lipid	17%	18%	20%	23%	19%	25%
Carbohydrates	14%	n/a	16%	18%	n/a	21%
Moisture	8%	n/a	8%	8%	n/a	8%
Ash	11%	9.9%	11%	11%	9.9%	11%
Total Phosphorus	1.6%	1.4%	1.6%	1.6%	1.1%	1.6%
Gross Energy	21.0MJ	22.62MJ	21.3MJ	21.7MJ	21.37MJ	21.7MJ
Digestible Energy	18.7MJ	19.27MJ	19.0MJ	19.3MJ	18.07MJ	19.3MJ

Table 6
Iterative feed specifications and associated feed requirements for *S. lalandi* fed 12, 15 or 18 MJ kg^{-1} diets and reared at 21–24 °C. Shaded boxes indicate possible practical diet specifications for different growth stages.

	Fish weight (g)							
	50	100	200	300	600	900	1000	2000
Dietary DP:DE (g DP MJ DE^{-1})	38	38	38	31	31	31	24	24
12 MJ DE kg^{-1} diet								
Estimated DP content of diet (g kg^{-1})	456.0	456.0	456.0	372.0	372.0	372.0	288.0	288.0
Feed requirement (g $fish^{-1}$ d^{-1})	2.60	4.28	7.07	9.48	15.68	21.06	22.73	37.67
Feed requirement (% BW^{-1} d^{-1})	5.19	4.28	3.53	3.16	2.61	2.34	2.27	1.88
Expected FCR	1.06	1.20	1.37	1.48	1.68	1.82	1.86	2.12
15 MJ DE kg^{-1} diet								
Estimated DP content of diet (g kg^{-1})	570.0	570.0	570.0	465.0	465.0	465.0	360.0	360.0
Feed requirement (g $fish^{-1}$ d^{-1})	2.08	3.43	5.66	7.59	12.54	16.85	18.19	30.14
Feed requirement (% BW^{-1} d^{-1})	4.16	3.43	2.83	2.53	2.09	1.87	1.82	1.51
Expected FCR	0.85	0.96	1.09	1.18	1.35	1.45	1.48	1.70
18 MJ DE kg^{-1} diet								
Estimated DP content of diet (g kg^{-1})	684.0	684.0	684.0	558.0	558.0	558.0	432.0	432.0
Feed requirement (g $fish^{-1}$ d^{-1})	1.73	2.86	4.71	6.32	10.45	14.04	15.16	25.11
Feed requirement (% BW^{-1} d^{-1})	3.46	2.86	2.36	2.11	1.74	1.56	1.52	1.26
Expected FCR	0.71	0.80	0.91	0.98	1.12	1.21	1.24	1.41

Feed requirement (g $fish^{-1}$ d^{-1}) = total daily energy requirement / DE content of diet (from Table 5).
Feed requirement (% BW^{-1} d^{-1}) = feed requirement (g) / BW (g) * 100.
FCR = feed requirement (g $fish^{-1}$ d^{-1}) / weight gain (g $fish^{-1}$ d^{-1}).
DP content of diet (g kg^{-1}) = DP:DE ratio • DE content of diet.

From (Booth, Allan, & Pirozzi, 2010)

HYPOTHESIS TO EXPLAIN POOR GROWTH AND ELEVATED MORTALITIES IN 2011 AND 2012 YEAR CLASS FISH AT CLEAN SEAS TUNA PTY LTD.

Dietary factors + Environmental cage based stressors

⇩

Immunosuppression- lowered mucosal immunity (skin/gill + gut); increased red cell fragility

⇩

Elevated prevalence & intensities of multi-pathogen disease, and osmoregulation problems

Array of pathologies summarised above. Detail in individual Year Class documents.

CONSISTENCY OF ASSOCIATIONS BETWEEN DETECTED INFECTIOUS DISEASE AGENTS, PATHOLOGICAL CHANGES TO YTK TISSUES AND MORTALITY

1) ENTERITIS

In the 2011 year class the enteritis appeared to begin prior to the observations of coccidian-like, and myxidia-like organisms. There was evidence of bacterial overgrowths in the gut lumen, and evidence of post-storm *Photobacterium damselae ssp damselae* systemic infections. Once the protozoa became involved there was an association between the intensity of intestinal pathology, and the level of protozoan infection.

In the 2012 year class, the enteritis which had a similar spectrum of pathological/architectural changes to the 2011 fish, was not associated commonly with coccidian or myxidia organisms. Only occasional fish presented with protozoal infections and some with bacterial overgrowths. This evidence suggests a non-infectious factor is involved. The negative viral screening results also suggest infectious disease is a less likely explanation of the problems observed.

There is insufficient evidence in my opinion, from a substantial amount of diagnostic sampling to implicate an infectious disease agent with the primary necessary causal role in generating the enteritis. The enteritis is however commonly associated with elevated mortalities and poor growth from affected cages, indicating that it is an important lesion.

There is evidence of non-infectious causes, dietary factors outlined below, which could be associated with the changes observed.

2) BRANCHITIS

All populations of fish have experienced epitheliocystis which has caused significant damage to the gills of affected fish. Severe outbreaks have correlated with spikes in mortality, but have not correlated with the chronic elevated mortalities. It is unclear if immunosuppression is playing a role in the intensity of these outbreaks.

All populations of fish have experienced moderate to at times heavy infestations of gill flukes, necessitating bathing with peroxide, or praziquantel. Whilst heavy infestations have been associated with clinical signs of pale gills, and increased mortalities, the level of gill infestation has not correlated with the chronic ongoing mortality observed in the populations. The external parasitic infestations cannot explain the internal lesions observed in the intestine and liver.

3) EPICARDITIS

A minority of moribund fish had inflammatory aggregates detected on their hearts. These were subsequently linked to myxozoan stages becoming lodged there. They are insufficient in number and in timing of appearance to explain the observed mortalities, or the slow growth observed in the populations. There is not a clear link between the myxozoan heart lesion and the intestinal lesion.

It is unclear whether immunocompromise affected the fishes capacity to control the myxozoan infections.

4) RENAL MYXOSPORIDIOSIS

All populations of fish developed these parasitic renal infections. The level of damage to the kidney appeared to be insufficient in its own right to be associated with killing the fish. Hence it was considered by pathologists to be a secondary consideration, and may be more related to the immunocompetence of the exposed fish.

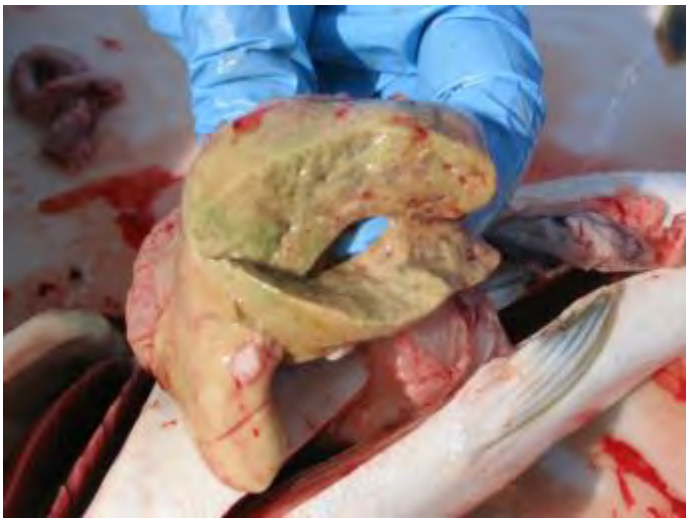
The pattern of mortalities does not align with the appearance and resolution of the renal myxosporidial infections. Hence this appears to be an insufficient cause of observed mortalities.

5) HEPATIC NECROSIS

The cause of the intermittently observed hepatic necrosis is not clear. It does not appear to be due to infectious diseases, with negative bacterial cultures, and negative viral diagnostic tests to date. Only occasionally have microsporidian been associated with the liver lesions, which have been mostly mild to moderate, and rarely severe.

The changes are more consistent with a toxic insult, and may be linked to oxidative stresses, due to dietary deficiencies. A non-infectious cause of the lesions is plausible.

In recent sampling (9/7/12) there has also been observed an increase in the prevalence of green liver, as illustrated below.



6) WATER QUALITY AND ALGAL BLOOMS

Monitoring station data demonstrates no association between dissolved oxygen levels and the long term mortality patterns. There is also no strong association between cage densities and mortality rates. There have been no lesions detected that would be consistent for exposure to toxic algal blooms.

DIETARY FACTORS IMPLICATED IN CONTRIBUTING TO IMMUNOSUPPRESSION AND ALTERED MUCOSAL IMMUNITY

(Li, Yin, Li, Kim, & Wu, 2007) reviewed the role of amino acids in immune function and identified many linkages between the adequacy and balance of amino acid supply and the functionality of the immune system. Given the range of pathologies exhibited, and the diet test results, it seems plausible that an amino acid deficiency of Tau may be contributing to the adequacy of the immune response. The authors made the following comments specifically on Tau.

“Tau is the most abundant free amino acid in lymphocytes and a potent antioxidant (Fang et al. 2002). Further, the reaction of Tau with hypochlorous acid, which is a microbicidal agent produced by activated monocytes and neutrophils, yields Tau chloramine (Wright et al. 1986). This long-lived oxidant reduces the production of proinflammatory cytokines (e.g. IL-1, IL-6 and TNFa) and prostaglandin E2 (Weiss et al. 1982; Chore¸z˙y et al. 2002), and increases histamine release from neutrophils of carrageenin-induced rats (Wojtecka-Lukasik et al. 2004).”

TAU’S MANY BIOLOGICAL ROLES

Tau is not listed as one of the essential amino acid for fish. However, there is emerging evidence thatYellowtail Kingfish may require high levels in their diets as they are not able to manufacture sufficient quantities endogenously. (Maita, Maekawa, Satoh, Futami, & Satoh, 2006) (Takagi, et al., 2006) (Takagi, Murata, Takanobu, Endo, Yamashita, & Ukawa, 2008).

It has many roles in the body which are incompletely described and the subject of ongoing research. One of its key roles is in the production of the bile salts, sodium taurochenodeoxycholate and sodium taurocholate. Tau is required for the rate limiting enzyme in the production of these bile salts. Bile salts are derivatives of cholesterol, which are highly effective detergents useful to solubilise lipids in the diets. By solubilising the lipids

it assists the lipase enzymes to break them into shorter chains, through hydrolysis, and also facilitates their absorption by the intestine. Tau deficiency in yellowtail (*Seriola quinqueradiata*) has been reported to decrease bile salt excretion.

Tau is also required for the activity of the rate limiting enzyme for the endogenous production of cholesterol. (Maita, Maekawa, Satoh, Futami, & Satoh, 2006), hence deficiency can lead to hypocholestrolaemia. (Maita, Aoki, Yamagata, Satoh, Okamoto, & Watanabe, 1998) indicated that hypocholestrolaemia was associated with reduced disease resistance in yellowtail.

Tau has also been associated with crossing the blood-brain barrier and influencing neurotransmission, membrane stabilisation, feedback inhibition of inflammatory cell respiratory burst, fat tissue regulation, recovery from osmotic shock and acting as an anti-oxidant protecting against the toxicity of various substances (eg metals).

In fish it has been associated with osmoregulation, and in yellowtail the concentration of Tau is related to serum osmolality. Histopathology investigations of Clean Seas YTK, are not able to discern whether fish were suffering from osmoregulatory problems, however it is notable that on many occasions the pathologists felt that the extent of visible lesions was insufficient to explain the high mortality rate in the population. The diagnostic picture may make more sense, were the fish to be dying of osmoregulatory failure. This is yet to be confirmed through further blood tests.

Preliminary testing indicates that cages with high mortality have a lower haematocrit, compared to lower mortality cages on different diets.

Supplementation has been shown to prevent oxidative stress induced by exercise.

Tau plays a role in aiding the movement of electrolytes (potassium, magnesium, calcium, sodium) in and out of the cell. It also plays a role in normal skeletal muscle function, with deficient animals having a reduced capacity for exercise. It is uncertain if this may explain the observation of the immediate post-mortem tetany of the hypertrophic intestines which was observed in cage 11-11 and cage 11-12.

The role of Tau in stabilising cell membranes of red blood cells may influence red cell fragility, and hence deficiency may induce a haemolytic anaemia. (Maita, Maekawa, Satoh, Futami, & Satoh, 2006) (Takagi, et al., 2006)

(Matsunari, Takeuchi, Takahashi, & Mushiake, 2005) identified that Tau played a role in the sulphur amino acid metabolism of yellowtail juveniles(0.5g). Hence the physiological impacts of Tau deficiency can spread into the many roles of sulphur amino acids also. These authors fed very high fish meal(65.1g/100g)krill meal(15g/100g)squid meal(10g/100g) given

a CP ~53% Lipid 11%. The best growth was achieved with Tau levels>1792mg/100g of diet over the first 3 weeks of the diet trial.

The bioavailability of Tau from fish meal sources appears variable, and addition rates recommended of crystalline Tau, appears to variable between papers and fish sizes/ages. This area appears to need more considered research.

YELLOWTAIL KINGFISH DIETS: DP:DE TAU and n-3 HUFA

Increasing lipid supply is likely to result in increased bile salt demand, and hence increased Tau demand to manufacture it. The high metabolic performance of YTK immediately places them at the high end of the demand spectrum for all micronutrients including Tau. The diet substitution trials for YTK indicate that growth performance is readily impaired with substitution of protein and lipid sources, more readily that occurs for many other better studied fish species. Diets currently being fed to YTK are energy dense typically > 18-19MJ, with much of this energy coming from lipid inclusions derived from poultry and fish oil. Diets for larger fish have generally reduced digestible protein content, and increased digestible energy.(See table 1 above) (Olivia-Teles, 2012) commented "On the contrary, with low DP/DE diets, fish will stop eating before ingesting an adequate amount of protein, thus compromising growth rate and eventually debilitating the animals. As there is now a trend for increasing dietary energy content for reducing feed intake per unit of growth and decrease feed losses, a reappraisal of dietary nutrient requirements may be required to reassure that adequate amounts of essential nutrients are included in the diets (Hardy 2001; Wilson 2002)."

Another effect of this high lipid intake may be to increase the Tau demand of the fish. And hypothetically this may induce or exacerbate Tau deficiency in the fish. (Takagi, et al., 2011) suggested that Tau deficient fish may also become n-3 HUFA deficient, due to decreased digestive absorption of lipid, via the decrease in bile acid secretion. The n-3 HUFA deficiency may then explain the increased fragility of red blood cells, and potentially the reduced immunocompetence which has been linked in other literature to lowered n-3 HUFA intakes (see commentary in FFVS CST 0023 YC 12 Health summary 130312). The diets fed to the YC 2012 fish, appear to be low in n-3 HUFA's to begin with, based on test results presented below. Hence any inhibition to bioavailability, may exacerbate the deficiency experienced by the fish.

The effect of sea cage based stressors in increasing dietary requirements of some nutrients may increase demand for n3-HUFA's under South Australian culture conditions which are at times outside the optimal thermal and oxygen saturation range for this pelagic species.

Tau levels tested in 1.5mm, 2.3mm, 3mm and 4mm diets from both Ridley and Skretting by CST have been variable between batches and between manufacturing companies. The results are still to be confirmed with additional testing at a European feed laboratory. Hence the figures should be interpreted with caution until the levels can be confirmed. For the benefit of the workshop discussions, and in the absence of information to the contrary, I will consider the tested levels, to be the actual levels, for comparison to the literature. Levels of Tau are quoted in mg/100g of feed on dry weight basis.

	Marine CST	Optima YT 100	Protec	Protec ME	Marine CST	Marine CST	Protec	Gemma Diamond
Taurine(free	191	223	245	158	127	234	218	331
Total n-3 P	1767	4180	1735	2028	3438	3370	3790	4610
EPA+DHA	1260	3530	1320	1620	2960	2800	3090	3870
Total LC PU	1380	3770	1418	1730	3130	3010	3350	4140
n-3:n-6	0.821478382	3.362831858	1.054711246	1.410292072	2.107909258	1.908267271	2.113775795	4.373814042

There has been a trend that diets appear to have lower Tau levels than recommended by Dr Masashi Maita, based on Japanese work, for juvenile YTK of 1%. Dr Maita also noted thatYellowtail Kingfish (*Seriola lalandi*) require higher levels of dietary protein, and lower levels of lipid in their diet compared to Japanese yellowtail (*Seriola quinqueradiata*). The diets being fed are all high in lipid content, giving relatively high DE values >18.5MJ.

(Takagi, et al., 2006) supplemented non-fish meal diets with >3390mg/100g of Tau and observed a clinical response in improved growth, PCV and red cell stability. Feeding >5280mg/100g resulted in further improvements in growth and red blood cell stability. In other results the same authors identified that supplementation of >2140mg/100g to a Soybean protein diet, delivered superior results to Tau levels of < 73mg/100g. The fishmeal diets contained 408mg/100g Tau which performed adequately. The authors noted that the dietary Tau requirement of yellowtail was substantially increased (10 fold) in soya substituted diets, above that of the 100% fishmeal diets. The Tau level in 100%FM diets may in itself be insufficient for requirements of YTK, as has been found to be the case in Japanese flounder. (Matsunari, Takeuchi, Takahashi, & Mushiake, 2005) reported that 0.5g yellowtail required >1000mg/100g of Tau. And (Park, Takeuchi, Seikai, & Yokoyama, 2001) report that Japanese flounder require 1500-2000mg/100g of Tau. The level for *Seriola lalandi* is not definitively known, but may be higher than Japanese Yellowtail. The lack of knowledge about bioavailability of Tau in various diet formulations is a further complicating factor for avoiding deficiencies. (Olivia-Teles, 2012)

(Sarker, Satoh, Kamata, Haga, & Yamamoto, 2012) found that over a 12 week feed trial that some yellowtail developed green livers at Tau levels of 260mg/100g of dry weight of feed. Diets containing more fishmeal, had levels of Tau of 360mg/100g, and no green livers were observed, and growth was superior, suggesting this is closer to the Tau requirement for yellowtail. The authors final word is a warning that protein substitution in yellowtail diets, is not a fully understood science. “More studies in this area with different size/age groups of fish are warranted before commercial formulations reach the market.”

In yellowtail studies the supplementation of Tau to deficient Soy Protein Concentrate diets resulted in correction of blood cells and hepatic function parameters within 4 weeks.

Of interest, (Jirsa, Davis, Stuart, & Drawbridge, 2011) fed diets containing SBM and SPC to Seriola lalandi, without attending to supplementation of methionine, lysine or Tau, and documented poor growth performance over short 6 week feeding trials. They did not observe enteritis. Clean Seas 2010 year class were fed similar soya diets and the survival was poor, growth poor, and the fish developed enteritis. The cause was not determined at the time, but in light of current research, may have been due to an amino acid deficiency. (See reports; FFVS Clean Seas second opinion final; FFVS Clean Seas YTK Mortality Nov 2010)

Diets have been based in part on research indicating optimal digestible protein: digestible energy ratios. (Booth, Allan, & Pirozzi, 2010) The diet trials behind this were short ~35 days. It is of interest that in (Maita, Maekawa, Satoh, Futami, & Satoh, 2006) there were no differences in the growth of fish in the trial after 30 days, but after 60 days significant differences emerged. There is a risk in my view that clinical deficiencies associated with modified diets may take longer than 5 weeks to clearly emerge. Hence future diet work needs to identify facilities that allow it to run longer trials, and look at blood and biochemical indicators which may alter before growth is statistically affected.

It is also apparent that the intestinal disease described in fish in sea cages has not been reported in fish held in tanks. Hence it is likely there are additional stressors that are playing a role in sea cage fish, which mean that feed trials in tanks may not reflect the actual performance achieved when fed under commercial sea cage conditions. Longer trials in sea cages may better predict a diets actual performance, outside of a research trial. (Olivia-Teles, 2012) acknowledges that supplementation of essential fatty acids, microminerals and amino acids above basal requirement can have benefits in aquaculture reared stock, due to the elevated demand created through husbandry stressors which cannot be completely eliminated. The review also suggests there may be benefits in prebiotics. CST diets have included prebiotics (such as mannin-oligosaccharides) and organic acids to date, which have failed to ameliorate the intestinal signs of disease, suggesting that a more fundamental deficiency/disease process is occurring.

There have been signs in some of the YC 12 fish of increased red cell turnover, with erythrophagia observed in the spleen and increased melanomacrophage centres there, associated with some iron pigments. It is possible that the renal proteinosis observed is also associated with the red cell fragility, through releasing haemoglobin into the plasma, which passes through the glomerulus of the kidney into the tubules, where the tubular cells have been resorbing it. (Takagi, et al., 2011) noted that in red sea bream, there was not necessarily haemosiderin deposits in the spleen, despite there being evidence of red blood cell haemolysis with lowered PCV's in the Tau deficient fish.

The fish also appear to have been immunosuppressed and vulnerable to infection by a range of parasitic and bacterial pathogens which has resulted in increased mortality. (Maita, Maekawa, Satoh, Futami, & Satoh, 2006) noted that Tau deficiency was associated with immunosuppression, and with hypocholestrolaemia. The low blood cholesterol levels were attributed to reduced intakes of cholesterol, and reduced endogenous production.

Cholestrol plays a key role in cell membranes and it seems feasible that a deficiency may result in compromised mucosal immunity of the intestine and skin. Dr Maita mentioned that Tau deficient yellowtail may experienced reduced skin mucous production, and suffer increased infections as a consequence. This compromised surface immunity of fish, may be contributing to the poor resilience of CST fish to gill and skin fluke infestations, in addition to the gut protozoan (coccidian/myxidia) infections.

CST YTK have exhibited signs of compromised intestinal mucosal immunity over the past few year classes at least. With many fish exhibiting an enteritis, with chronic changes to the

structure of the gut. The association of changes with pathogens in the gut has been variable- with bacteria and protozoa being associated in some, but not all, groups of affected fish. It is the opinion of the veterinary pathologist Dr Dennis, that the bacterial overgrowths in the intestine do not represent the primary cause of the enteritis, rather, are a secondary manifestation.

The shortage of n3-HUFA's in the diet, and ratio's of some diets being low n3:n6, is known to be pro-inflammatory and associated with increased risk of inflammatory bowel disease.

GUT MICROBIAL DIVERSITY

Gut lumen swabs onto multiple types of media have revealed a very low biodiversity of culturable bacteria in the 2012 year class of fish which have an enteritis- often monocultures of *Vibrio scopthalmi*. The cause of this dysbiota is not known. It has also been observed in investigations in 2010 on the 2009 year class which were suffering severe lymphocytic enteritis. It may be a product of the compromised intestinal mucosal immunity, or associated with some other feed factor, such as a sensitivity to a sanitiser (anti-fungal). Further investigations of whether there are diet based substances that are exerting antimicrobial properties are warranted.

LYSINE AND CAUDAL FIN EROSION

Lysine deficiency has been reported in the literature to cause caudal fin erosion. (Latremouille, 2003) This lesion was prominent in recent sampling of the Port Lincoln 2012 year class. The diet testing to date, does not appear to suggest that diets are deficient in this essential amino acid. (Ruchimat, Masumoto, Hosokawa, Itoh, & Shimeno, 1997) suggest that 1.78% of dry diet, or 4.13% of the dietary protein should be lysine to meet requirements for weight gain and feed efficiency.

CONCLUSIONS

The severity of losses experienced in the Arno Bay 2012 outer zone fish are I believe the compounding of several effects. The losses are substantially higher than previous years due to the compounding nature of the stressors including:

1) Inability to provide sufficient feed to fish, due to rough weather reducing site access to remote area.

2) Rough weather leading to increased metabolic demands on juvenile fish.

3) Longer transportation for fish from Port Augusta hatchery, compared to previous stocking in Whyalla.

4) Diets deficient in Tau and with insufficient n3-HUFA's

5) Diets with insufficient digestible protein, to the digestible energy ratio (or excess energy for the amount of protein present)

The losses in some of the Port Lincoln 2012 fish have slightly less severe due perhaps to improved diets (requested higher fish meal/fish oil content), - see graphs in Appendix 1. The

losses in the cage fed Ridley feed (11-12) have still been some of the worst in the company's history with already >40% mortality from the initial stocked population, which went in the water in early 2012. Growth has also been substantially under the previous benchmarks. Improved environmental conditions at the Port Lincoln site that have allowed regular feeding, bathes and net changes when required were insufficient by themselves to stop the elevation in mortalities. Further substantial improvement is likely with optimisation of the diets with respect to fatty acid and amino acid supply, matched to fish size and seasonal conditions.

The losses observed in 2011 year class fish, are associated with similar pathologies to those in the 2012 year class, with greater prominence of the role of gut protozoa. It is plausible that the same dietary deficiencies are affecting the mucosal immunity of these fish- however data is yet to be obtained to confirm this suspicion. The epidemiological picture is compelling in suggesting that diet is playing a role in the health of the fish. Given the striking difference in survival between two different diet groups on the same site, it suggests that immunocompromise is playing a large role in facilitating the ongoing gut protozoal infections. And, that should this immunocompromise be controlled, there is a reasonable prospect of good YTK production, even in the presence of the gut protozoa in the environment.

It should be possible to return to the high performance experienced in the first 2 years of the industry, with rapid growth, efficient FCR's and low mortality (<20% across entire grow-out period).

It is more uncertain how effective the dietary improvements will be at preventing the myxozoan infections of muscle, brain and kidney. Given wild kingfish are reported to get significant Kudoa sp infections it would appear that these organisms can be genuine pathogens/parasites of YTK. And as such, are likely to pose ongoing challenges to management. Exploration of effective treatments is likely to be of value in the future, given the equivocal results from experimental treatments thus far.

OBSERVATIONS THAT DO NOT APPEAR TO FIT NEATLY WITH TAU DEFICIENCY BEING THE ONLY PROBLEM WITH YTK DIETS

1) Literature does not identify enteritis as a component of the syndrome of Tau deficiency, however it is common across affected CST fish populations

2) The observation of green livers in affected fish populations has been relatively rare, and typically segmental rather than throughout the entire organ and common as reported with yellowtail Tau deficiency. However, in sampling undertaken on 9-10 July 2012, in cage 11-11 and 12-11, that had been starved for 4 days due to towing between sites, the prevalence of green liver was much higher (>90%), and was of the same appearance as Tau deficient fish in Japan, according to Dr Maita. In cages 11-12 and 12-12 which had been on feed, the prevalence of green liver was lower~ 4/10 fish necropsied.

FURTHER INVESTIGATIONS OF TAU & N3-HUFA DEFICIENCY HYPOTHESIS

1) Haematology

Sample convenience captured fish (minimum 10) from each population (PL YC 12; AB YC 12, AB YC 11). Collect blood into anticoagulant tubes.

Measure: PCV(Haematocrit); MCV; haemoglobin; MCHC; MCH (underway in part)

2) Biochemistry

From spun down plasma of convenience sampled fish from each population

Measure: cholesterol; bilirubin; biliverdin; ALP; triglycerides; BUN (underway in part)

3) Measuring tissue levels of Tau (liver and muscle) (underway)

4) Treatment monitoring/response of 1% crystalline Tau addition to diets, through haematology and tissue testing (underway)

5) Investigate testing options for lactic acid in intestinal mucosa/muscularis, in relation to the observed post-mortem tetany compared to normal ATP-ase rigor mortis. (not commenced)

6) Testing the diet for anti-microbial activity, and testing feed sanitisers for impact on common gut flora. (proposed)

KEY QUESTIONS FOR NUTRITIONISTS/VETERINARIANS/PHYSIOLOGISTS

1) What level of fish meal origin Tau is sufficient in diets for fish of different sizes in different water temperatures ?

2) What level of supplementation of crystalline Tau is therefore required ?

3) Is the level of Tau supplementation required altered by other dietary components- eg SPC affecting bioavailability?

4) What is the optimal DP level in diets for YTK of different sizes, in different water temperatures ?

5) What is the optimal DE level in diets for fish of different sizes ?

6) Changes to diets were requested to increase FM/FO inclusion for the Port Lincoln 2012 fish. Why have the analysis results not reflected an increase in n-3 fatty acids and Tau increase, compared to previous formulations?

How can we modify n3:n6 ratio cost effectively in diets?

Can meat meal be used to boost Tau levels?

7) What is the best method for identification of the gut protozoa which have been labelled coccidian and myxidia based on histological appearance?

8) Is it feasible to undertake infection trials with these organisms on fish which are on a range of diets, to explore the role of diet in assisting infection?

9) Are treatment trials for these organisms able to be organised/ warranted and commercially viable?

10) Are feed sanitisers used in diets? If so which diets, and are they capable of modifying gut flora?

11) Can a rapid testing methodology to assess osmoregulatory status/stress be developed?

REFERENCES

Booth, M., Allan, G., & Pirozzi, I. (2010). Estimation of digestible protein and energy requirements ofYellowtail Kingfish Seriola lalandi using a factorial approach. *Aquaculture , 307*, 247-259.

Jirsa, D., Davis, A., Stuart, K., & Drawbridge, M. (2011). Development of a practical soy-based diet for California yellowtail, Seriola lalandi. *Aquaculture Nutrition , 17*, 869-874.

Latremouille, D. (2003). Fin Erosion in Aquaculture and Natural Environments. *Reviews in Fisheris Science , 11* (4), 315-335.

Li, P., Yin, Y.-L., Li, D., Kim, S., & Wu, G. (2007). Amino acids and immune function. *British Journal of Nutrition , 98*, 237-252.

Maita, M., Aoki, H., Yamagata, Y., Satoh, S., Okamoto, N., & Watanabe, T. (1998). Plasma biochemistry and disease resistance in yellowtail fed a non-fish meal diet. *Fish Pathology , 33* (2), 59-63.

Maita, M., Maekawa, J., Satoh, K.-I., Futami, K., & Satoh, S. (2006). Disease resistance and hypocholesterolemia in yellowtail Seriola quinqueradiata fed a non-fishmeal diet. *Fisheries Science , 72*, 513-519.

Matsunari, H., Takeuchi, T., Takahashi, M., & Mushiake, K. (2005). Effect of dietary Tau supplementation on growth performance of yellowtail juveniles Seriola quinqueradiata. *Fisheries Science , 71*, 1131-1135.

Matsunari, H., Takeuchi, T., Takahashi, M., & Mushiake, K. (2005). Effect of dietary Tau supplmentation on growth performance of yellowtail juveniles Seriola quinqueradiata. *Fisheries Science , 71*, 1131-1135.

Olivia-Teles, A. (2012). Nutrition and health of aquaculture fish. *Journal of Fish Diseases , 35*, 83-108.

Park, G.-S., Takeuchi, T., Seikai, T., & Yokoyama, M. (2001). The effects of dietary Tau on growth and Tau levels in whole body of juvenile Japanese flounder Paralichthys olivaceus. *Nippon Suisan Gakkaishi , 67*, 238-243.

Ruchimat, T., Masumoto, T., Hosokawa, I., Itoh, Y., & Shimeno, S. (1997). Quantitative lysine requirement of yellowtail (Seriolae quinqueradiata). *Aquaculture , 158*, 331-339.

Sarker, M., Satoh, S., Kamata, K., Haga, Y., & Yamamoto, Y. (2012). Partial replacement of fish meal with plant protein sources using organic acids to practical diets for juvenile yellowtail, Seriola quinqueradiata. *Aquaculture Nutrition , 18*, 81-89.

Takagi, S., Murata, H., Goto, T., Hatate, H., Endo, M., Yamashita, H., et al. (2011). Role of Tau deficiency in inducing green liver symptom and effect of dietary Tau supplementation in improving growth in juvenile red sea bream Pagrus major fed non-fishmeal diets based on soy protein concentrate. *Fisheries Science , 77*, 235-244.

Takagi, S., Murata, H., Goto, T., Hayashi, M., Hatate, H., Endo, M., et al. (2006). Hemolytic suppression roles of Tau in yellowtail Seriola quinqueradiata fed non-fishmeal diet based on soybean protein. *Fisheries Science* , 546-555.

Takagi, S., Murata, H., Takanobu, G., Endo, M., Yamashita, H., & Ukawa, M. (2008). Tau is an essential nutrient for yellowtail Seriola quinqueradiata fed non-fish meal diets based on soy protein concentrate. *Aquaculture* , 198-205.

ACKNOWLEDGEMENTS

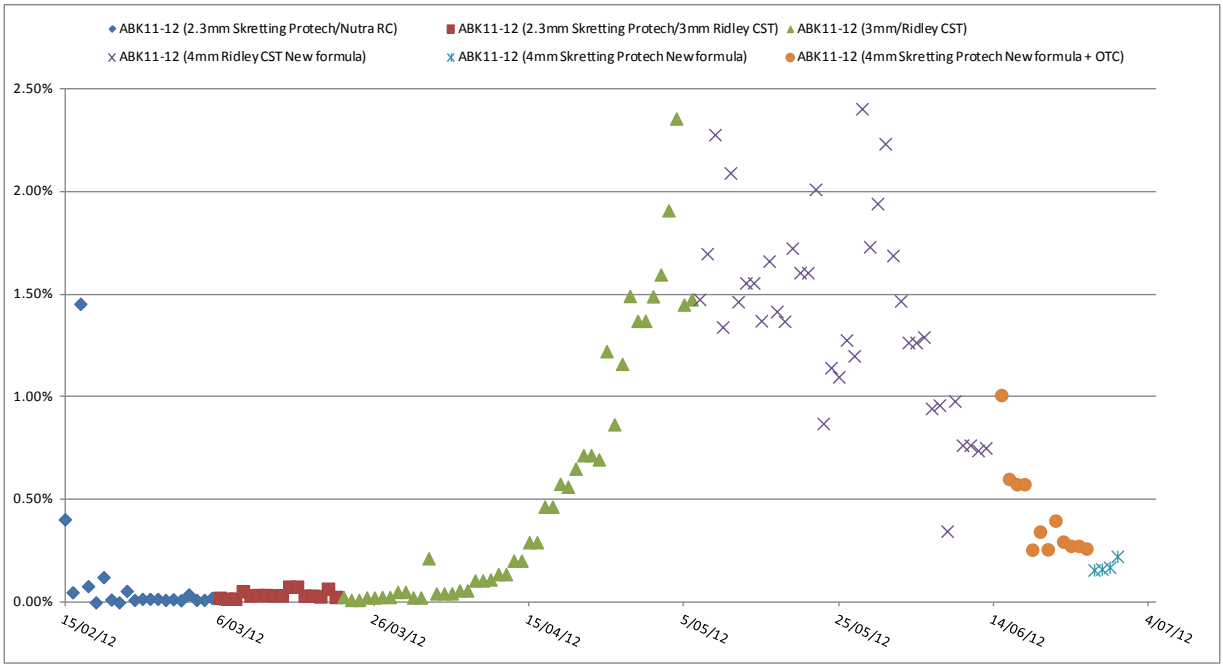
Mike Thompson/Sam Feige for mortality/feed graphs (Appendix 1, 2)

Trent D'Antignana for communications with other scientists, and Tau literature review (Appendix 3)

Damian Critchley, Ben Underdown, Tony Barton, Haydn Ramsay, Philip Nielsen for assistance in stock sampling for disease investigations.

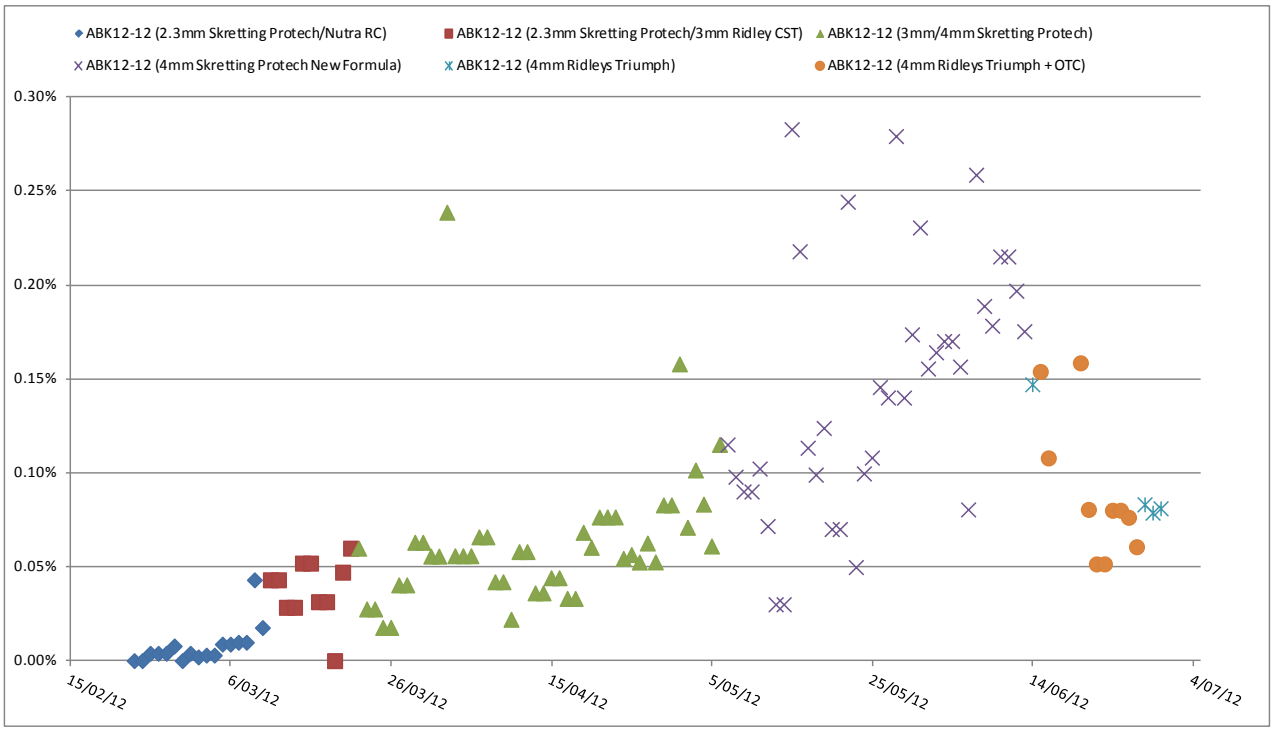
APPENDIX 1- Mike Thompson Mortality/Feed Type Graphs

Figure 1: Port Lincoln 2012 cage ABK11-12 (daily mortality against diet)



- In cage ABK11-12, switching to a new formulation (100% fish oil and high fish meal diet such as 4mm Ridleys CST New Formula and 4mm Skretting Protech New formula has coincided with a reduction in daily mortality, BUT there is some indication that the daily mortality rate may have been reaching it's peak just before the diet was switched to a new formula).
- The addition of the antibiotic oxytetracycline (OTC) was done at a time when the daily mortality rate was already reducing, which confounds any conclusion regarding it's efficacy.
- The daily pattern observed over the coming weeks may confirm if the antibiotic has helped to reduce the mortality or not, e.g. if the mortality rate increases now that the use of oxytetracycline has been dis-continued.

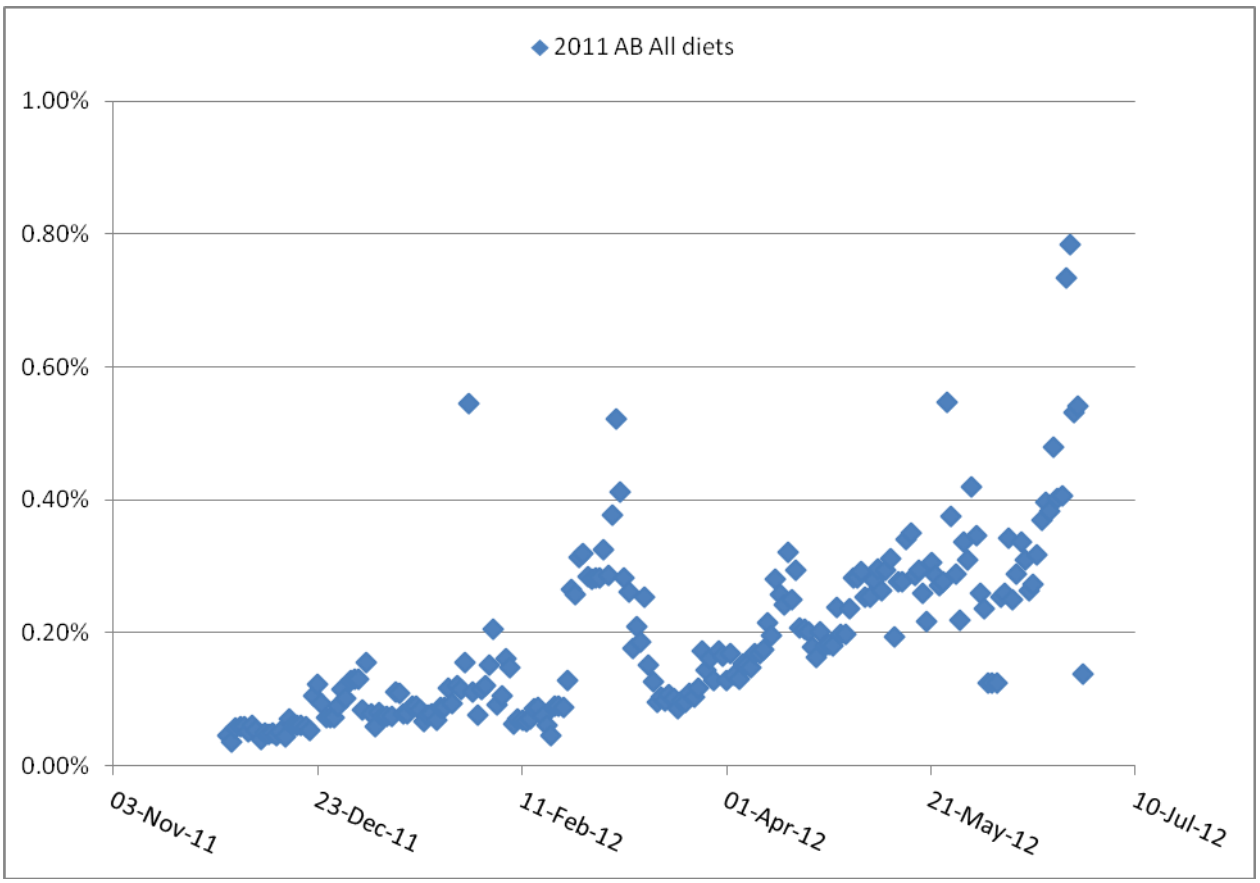
Figure 2: Port Lincoln 2012 cage ABK12-12 (daily mortality against diet)



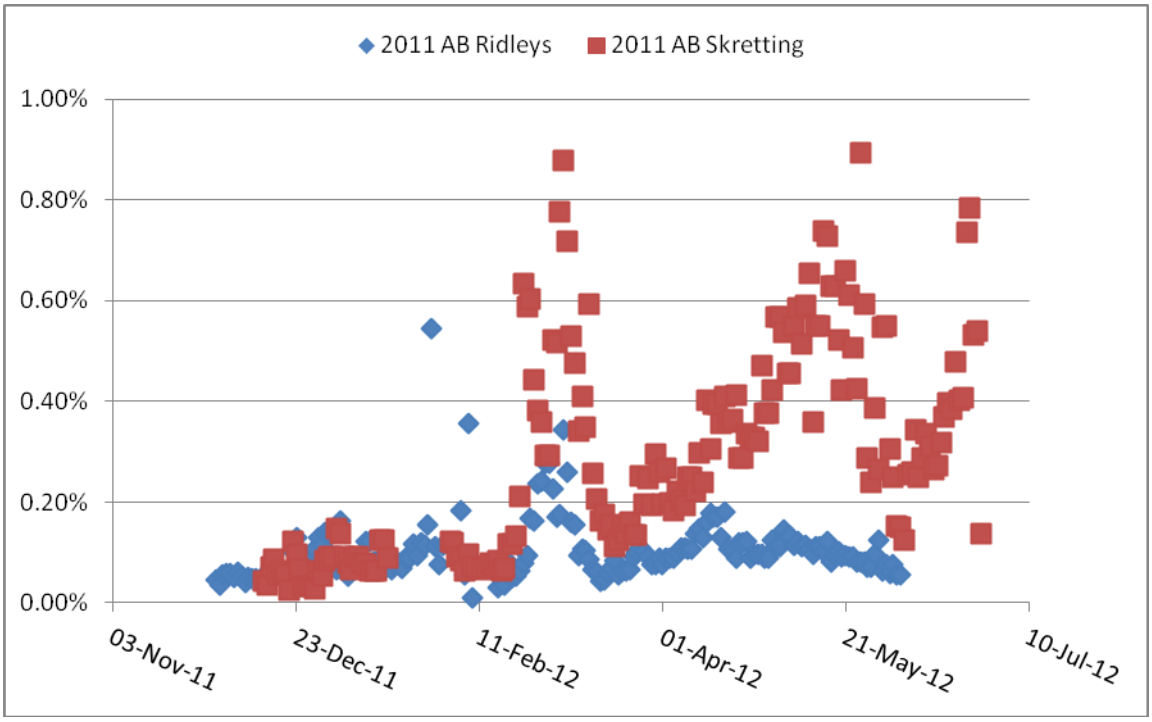
- In contrast with cage ABK11-12, in cage ABK12-12, switching to the new formulation (4mm Skretting Protech New formula) has not led to a reduction in daily mortality.
- However, the combined effect of the addition of the antibiotic oxytetracycline (OTC) and a switch to a 100% fishmeal and 100% fish oil diet (Ridleys 4mm Triumph) does appear to have led to a reduction in daily mortality during the course of the antibiotic course.
- As with cage ABK11-12, the daily mortality patterns in the coming days and weeks may help determine whether the addition of oxytetracycline or the new diet was the prime reason for the reduction in daily mortality.

APPENDIX 2: Summary 2011 mortality vs feed source- by Mike Thompson

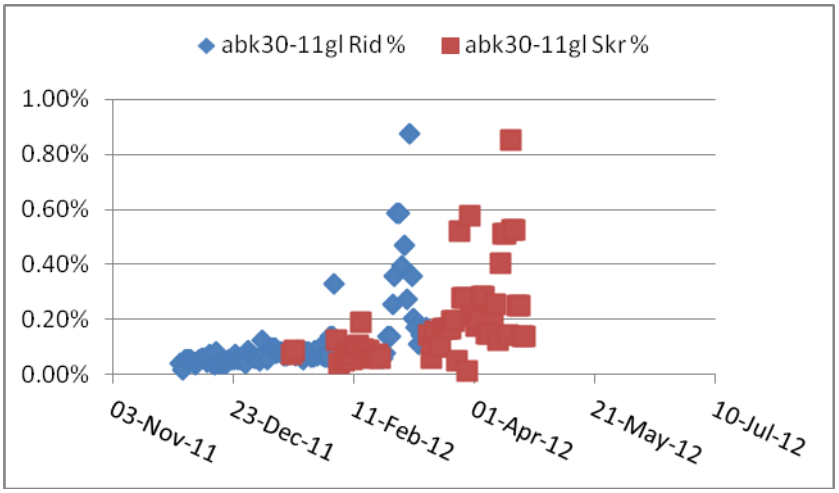
Arno Bay 2011 Generation – Mortality versus diet type (December 2011 to June 2012)

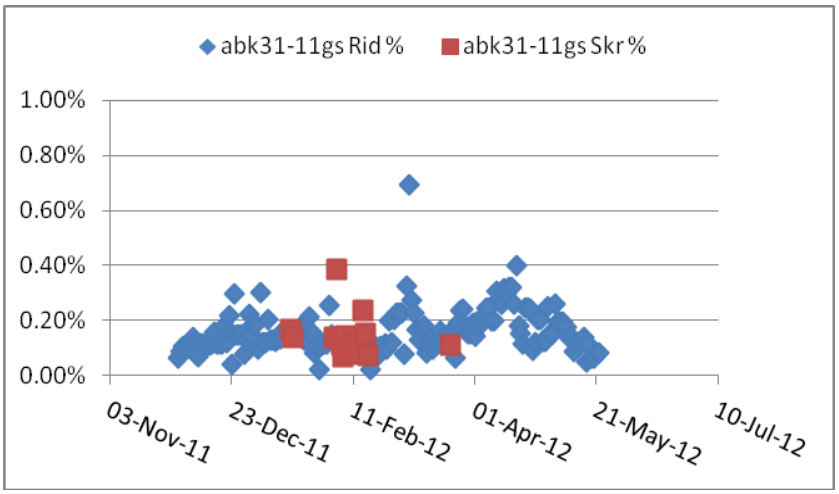


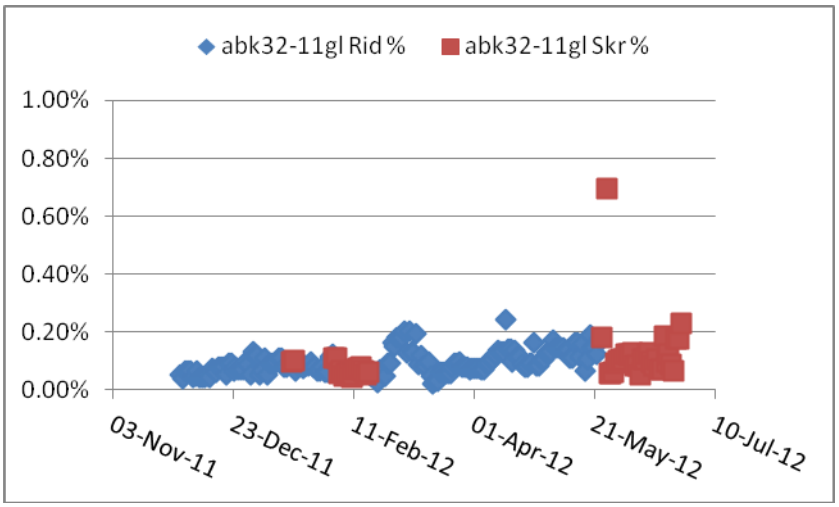
- Chart showing the average mortality rate of **ALL** 2011 Arno Bay cages **irrespective of diet**
- Note the peaks and troughs in mortality patterns



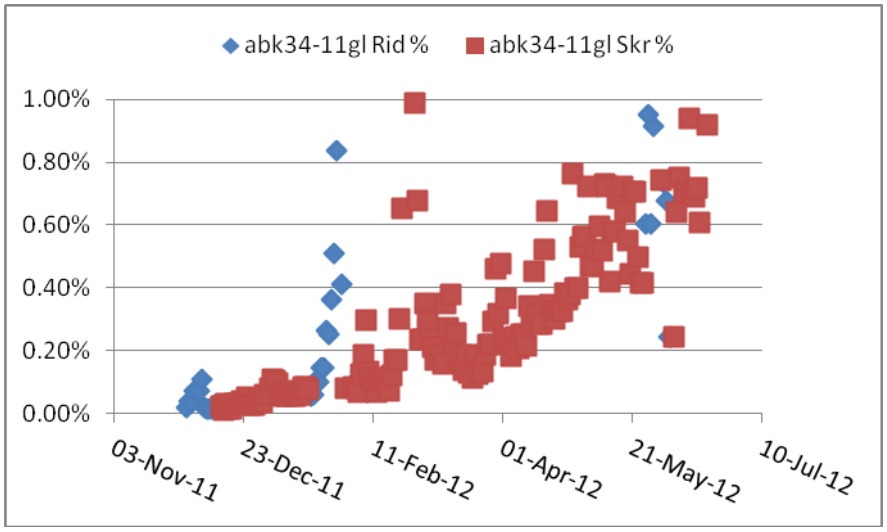
- Chart showing the average mortality rate of 2011 Arno Bay cages on Skretting and Ridleys diets
- There appears to be a divergence in cages from about mid February onwards when Skretting cages have had a consistently higher mortality rate than those fed Ridleys
- Notes:
    - some cages were fed a mixture of Ridleys and Skretting on the same day (in which case they have been identified as a Skretting cage for that day
    - on some days, cages switched from one diet to another due to short term availability issues and on these occasions these cages have been removed from the analysis
    - all cages have been on Skretting diet medicated with amprolium / salinomycin since 6th June (which results in the mortality rate in the Skretting cages appearing to drop after this date (due to the cages that were previously fed on Ridleys having a lower mortality rate)
    - A cage by cage analysis follows

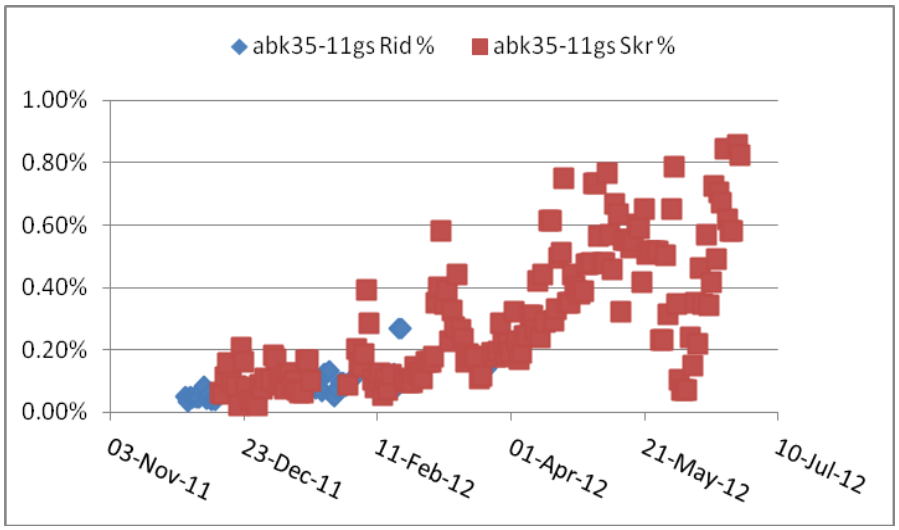


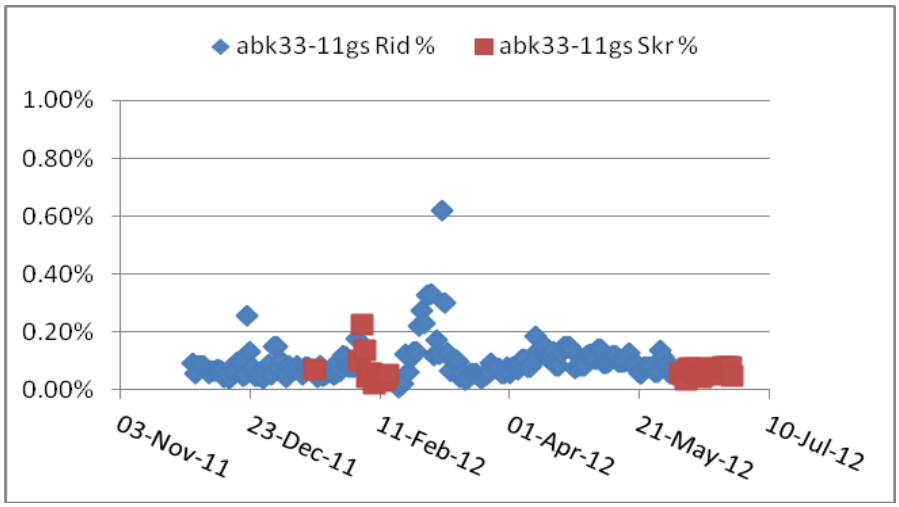


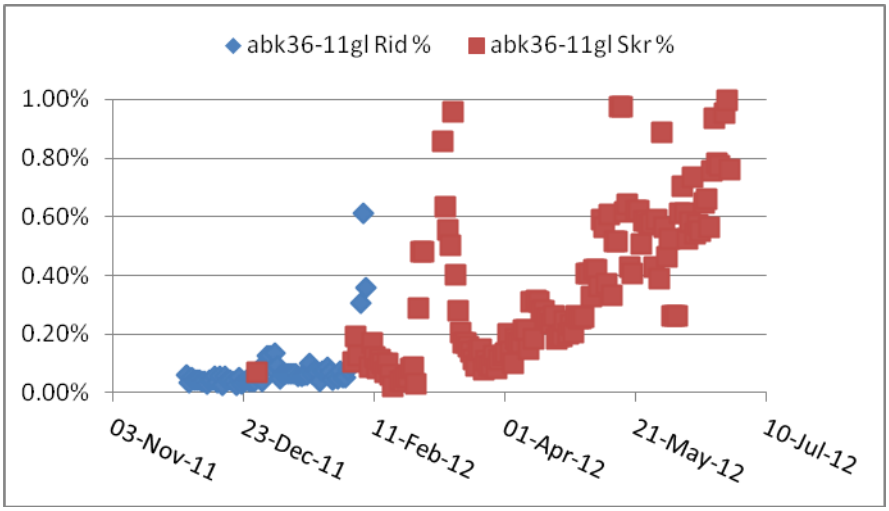


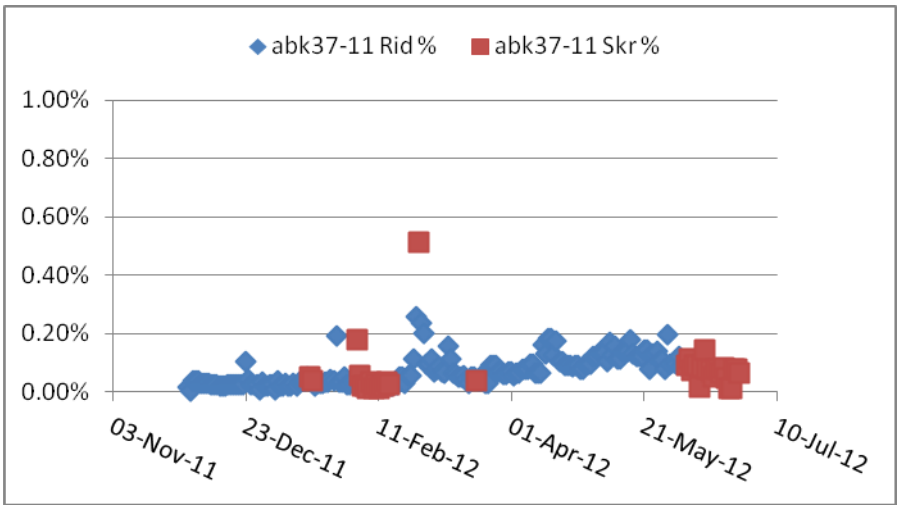
These charts have been copied and pasted from the attached file in an attempt to line up both axes

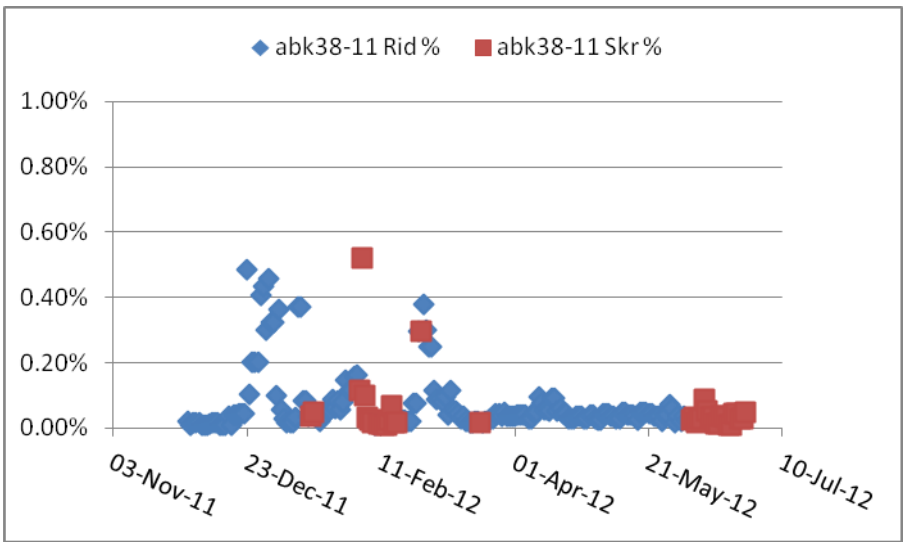

abk34-11gl Rid %
abk34-11gl Skr %
1.00%
0.80%
0.60%
0.40%
0.20%
0.00%
03-Nov-11
23-Dec-11
11-Feb-12
01-Apr-12
21-May-12
10-Jul-12


abk35-11gs Rid %
abk35-11gs Skr %
1.00%
0.80%
0.60%
0.40%
0.20%
0.00%
03-Nov-11
23-Dec-11
11-Feb-12
01-Apr-12
21-May-12
10-Jul-12

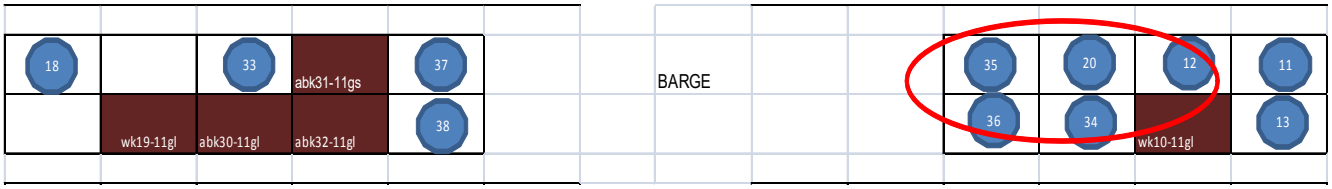

abk33-11gs Rid %
abk33-11gs Skr %
1.00%
0.80%
0.60%
0.40%
0.20%
0.00%
03-Nov-11
23-Dec-11
11-Feb-12
01-Apr-12
21-May-12
10-Jul-12


abk36-11gl Rid %
abk36-11gl Skr %
1.00%
0.80%
0.60%
0.40%
0.20%
0.00%
03-Nov-11
23-Dec-11
11-Feb-12
01-Apr-12
21-May-12
10-Jul-12


abk37-11 Rid %
abk37-11 Skr %
1.00%
0.80%
0.60%
0.40%
0.20%
0.00%
03-Nov-11
23-Dec-11
11-Feb-12
01-Apr-12
21-May-12
10-Jul-12


abk38-11 Rid %
abk38-11 Skr %
1.00%
0.80%
0.60%
0.40%
0.20%
0.00%
03-Nov-11
23-Dec-11
11-Feb-12
01-Apr-12
21-May-12
10-Jul-12

- The graphic below shows the relative location of all cages which also indicates that all of the current high mortality cages (12, 20, 34, 35 and 36) are clustered together to the East side of the grid



APPENDIX 3: Summary Report on Tau- by T D'Antignana

Summary draft report on Tau from rapid literature review.

Prepared by Trent D'Antignana

This summary report has been prepared in response to discussions with Dr Masashi Matia from the Tokyo University of Fisheries and Technology. After reviewing our disease summary reports Masashi suggested that the disease picture may be due to Tau deficiencies as a result of FM substitution. Work conducted by Masashi et al (1997) demonstrated those yellowtail conditioned on a non-FM diet are susceptible to pathogens. The researchers observed that among the experimental groups, fish fed a non-FM diet showed the highest mortality from *L. garvieae*. However in the study where fish were reared in adjoining net cages, fish fed the same diet supplemented with cholesterol or Tau appeared to improved disease resistance. On examination, they attributed the decrease in disease resistance in the non-FM diets due to anaemia and that this was alleviated in diets which were substituted with Tau.

On further discussions with Masashi he suggested that if YTK are fed diets containing <30% FM, then Tau will be insufficient for normal growth and health. Typical symptoms of Tau deficiency being haemolytic anaemia and potentially green liver. He also suggested that Tau deficiency could result in reduced mucus excretion on the body resulting in increased infection with parasites. If FM was to be replaced then supplementation with 1% crystalline Tau should be sufficient to prevent a decrease in disease resistance.

Considering this information I have reviewed more than 30 papers primarily with YTK on amino acids, organic acids, Tau and fish meal replacement to gain a further understanding of the role dietary changes may be having on the immuno-competence of our fish. The most interesting points from the papers are outlined below.

- YTK and Bluefin tuna are not able to synthesise Tau effectively.
- Verakunpiriya et al (1997) revealed astaxanthin improved spawning performance (egg quality and final no. of normal larvae) and they suggested that the optimum supplemental level was approx. 30mg/kg for yellowtail broodstock.
- Matsunari et al (2006) showed that ovarian maturation accelerated significantly with increased dietary Tau as did the % of buoyant eggs, fertilised eggs and hatching rate. The

results suggested that the addition of at least 1.0% Tau to the FM based diet of YTK broodstock was beneficial.

- Non protein bound free amino acids (FAA) are significant energy substrates for developing embryos and newly hatched larvae of marine fish with pelagic eggs (Ronnestad et al 1999). In fact 60-90% of energy requirements of embryos and early larvae are met through FAA catabolism. Moran et al (2007) examined the importance of FAA in the development of YTK incubated at 17oC (ambient/spawning temp in NZ) 19, 21 and 23oC and found the following:
    - Time to hatch was negatively correlated to incubation temp, with 50% hatch occurring at 50, 65, 80 and 102 hrs. post fertilisation @ 23, 21, 19 and 17oC.
    - Survival rates of larvae at hatch were 71%, 67%, 38% and 36% @ 17, 19, 21 and 23oC. I.e. almost double the survival at 17oC than at 23oC
    - The predominant FAA in yolk-sac larvae were glycine, serine, valine, and unresolved arginine/Tau.
    - YTK hatch with a shorter body length at higher incubation temperatures.
    - FAA decrease rapidly during embryogenesis and were near exhausted at hatch. Suggests that early YTK development is reliant on FAA as the predominant energy source.
    - YTK eggs incubated at 23oC appeared to undergo a different pattern of substrate utilisation compared to lower incubation temperatures, possibly indicative of abnormal physiological development at a temperature above that which is routinely encountered in the wild.
- Stoh et al (2001) reported that P supplementation is necessary in FM based diets for YTK. Similarly Shimeno et al (1994) reported that it is necessary to supplement FM based feed for fingerling yellowtail with P at a level of 3.4mg/g. They suggested that yellowtail could partially utilise P contained in FM. The minimum dietary P required for optimal growth of YTK was suggested as 6.7g/kg dry diet by Masumoto 2002. It is considered that inorganic water soluble P supplementation is needed for FM-based diets to satisfy the requirement of this mineral.
- Matsuari et al (2003) examining the Tau content in early growth stages of artificially reared YTK to those in the wild demonstrated that Tau has an important role compared to other free AA, suggesting live food and diets used for YTK culture do not satisfy the Tau requirement for YTK. In this study the commercial diet contained 4.39 (g/kg dry wt. basis) of Tau.
- FM is highly variable in Tau ranging between 4.5 - 10g/kg of fish meal. Its content in plant protein is poor. Blood meal has undetectable levels of nucleotides and FAA and 83% of its peptides are larger than 10kDa. It also has the lowest protein solubility and Tau content. Hydrolysed feather meal is not much better (Appendix 1 and 2).

- The digestibility of proteins decreases when significant ratios of the protein-bound amino acids occur in the D-configuration. Caspo et al (2008) found that the amount of the D-amino acids increased significantly when the extrusion temperature was increased from 101-140^{o}C. No information available on the availability of Tau as a result of extrusion/temperature.

- Extrusion may negatively affect availability of amino acids such as lysine and arginine. This may induce the appearance of chronic subclinical deficiencies that negatively affect fish performance and weakens the animals, making then more susceptible to disease problems Oliva-Teles (2012). When given indispensable amino acid (IAA) deficient diets, fish display reduced growth and anorexia. For adequate performance IAA/DAA (dispensable AA) ratio in fish diets must be kept within the 50-60/50-40 ratio as either lower or higher ratios will negatively affect performance. Protein and AA deficiencies can impair immune function and increase the susceptibility of the fish to infectious diseases – through a reduction in plasma AA.

- Sarker et al (2012) found growth and feed performance was lower in the low FM diets (23% FM) compared with the other dietary groups (30-50% FM) indicating that Tau may be deficient in the low FM diets. In the low FM diet Tau was 1.8g/kg while the other diets contained higher levels of Tau (30% FM 2.3g/kg and 50% FM- 3.6g/kg) suggesting Tau supplementation is necessary for maintaining normal growth performances in low FM diets of YTK. Green liver was absent in fish fed diets containg 50% FM but present in fish fed diets containing ≥30% FM. This was attributed to Tau deficiency.

- Takagi et al (2005,2006a, 2006b,2008) observed physiological abnormalities and inferior performance of yellowtail fed non-FM diets based on soy protein concentrate and concluded it was a Tau deficiency. Tau supplementation to the 100% SPC diet (58% protein) dramatically improved the physiological state of yellowtail. In addition growth and feed utilisation were improved in proportion to the increase up to 4.5% (45g/kg) Tau supplementation in the SPC diet. In the 100% FM diet (58% protein) the dietary Tau content was between 2.17 and 3.36g/kg compared to the 100% SPC which had between 36.5 and 43g/kg as crystalline Tau. Despite the approx. 10-20 fold increase in the Tau level in the SPC diet, the performance of the SCP diet was still below that of the FM diet. Suggests that crystalline Tau is far less biologically effective than the endogenous form. Alternatively this result suggests that Tau requirements of fish fed SPC diets may become elevated due to an influence of dietary soybean protein, causing Tau to be excreted.

- Watanabe et al 1994, Maita et al, 1997 and Aoki et al 2000 have reported that yellowtail can be reared for up to 6 to 12 weeks with diets based on alternative protein sources containing 10% or less FM and growth and diet utilisation of such diets were comparable to fish fed a FM diet. However when the rearing period was further extended the performances of the fish fed the low FM diets was inferior in addition to the development of anaemia and green liver syndrome.

- Matsunari et al (2005) suggested that Tau requirements of juvenile YTK (0.5g to 10g) fed FM based diets are more than 10g/kg diet, but on closer examination of their results D'Antigana

does not believe the results support their claims. It should however be noted that the baseline level of Tau in their control diets was 3.9g/kg.

- Watanabe et al (1998) found that almost all YTK fed a non-FM diet supplemented with crystalline amino acids (AA profile of the FM substituted diets was similar to the control diet) had green liver. The symptom was found to be caused by occlusion of the bile duct due to a parasitic mucosporozoa. Mortality of 10-32% was observed in fish fed non fish meal diets, with most morts displaying green liver. Availability of the crystalline amino acids could have contributed to the poor performance of the YTK.

- FM and animal by-products are rich sources of Tau

- Cholesterol is rich in FM, while only low levels are present in most plant sources

- The requirement for EPA and DHA in combination is within the range of 0.8-1.2% of the diet for juvenile cobia (41g) (Chou et al, 2001)

- Dietary fatty acid composition influences immune response by determining which eicosanoid precursors are present in the cell membrane, with n-6 PUFA rich diets enhancing immune response and n-3 PUFA-rich diets being immunosuppressive. Replacing fish oil with vegetable oils in fish diets has effects on the FA composition and the ratio of the n-3/n-6 HUFA. This may affect the fish health status and resistance to disease. Low ratio of n-3/n-6 FA caused severe heart lesions in salmon (Bell et al 1991).

- Cobia fed FM substituted diets (these diets contained 29% FM and 40% yeast protein) supplemented with 0.5% crystalline Tau had significantly higher feed efficiency compared to a 100% FM non supplemented diet (Lunger et al 2007). However fish performance was significantly reduced in the substituted diets (again theses diets still contained 29% FM; 0.24% natural Tau) when crystalline Tau was not added. The author suggests that Tau supplementation is necessary for carnivorous fish sp. when fed diets with alternative protein sources. Cobia may need 0.4-0.65% Tau in the diet.

- Growth and health promoting effects of dietary arginine beyond meeting requirement for protein synthesis has been reported in some fish. Survival of channel catfish in response to challenge with *E. ictaluri* critically depended upon dietary arginine levels (Buentello and Gatlin, 2001). In Yellowtail weight gain, feed efficiency, protein efficiency and nitrogen retention increased with increasing level of arginine up to 1.56% in diet taking into consideration that the digestibility of fish meal is 92.5% (Ruchimat, et al 1998).

- Lysine and methionine are often the most limiting AA in ingredients used in commercial fish feeds especially when fishmeal is replaced by plant protein sources. Dietary lysine levels critically affect fish growth performance and health (Peng et al 2009). For YTK dietary lysine should be 1.78% of the diet or 4.13% of dietary protein (Ruchimat et al 1997) for methionine levels should be 1.11% of the diet or 2.56% of the dietary protein. Low methionine levels resulted in anaemia (Ruchimat et al 1997).

- Green liver syndrome in fish occurs due to several causes such as adverse effects to inferior feed quality, bile duct occlusion by parasites, reduction of bile secretion from the liver into the bile duct due to starvation, increase of haemolysis caused by the bacterial disease "bacterial haemolytic jaundice", oxidative stress induced by phenyl hydrazine and prolonged period of low water temperature. It is also caused by Tau deficiency as a result of feeding diets low in FM (Matia et al, 1997; Takagi et al 2010).

- Tau has been implicated in osmotic stabilization of juvenile (0.5-15g) marine fish. In juvenile Japanese flounder they require 15mg/kg of Tau (total volume) in their diets, even though a combined mix of fish, krill and squid meal are the main sources of protein in the diets.

In summary it appears that amino acid requirements require specific attention when replacing fish meal with alternative protein sources. It is obvious when reviewing the literature that YTK are extremely susceptible to dietary changes as a result of fish meal substitution. In nearly all instances when FM was replaced with another protein fish performance was negatively affected. Only though careful balancing was the performance of the fish improved when using an alternative protein source. In light of this it would appear that the nutrition of YTK can easily become a limiting factor if dietary formulations are in appropriate. It should be noted that many authors have observed that YTK reared on FM alternative diets generally do quite well in the short term (6-12 weeks), but performance decrease's when the rearing period is extended.

Considering the evidence it appears that Tau is an important amino acid for brood stock, larval and fingerling performance. It would appear that suboptimal performance will be observed at Tau levels <1.8g/kg. In predominately FM based diets Tau levels of 2.5 – 4g/kg are probably sufficient of optimal YTK performance, however the Tau level should be increased to at least 5g/kg through the use of crystalline Tau if substituting FM with alternative proteins. If SPC is to be considered as an alternative protein source then the level of crystalline Tau in the diet may need to be as high as 5%. Furthermore the literature suggests that Tau should be included in to broodstock diets at a level of 1%. The evidence also suggests that live feeds and early weaning diets for YTK should be enriched with Tau at 5-10g/kg of diet. Considering the recent results from AsureQuality it would appear that the majority of our diets have been either deficient in Tau or on the verge, thus Tau may be a factor limiting fish performance. Furthermore reading the literature, the following should also be considered:

- YTK dietary lysine requirements are 1.78% of the diet

- YTK dietary methionine requirement is 1.11% of the diet

- YTK dietary Arginine requirement is approx. 1.56% of the diet

- The IAA:DAA ratio in the feed should be kept within 50-60/50-40 to maintain optimum performance.

- The juvenile YTK requirement for EPA/DHA in combination is probably within the range of 0.8-1.2% of the diet.

- FM is highly variable in its Tau content and Tau analysis by the feed suppliers should be routine.

- Given egg incubation temperatures appear to play a significant role on embryogenesis and physiological development, time should be given to examine production records to determine whether our incubation temps have changed over the course of time and contributed to poor performance

- As replacement of FO with poultry oil and FM with alternative proteins can lead to hypercholesterolemia (Bowyer et al 2012 and Maita et al 2007), consideration should be given to determining the cholesterol level in the diet, blood plasma and liver of YTK.

- The histopathology report on gut physiology from the YTK feed management project has been submitted. The project examined whether FM substitution with Soya and FO substitution with canola oil and poultry oil caused physiological differences in YTK gut physiology after 6 weeks of feeding. In brief substituting FM and FO with alternative sources appeared to have no detrimental effect on gut health during the short term study. However, an increase in goblet cell proliferation and a reduction in supranuclear vacuolisation were observed in the initial fish prior to feeding the experimental diets. These physiological characteristics were also more sever in the larger fish (100g) than the smaller fish (22g), and suggest the fish were suffering from a mild inflammatory response. As a salmon assessment scale was used and no pathology from wild fish was available the abnormal abundance in goblet cells and a reduction in supranuclear vacuolisation needs to be viewed with caution. Alternatively, these results may suggest that the fish were predisposed to an inflammatory stressor from a young age (5-10g) and that the impact of the stressor increased with time. As the fish were fed commercial diets (Ridleys and Skretting) prior to the feed experiments these diets may have contributed to the observed pathology as no gut parasites were witnessed. Based on these results, it is recommended that the histology slides from this study are reviewed by Michelle Dennis or Brian Jones and that samples from wild fish are sourced to determine whether the observed pathology is abnormal for healthy YTK.

Appendix 9.6a – Considerations and comparative clinical pathology (Mark Shepard)

Enteritis – considerations & comparative clinical pathology

Mark Sheppard, B.Sc, DVM

July 12, 2012

Australian Seafood CRC –
Yellowtail kingfish workshop

What do we know about enteritis?

- It's inflammation & it's costly – performance, growth, FCR, survival, labour intensive (& diagnostically)
- Complex & multi-factoral (many avenues of pathogenesis to consider)
  - physical, functional, host, ecosystem, nutrition, stressors, opportunism, infections, metabolic, inflammatory, immunity, mal-absorptive digestion…
- Primary cause… secondary, & tertiary associations
- Progressive – acute to chronic…. moribund & agonal findings
- Widespread – numerous fish species and stocks
- Diarrhoea, dehydration, osmotic ionic imbalance, starve-outs, +/- toxemia
- A lengthening history – (mid 90's was the S.H.I.T.S.S.)

sVs

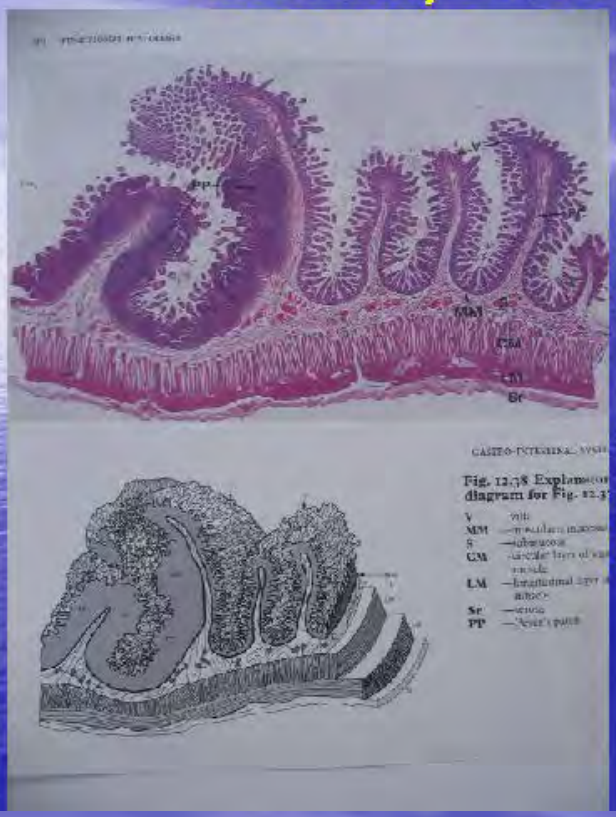
General signs of enteritis

- Intermittent bouts – acute, sub-acute, or chronic:
- Red intestines, often thickened
- Mucosal changes, damaged epithelium
- Necrosis, ulceration, bleeding, spasms? pain?… anxiety?
- Secondary infection(s)
- Malabsorption, maldigestion
- Osmotic diarrhoea
- Lethargy & illness
- Anaemia & blood chemistry
- Dehydration, poor colour

Crohn's disease (auto-immune)
Irritable bowel syndr (diet/stress)
Coeliacs disease (gluten reaction)
Ulcerative colitis (dogs)
Food allergies, pancreatic insufficiency
Johnes para-T. (infectious)
Swine haemorrhagic necrotic enteritis
Avian, bovine & horse SBM anti-nutritional syndromes
Infectious coccidiosis

sVs

Anatomy of the intestine



Lumin ('luminal digestion')

PC = plica circularis (rings & rugae)

V = villi (mucosal epithelium, connective tissue / lamina propria) = 'membrane digestion'

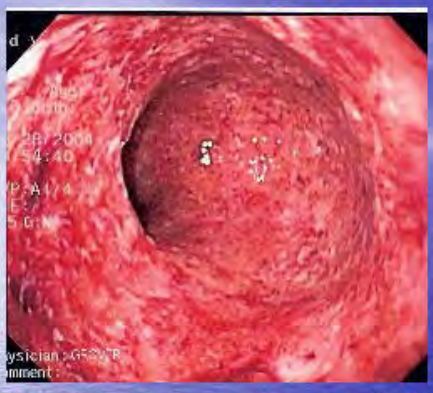
MM = muscularis mucosa

S = sub-mucosa

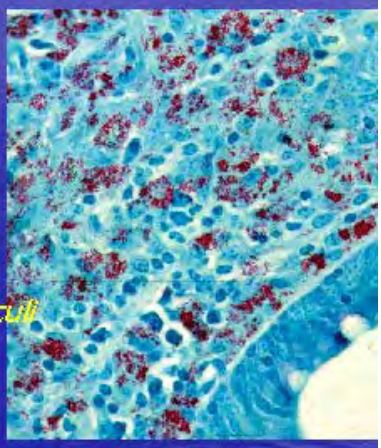
PP = Peyer's patch (lymphocytic; leucocytes)

sVs

Inflammed intestines



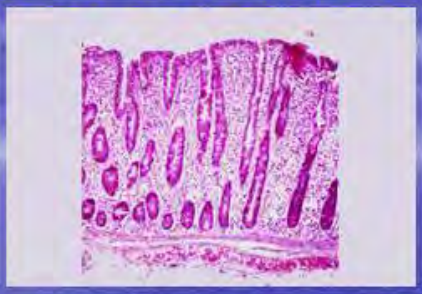
Crohn's intestine

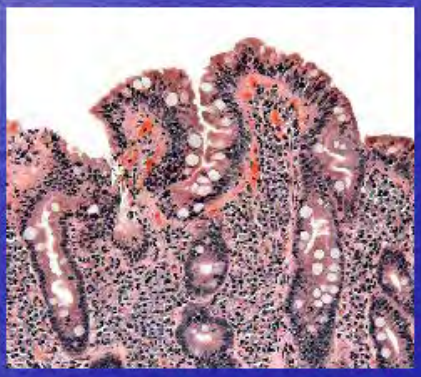


Johnes intestine (sheep)
Mycobactrium paratuberculi
M. johnei)

Normal PC, rugae & mucosa

Inflammed Intestines (histology)



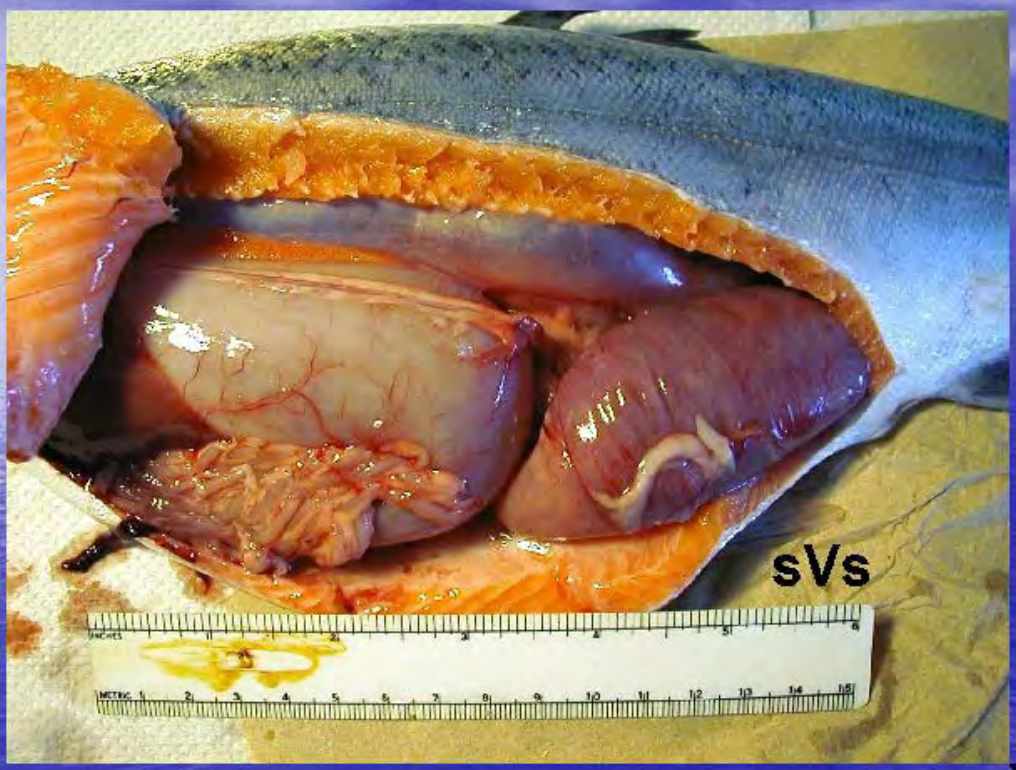


Coeliacs' response to gluten (etc.)

Crohn's disease

Chinook salmon in Canada

sVs



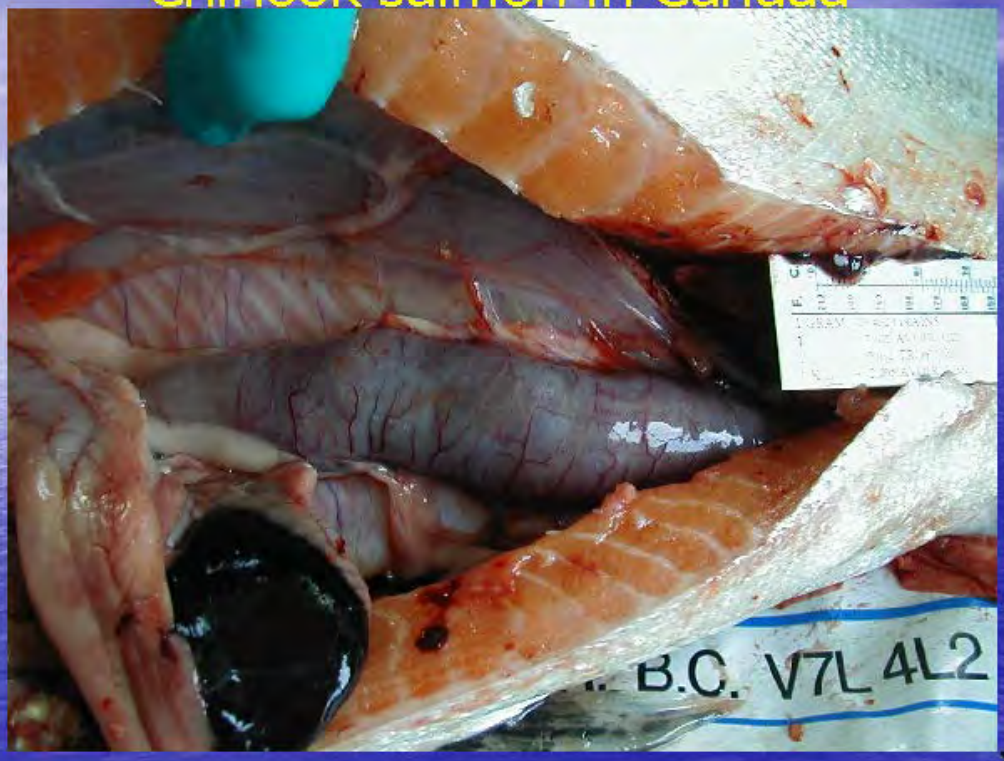
sVs

Related to feed manufacturing
(ingredients &/or extrusion protocols?)

- gastric dilatation & paralytic ileus
- osmotic imbalance
- excessive drinking of sea water
- intestinal torsion & necrosis

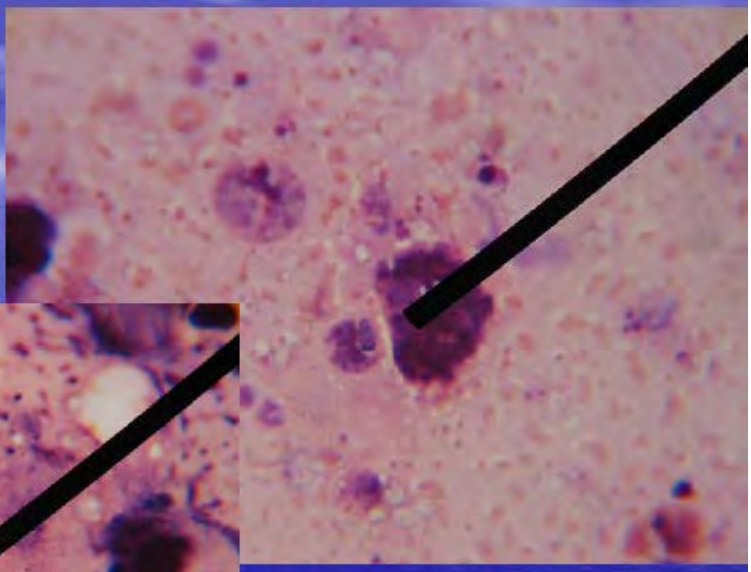
sVs

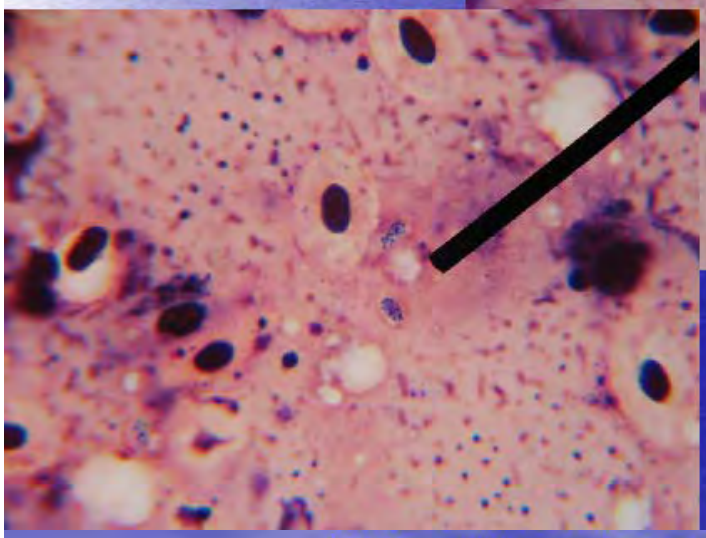
Chinook salmon in Canada



sVs

Microsporidiosis.
Retroviral.
Inflammation.
"A syndrome"





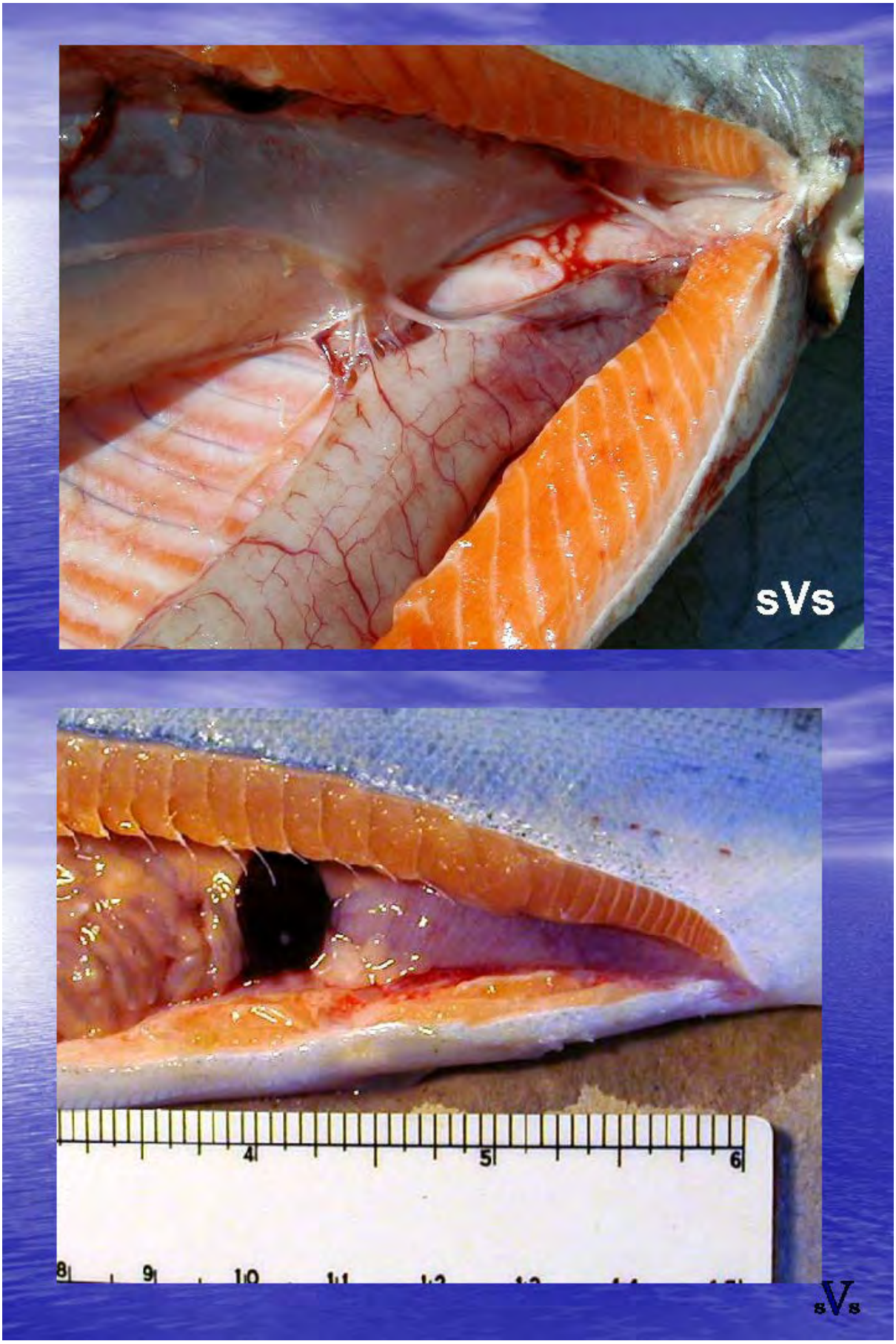
vs

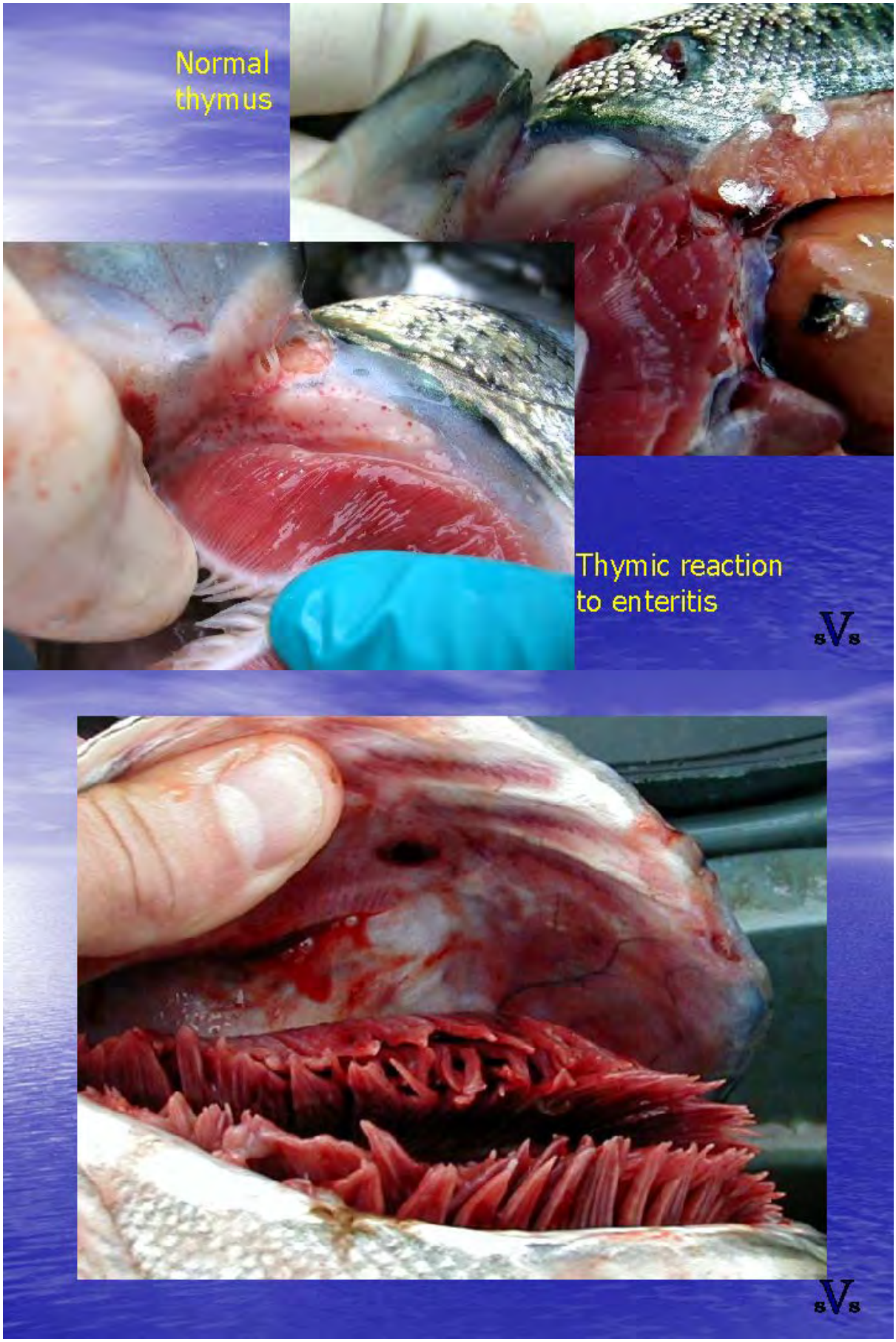
Atlantic salmon in BC

vs


sVs


sVs

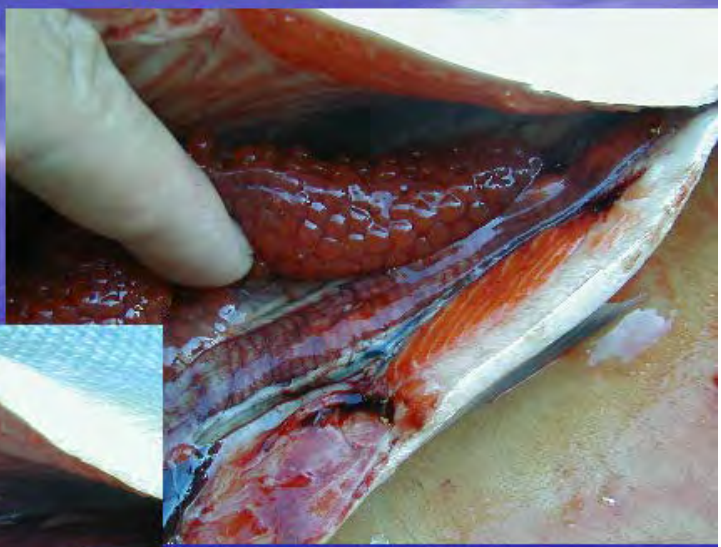

sVs
4
5
6
8
9
10
sVs

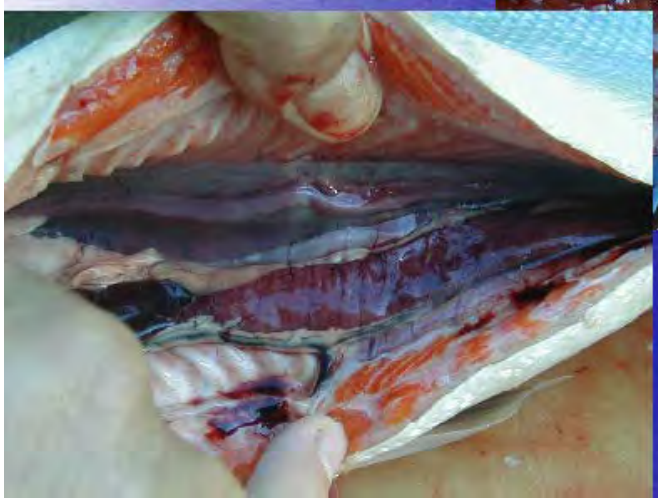

Normal thymus
Thymic reaction to enteritis
Vs
Vs

Bacterial enteritis? Mono-cultures

vs

Wild sockeye salmon

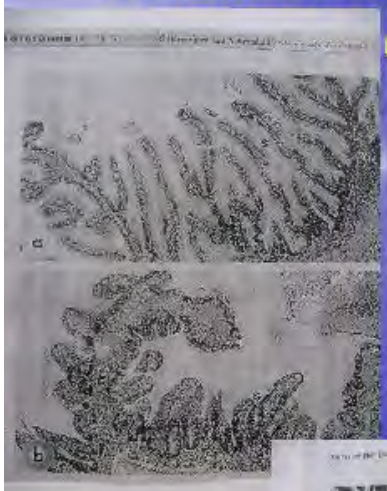




vs

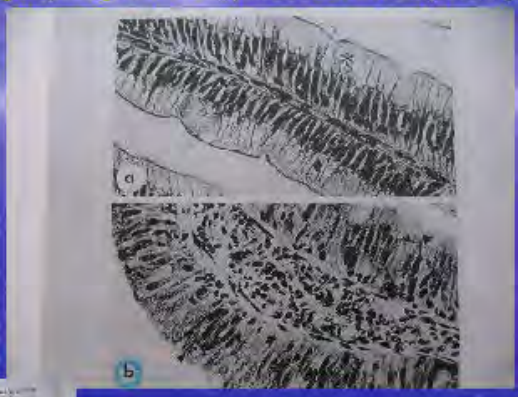
Salmonid mucosal reaction to soy products

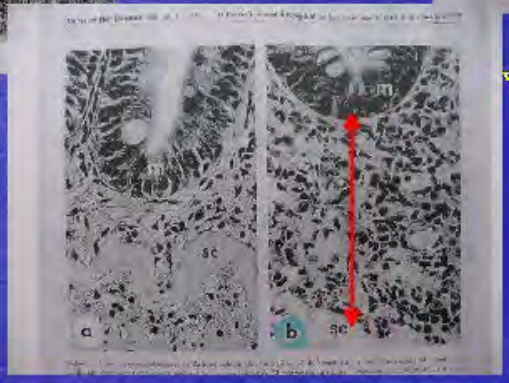
(Atlantic salmon, Baeverfojord/Krogdahl 1996; Uran PA thesis , 2008)



a) Normal

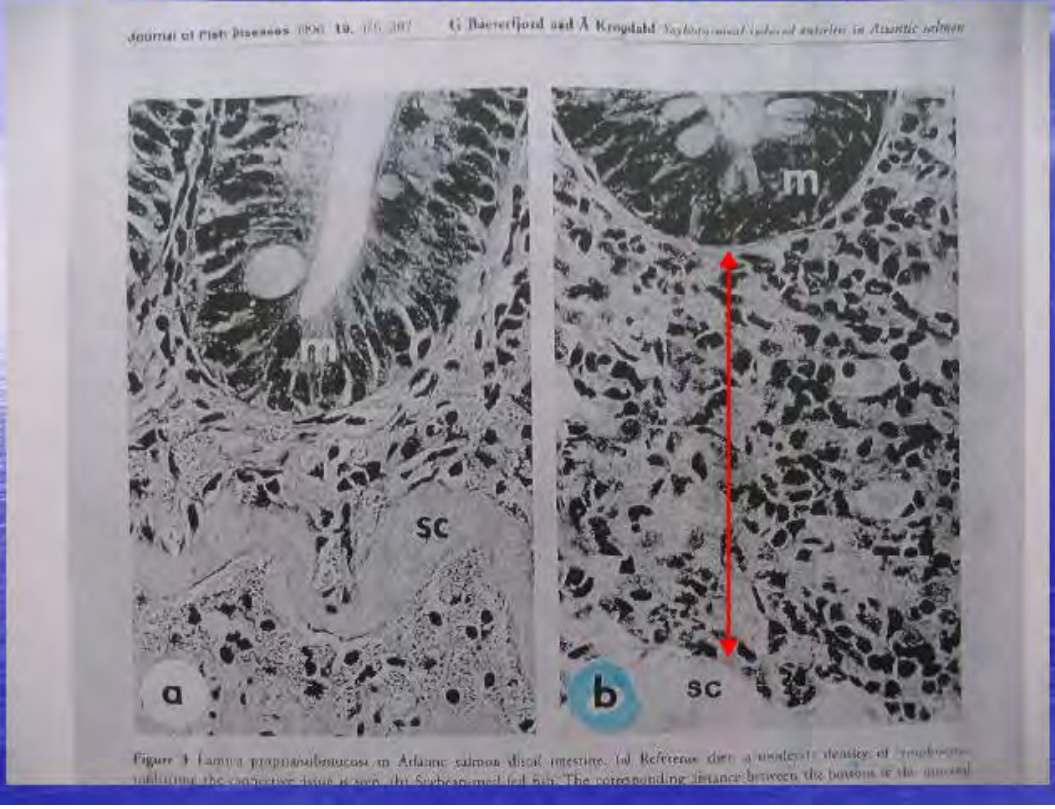
b) Exposed / Affected



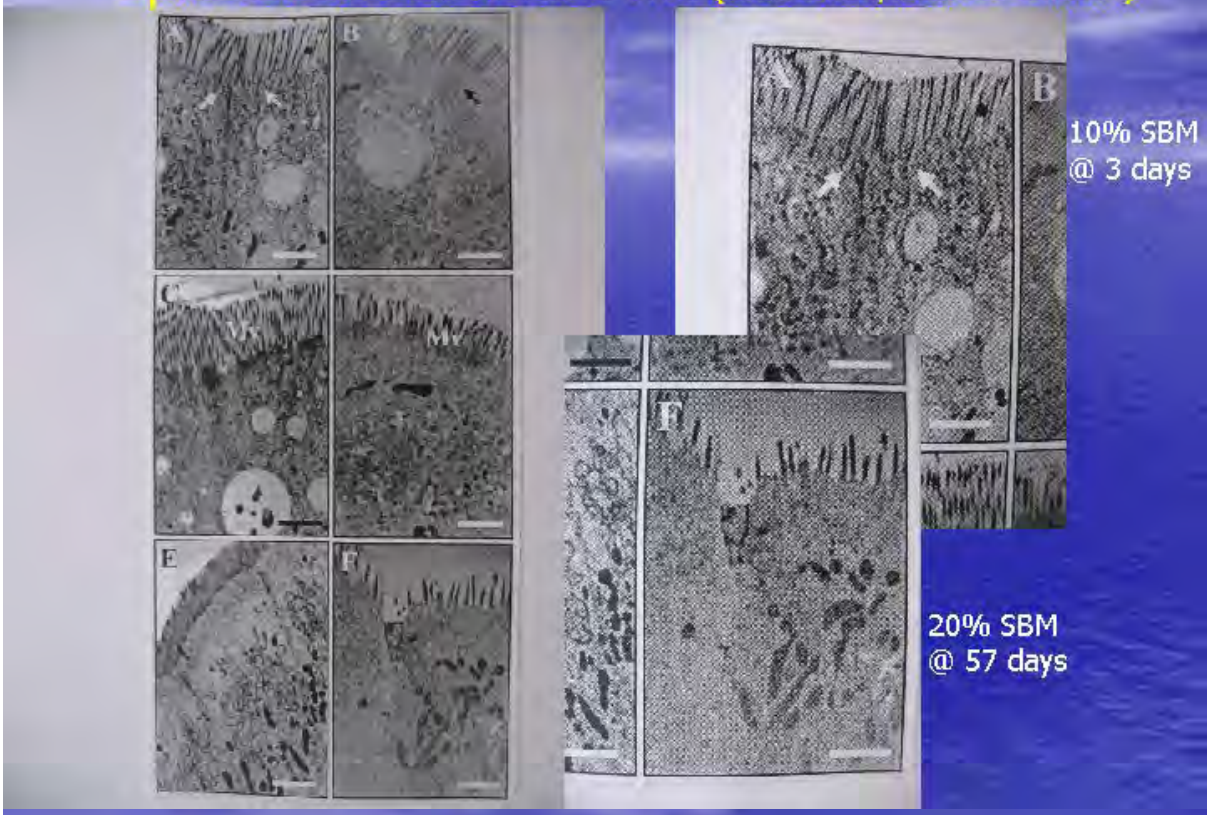


"Non- infectious, sub-acute enteritis, and a pathogenesis involving immunological mechanisms"

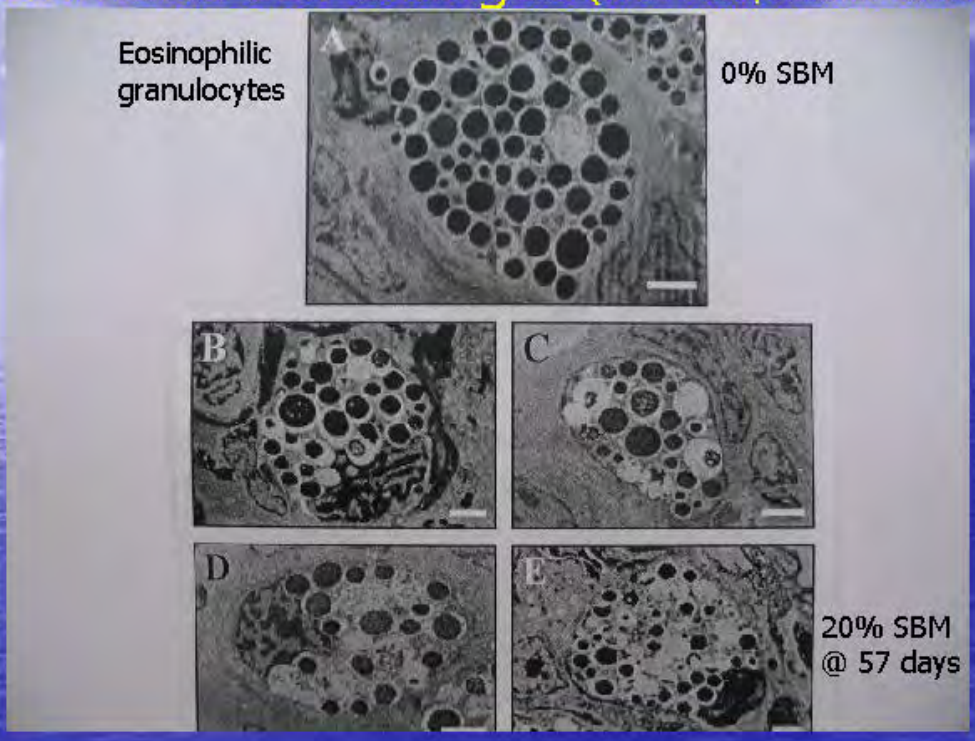
Thickened intestinal mucosa (Atlantic salmon)



Epithelium & microvilli (Uran 2008, Atlantic salmon)



Immune cell changes (Uran 2008, Atlantic salmon)



Yellowtail (hamachi, buri…) *Seriola quinqueradiata*

vs

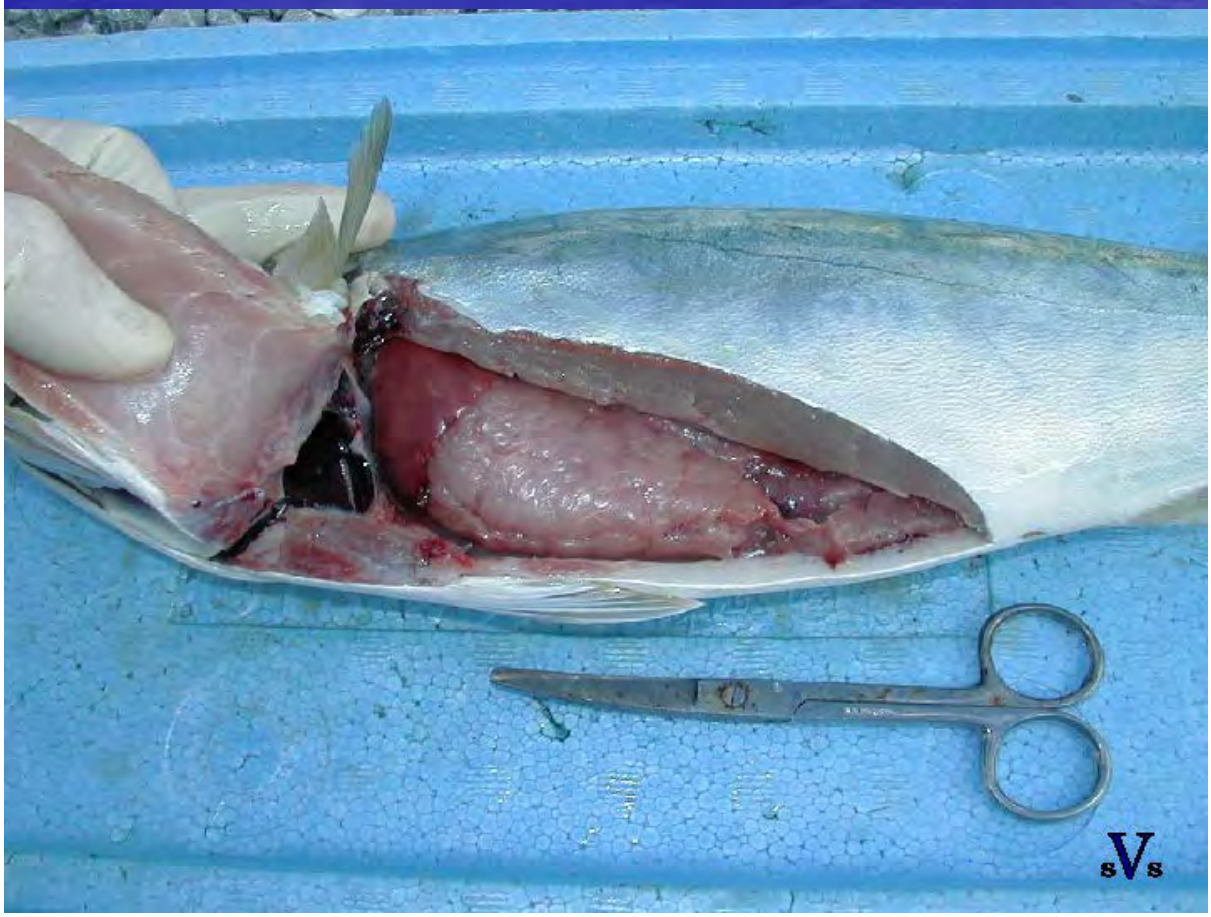
"Kingfish" (hiramasa, gold-striped amberjack) *Seriola lalandi*

vs

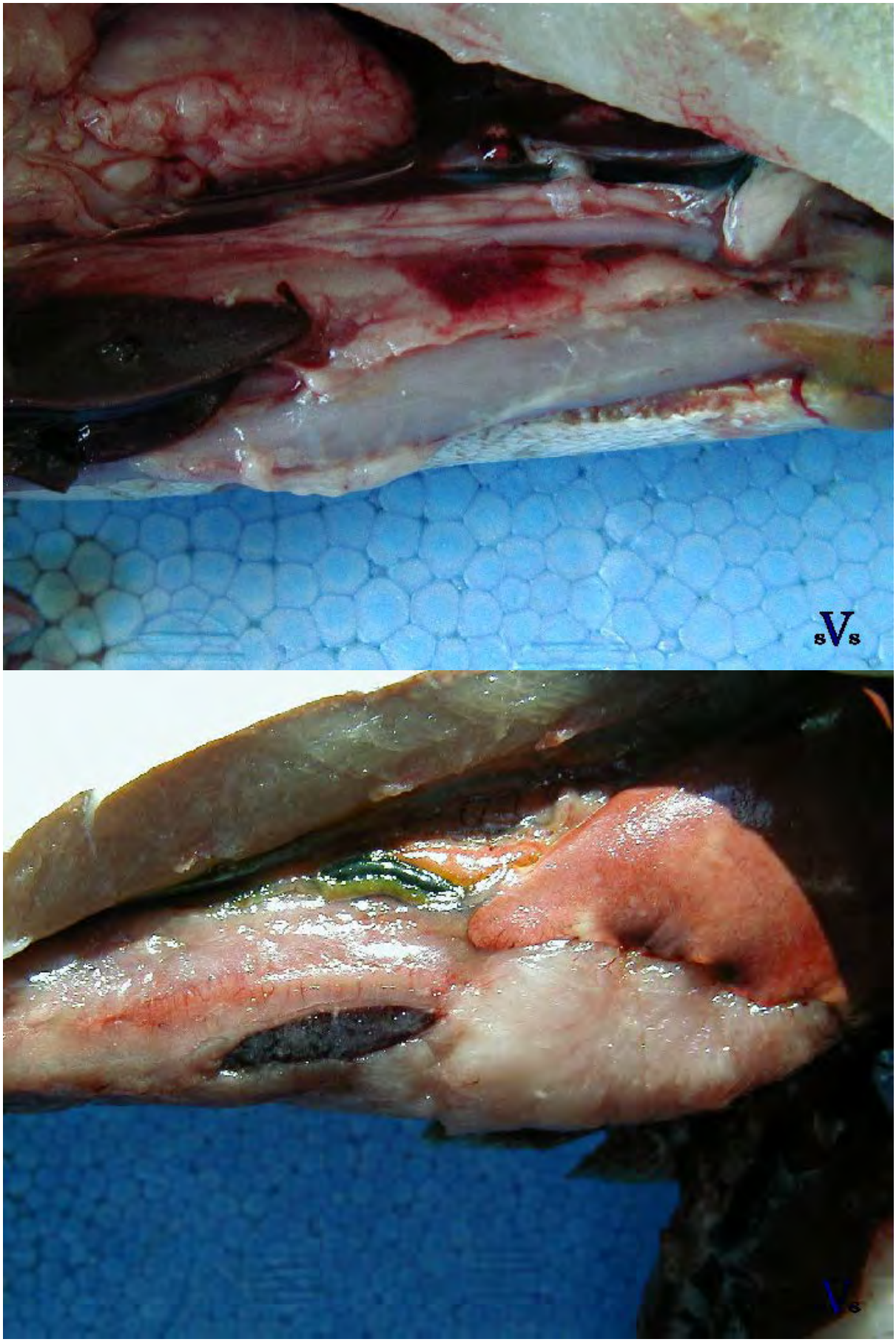
Necrotic enteritis (winter syndrome, initially)

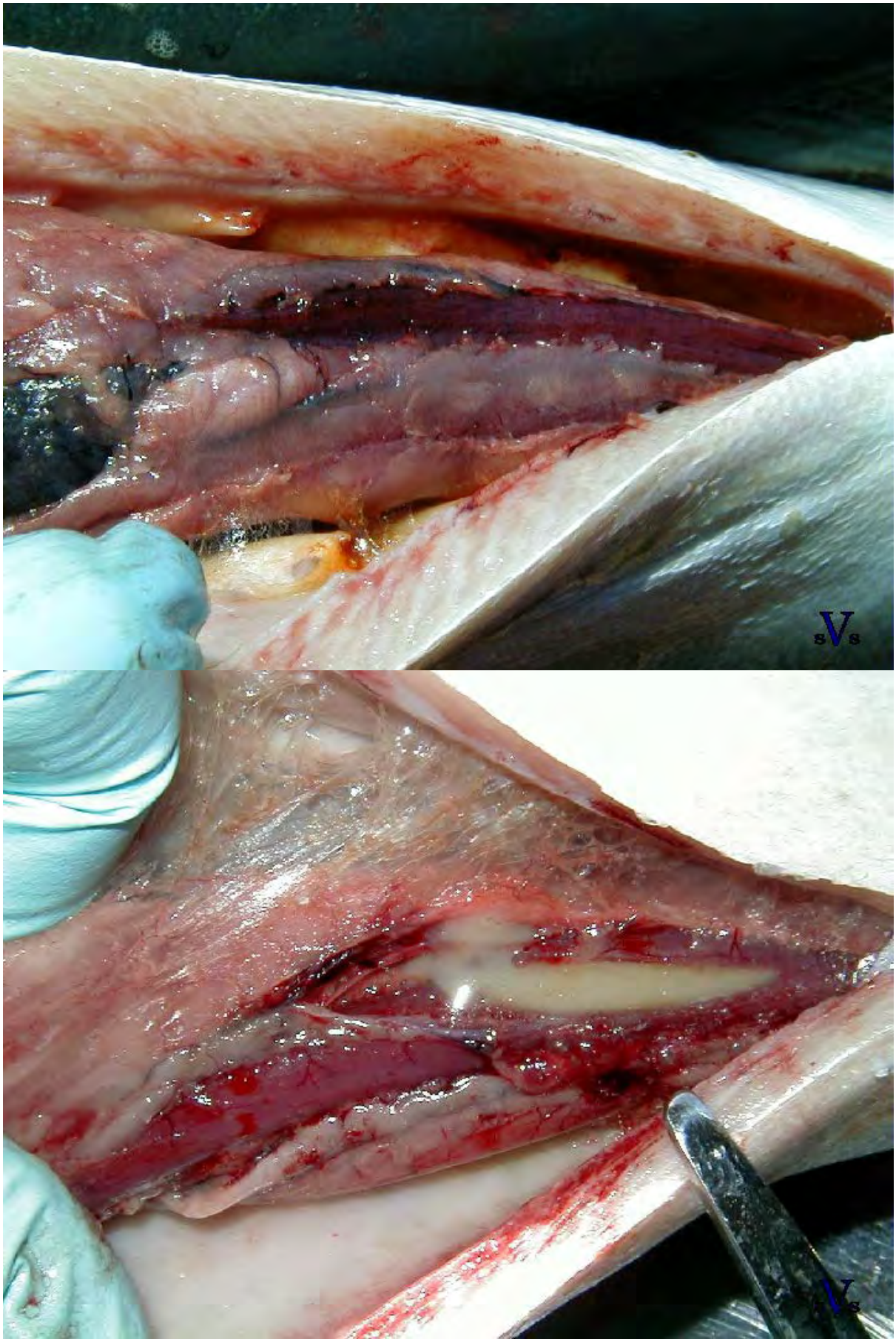
- Diagnosis by examination (thin red gut), histology
- Moderate mortality in cool seasons (13-18C)
- Confounding factors:
  - temp, stress, immunity, physiology, metabolism, feed, opportunism…
- Antibiotics are only marginally & transiently effective

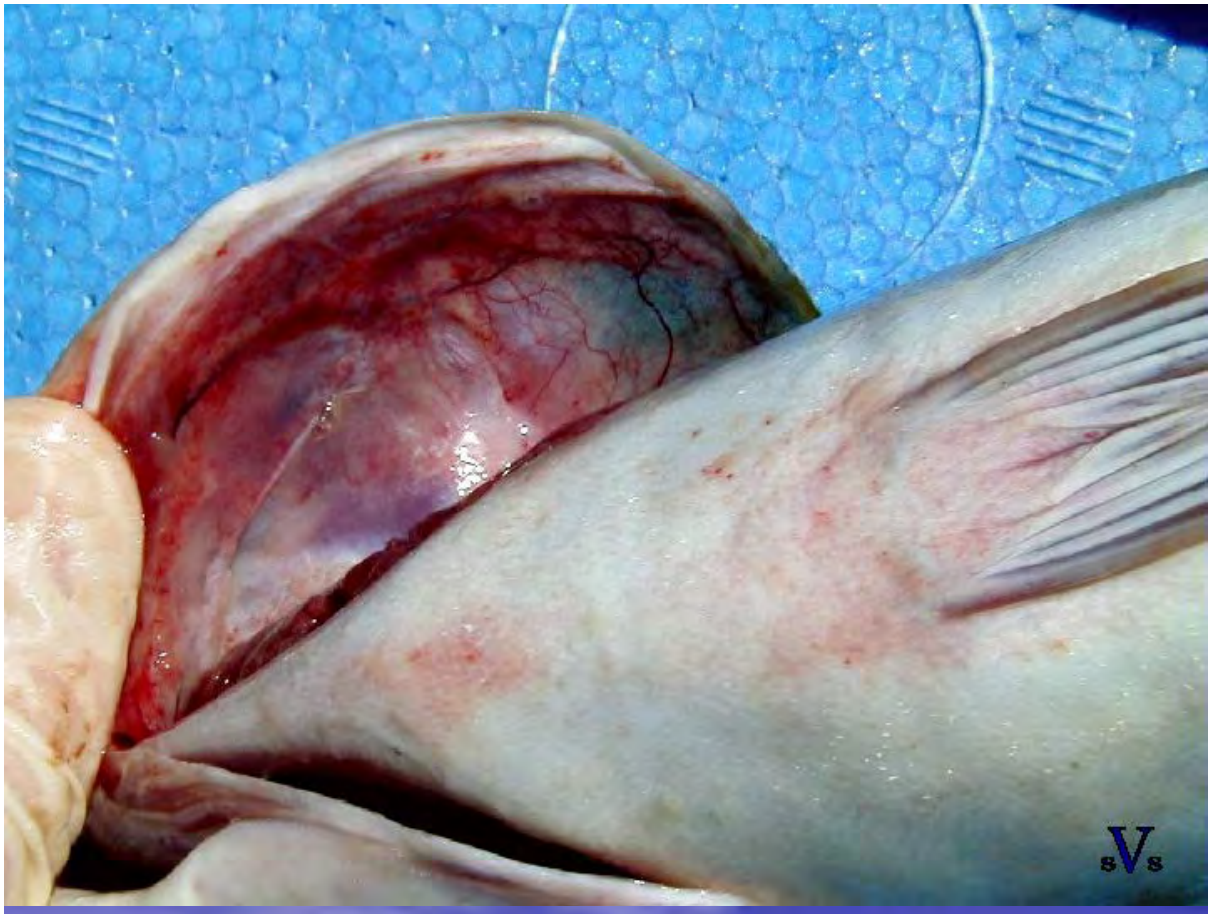
sVs



sVs

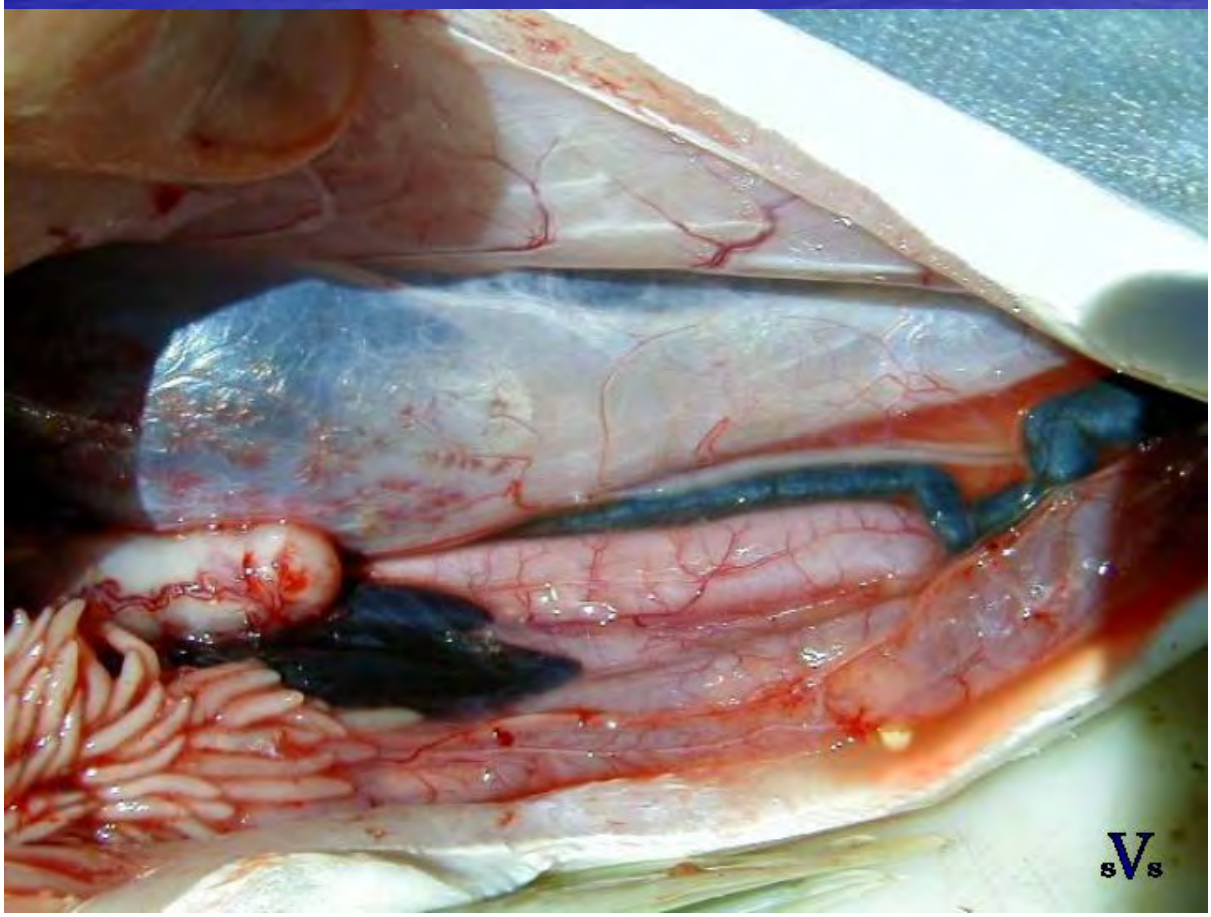

sVs
Vs

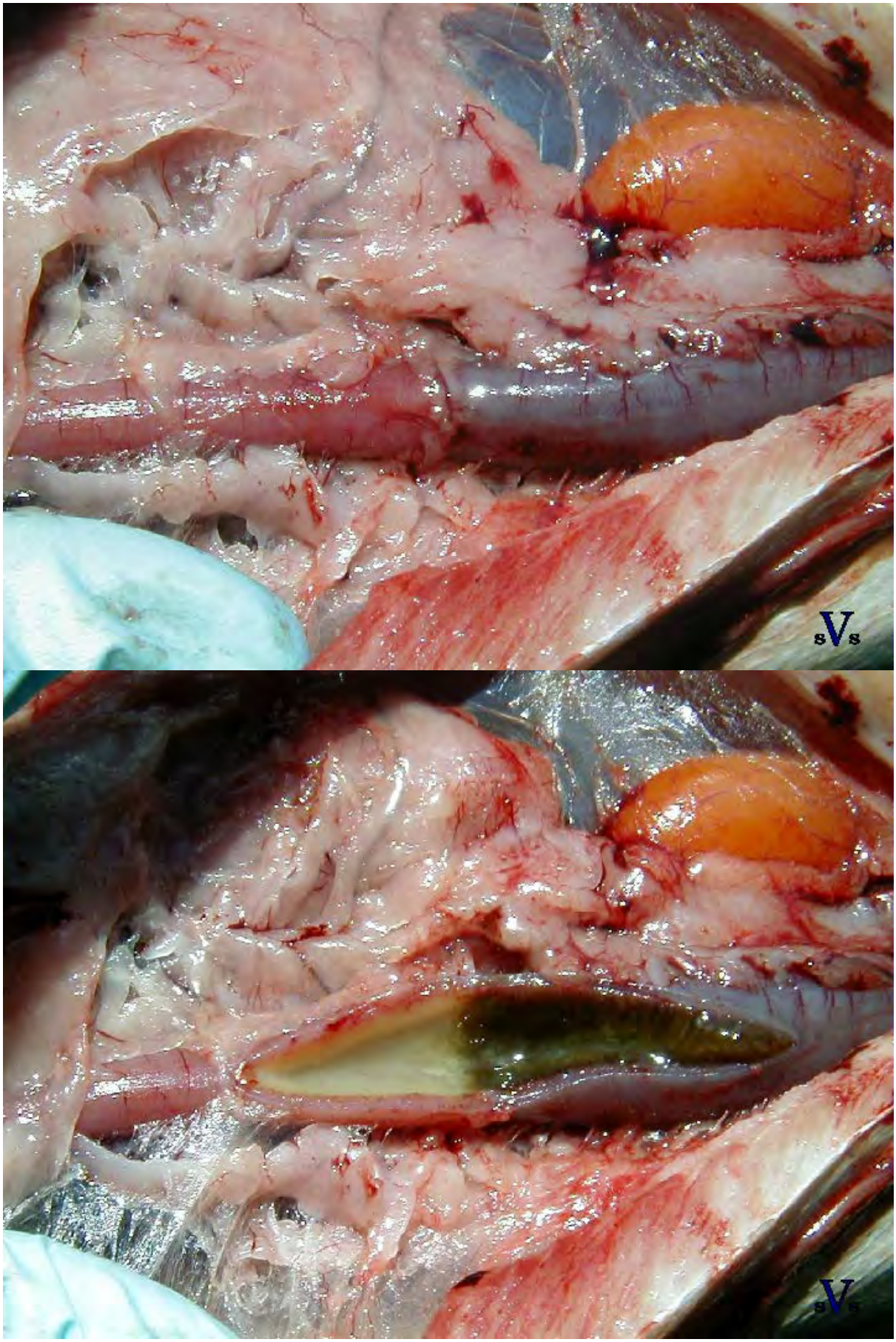

sVs
Vs



Enteritis with bacterial overgrowth


ULTRA
sVs

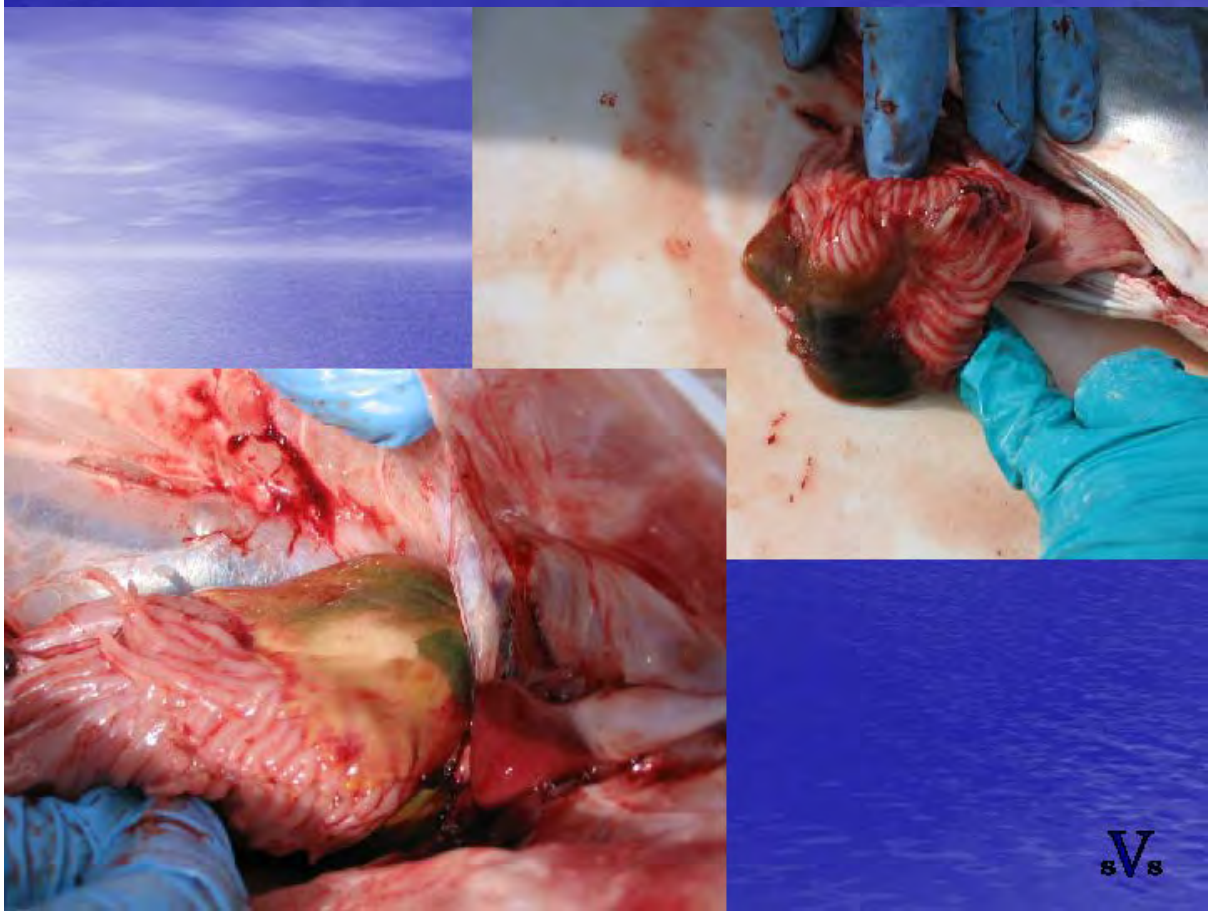

sVs


sVs
sVs


CST FFVS 2010

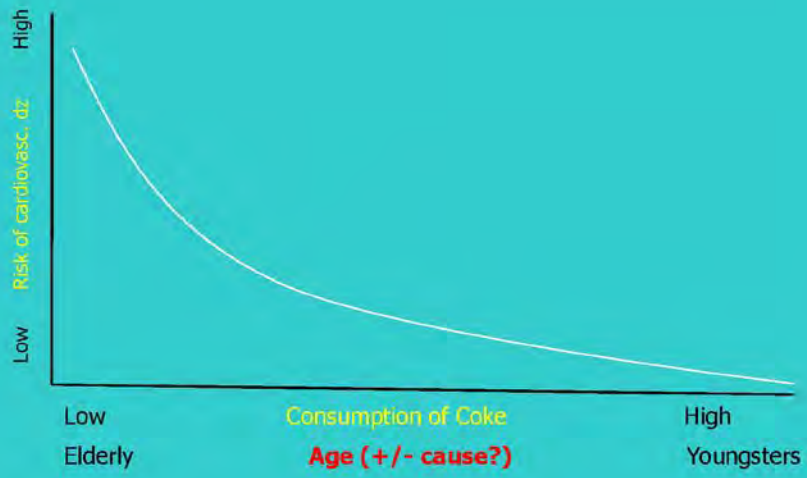
"Coccidial-like, protozoal enteritis"

(CST FFVS Report 08-09-2011)

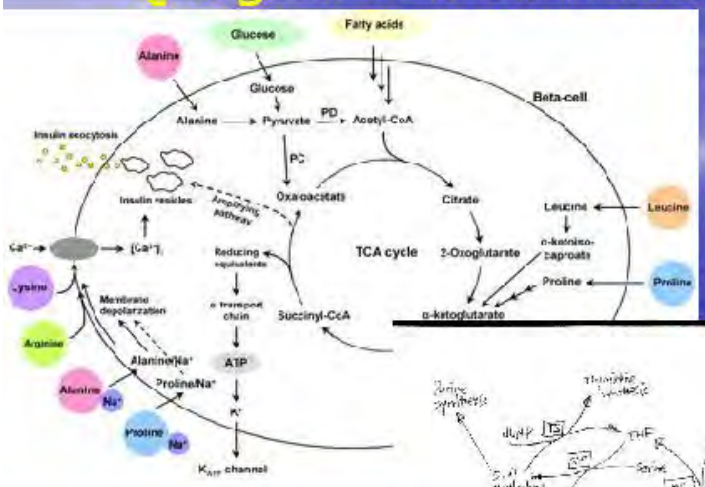


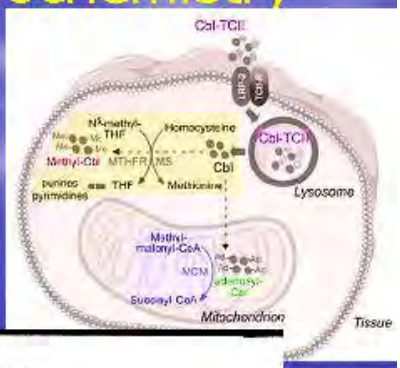
vs

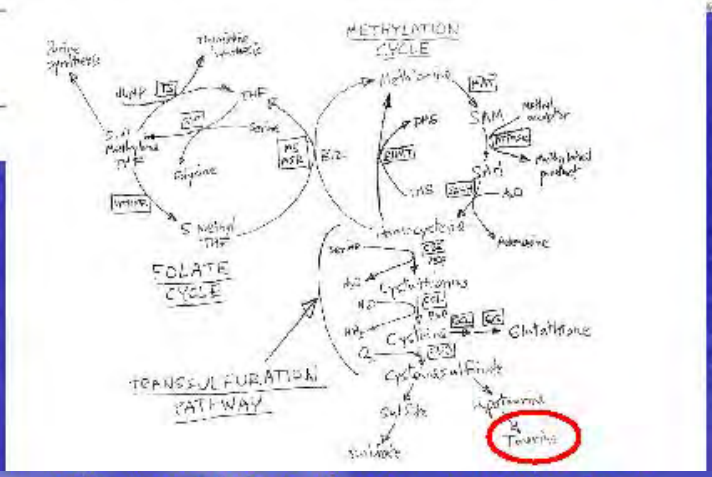
Association vs. Causation



Quagmire A: nutritional biochemistry



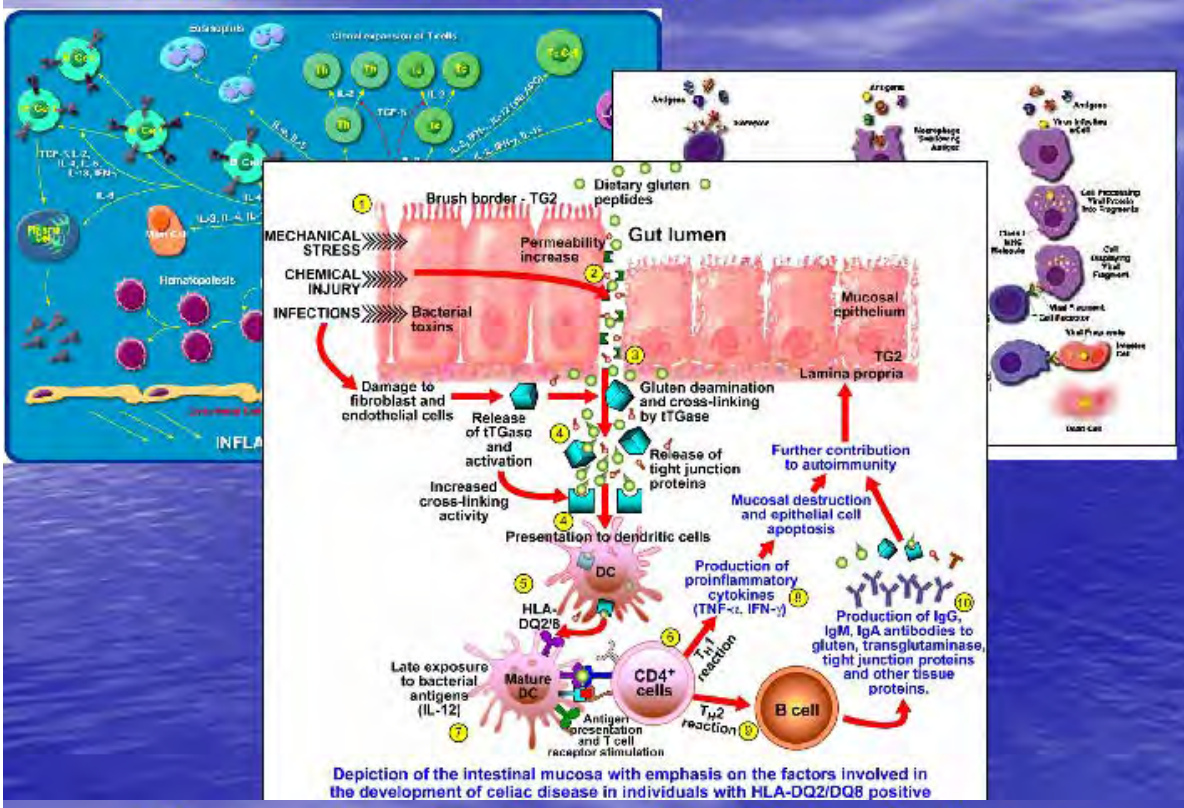




The virtues of methionine, cysteine… specifically, taurine

- a sulfonic acid = essential to fish, particularly *Seriolae* (>3%)
- conjugates with bile acids
- emulsification and absorption of lipids
- membrane stabilization (fluidity)
- facilitates ion gradients (K & Mg kept in; Na out)
- calcium homeostasis
- anti-oxidant qualities
- mucogenic?
- recovery from osmotic shock
-

Quagmire B: immunity & inflammation



Quagmire C: the effect(s) of stress

sVs

Chronic stress

STRESS (cortisol)

Elevated FCRs

Reproduction failures

IMMUNO-SUPPRESSION of:

- **Non-specific (natural) immunity**
- **Specific (acquired) immunity**

DISEASE & MORTALITY

vs

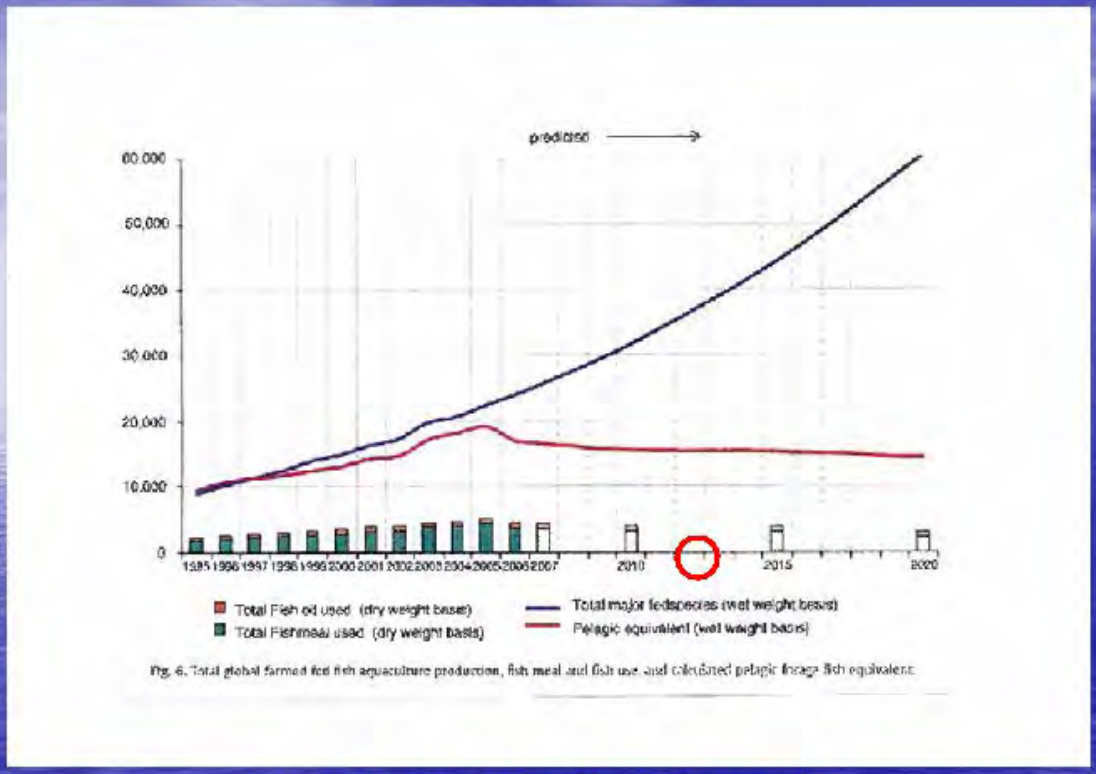


Fig. 6. Total global farmed fed fish aquaculture production, fish meal and fish use and calculated pelagic forage fish equivalent

Source: Tacon & Metian (Aquaculture, 2008):
Global overview fish meal & fish oil trends

Quagmire D: Nutrition (anti-nutrition): fish meal/oil vs. alternatives (soy or other)

- Sustainable production vs. digestive complexity
- *Seriolae* vs. salmonids (Yokoyama et al., 2008)
- Amino acid deficiencies / imbalances / absorptions & availability
- Balance & availability of LC polyunsaturated fatty acids… at warm vs. cool temperatures (Bowyer et al., 2012)
- feed transport & storage: oxidation, altered chemistry, rancidity, depletion of anti-oxidants, fungus?

sVs

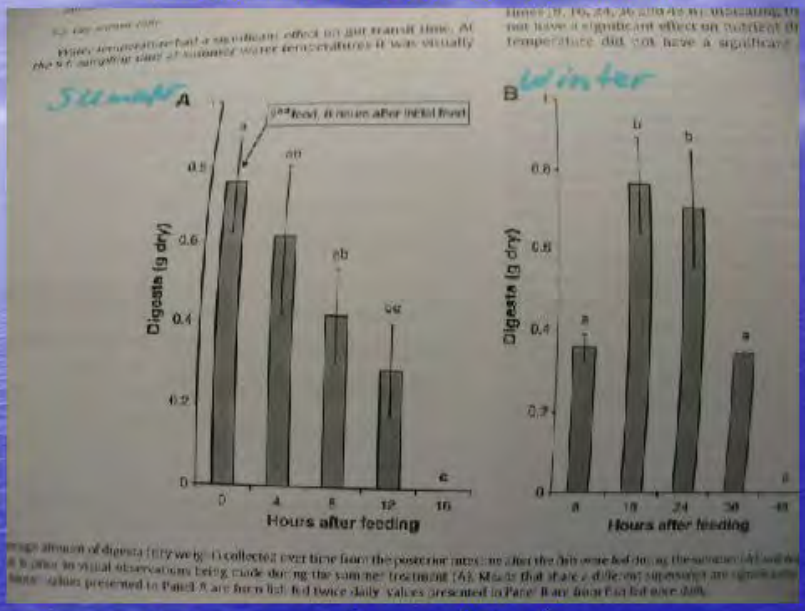
Anti-nutrition: fish meal/oil vs. alternatives (soy or other)

- ANFs not just in soy bean products (wheat gluten, corn, canola…)
- Non-starch polysaccharides & oligosaccarhides (raffinose, stachyose) may interfere with the digestion of other nutrients so elevate osmotic concentrations of digesta = restricts water absorption by the gut.
- Mal-absorption & enterocytic sloughing = selective loss of albumin, typical of protein-losing enteropathies (J.Del-Pozo et al., 2010, RBT)

sVs

Gut transit time

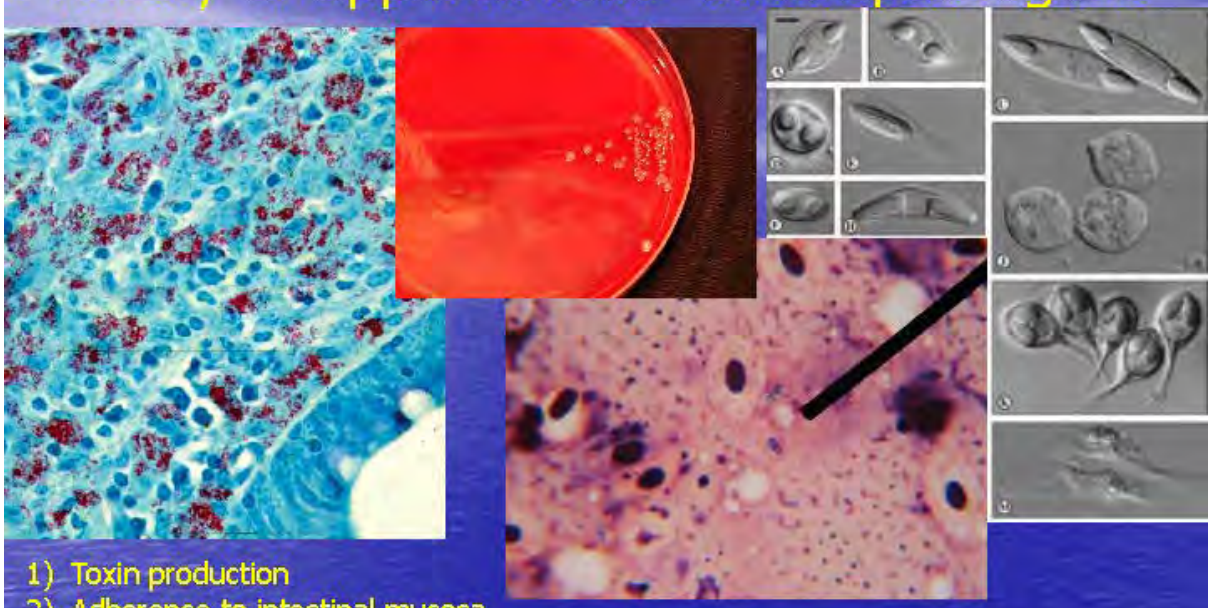
(Miegel et al., 2010, *Seriola lalandi*)



Temperature, age, species, meal size/frequency, ingredient digestibility & exposure time, gut motility…


Environmental interactions with
wild animals (+ pathogens)

Primary or opportunistic "enteropathogens"



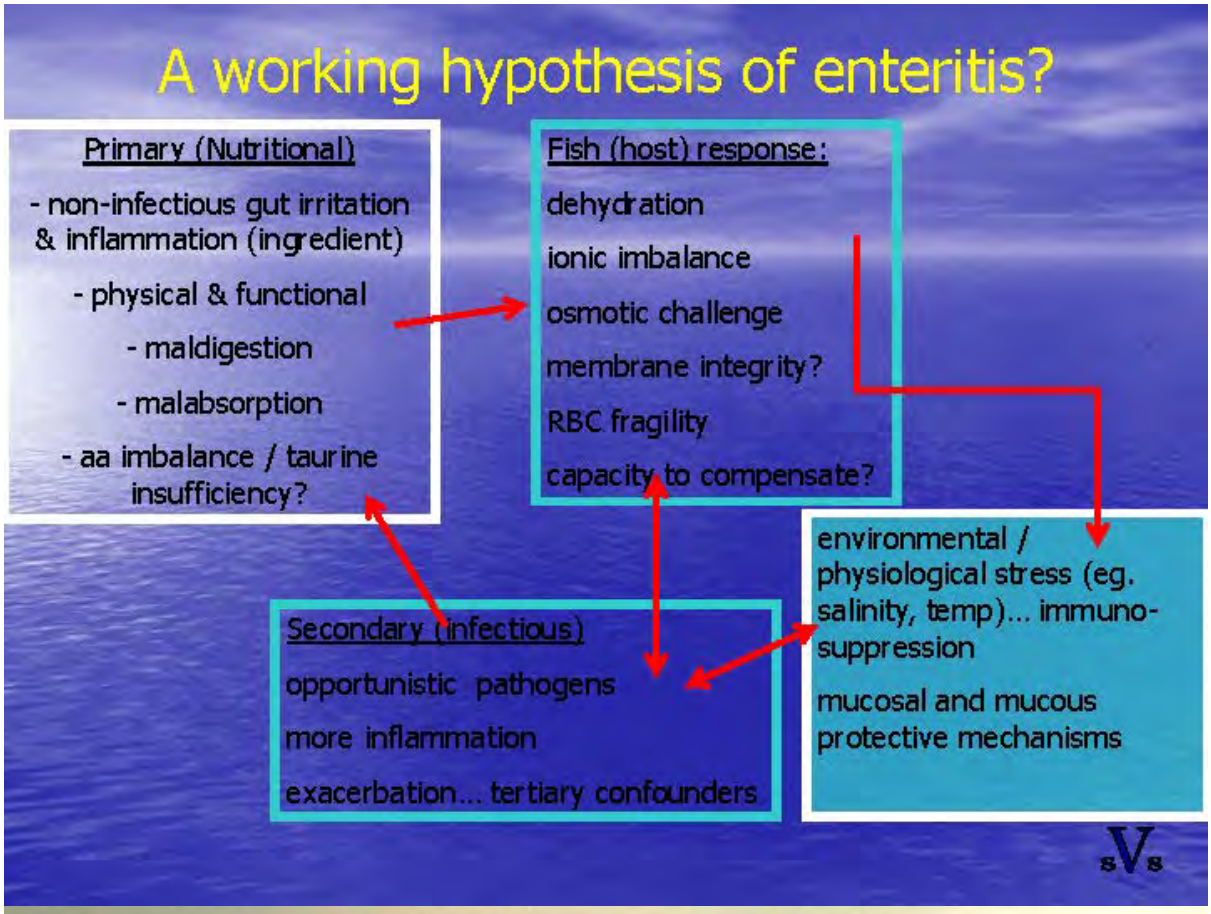
1) Toxin production
2) Adherence to intestinal mucosa
3) Invasion & inflammation of gut wall
4) Disruption of structure or function of mucosal villous cells

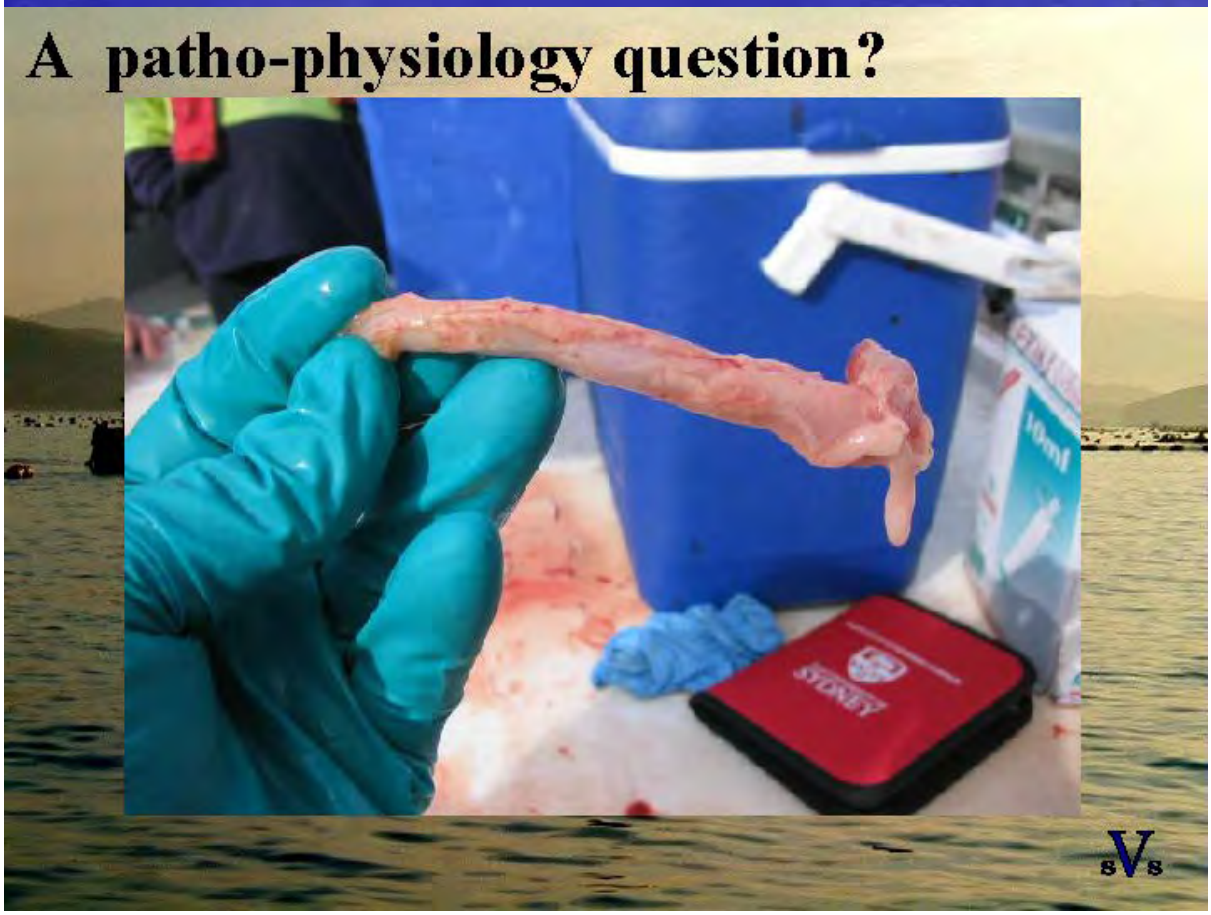
Tx: *"eliminate possible causal or aggravating agents"*
(Gregg & Nassar, 1999)

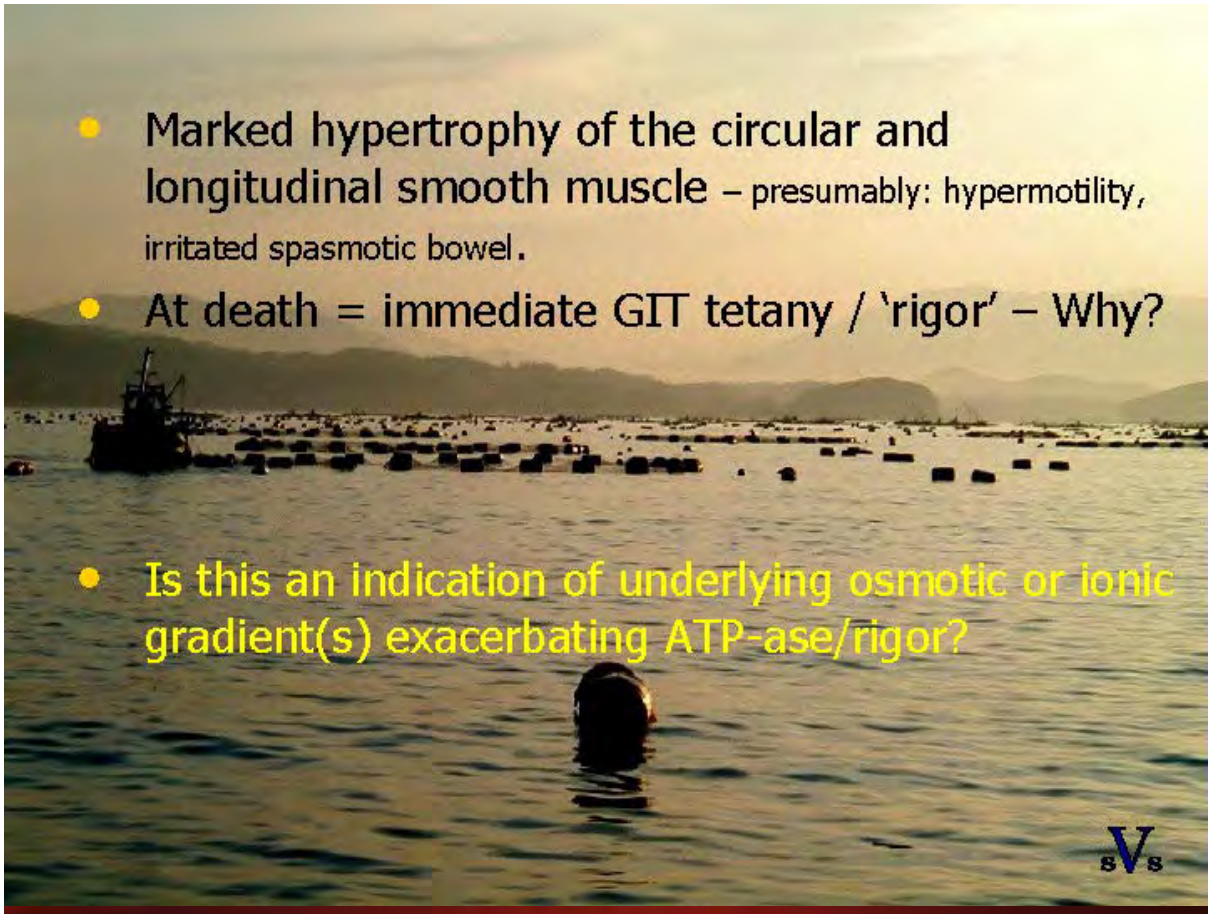
Vs

What do we know about YTK enteritis?

- It's inflammation & it's costly
- Complex & multi-factoral
- Physical & functional pathology
- Progressive, insidious
- Primary cause... exacerbation
- Widespread
- Chronic diarrhoea, dehydration = osmotic imbalance


A working hypothesis of enteritis?
Primary (Nutritional)
- non-infectious gut irritation & inflammation (ingredient)
- physical & functional
- maldigestion
- malabsorption
- aa imbalance / taurine insufficiency?
Fish (host) response:
dehydration
ionic imbalance
osmotic challenge
membrane integrity?
RBC fragility
capacity to compensate?
environmental / physiological stress (eg. salinity, temp)... immuno-suppression
mucosal and mucous protective mechanisms
Secondary (infectious)
opportunistic pathogens
more inflammation
exacerbation... tertiary confounders
sVs


A patho-physiology question?
sVs


Marked hypertrophy of the circular and longitudinal smooth muscle – presumably: hypermotility, irritated spasmotic bowel.
At death = immediate GIT tetany / 'rigor' – Why?
Is this an indication of underlying osmotic or ionic gradient(s) exacerbating ATP-ase/rigor?
sVs


Discussion?
Mark Sheppard, B.Sc, DVM
Sakana Veterinary Service Ltd., Canada
www.sakanavetservice.com svsbook@shaw.ca
sVs

Appendix 9.6b:Yellowtail Kingfish health workshop (Matt Landos)

Australian Seafood CRC Clean Seas Tuna Pty Ltd Yellowtail kingfish health workshop

Dr Matt Landos[1] BVSc(HonsI)MANZCVS (Aquatic Animal Health Chapter)
Matty.landos@gmail.com
Dr Christine Huynh BVSc
Future Fisheries Veterinary Service Pty Ltd

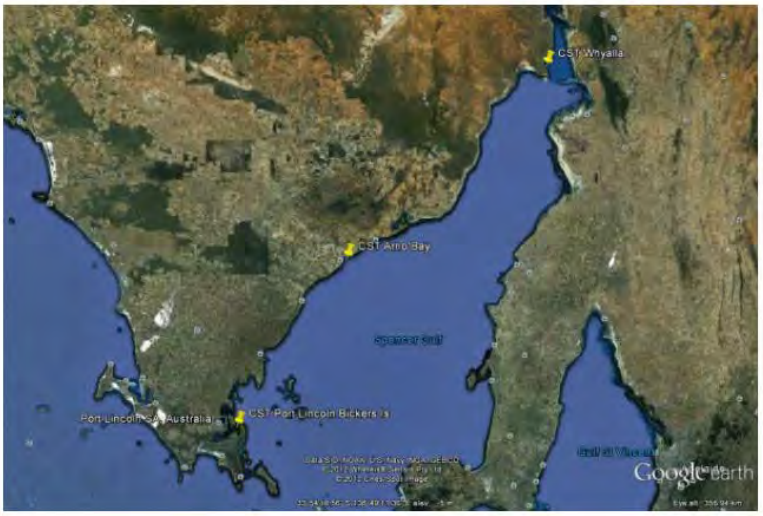
YTK Culture History

- Clean Seas first two crops in 2006 2007 grew rapidly, with <20% mortality from stocking to harvest at 22-30 months of age.
- The company expanded on the strength of the results.
- Both feed companies have supplied various amounts of feed in many different dietary formulations over the years of operation.
- Full disclosure of feed formulation has never been available.

Problem outline

- Poor growth and elevated mortality since at least early 2010.
- Affected year classes of fish: 2009; 2010; 2011; 2012
- Size of affected fish: 30g-3000g
- Sites affected: Whyalla; Arno Bay; Port Lincoln
- The 2011 and 2012 crops have been the worst performing in company history by far- slowest growth, highest mortality
- FFVS commenced investigation in late 2010 following on from Panaquatic

Farm locations



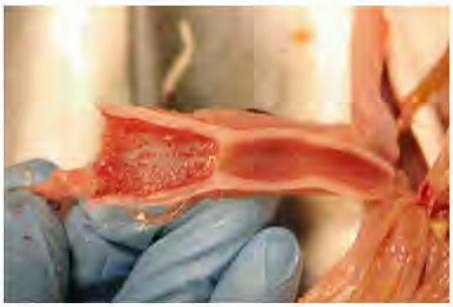
- All- Shallow
- Port Lincoln- mussel and tuna, limited sites
- Arno- exposed to swell/wind
- Whyalla- high salinity, low DO, industrial proximity

Investigation methods

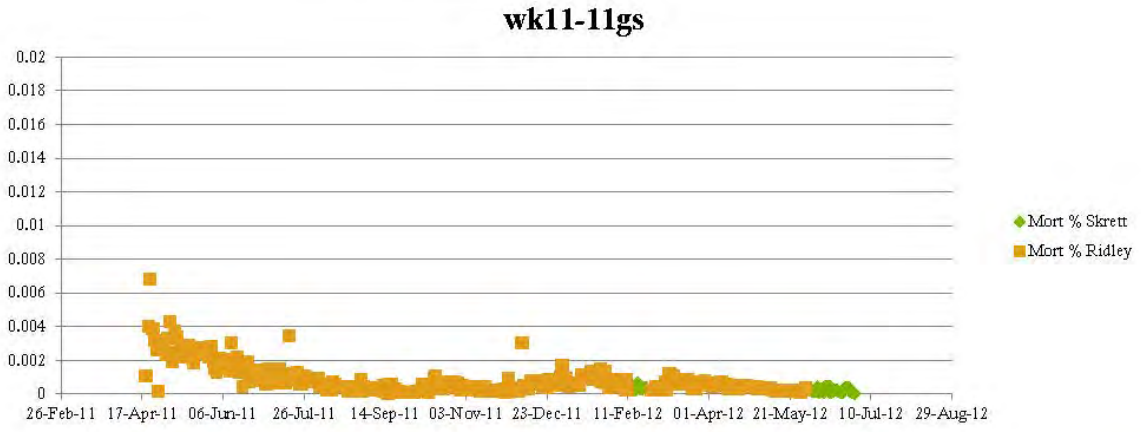
- Clinical signs
- Data analysis of mortalities, water quality, epidemiology
- Gross Pathology- Necropsy
- Microbiology
- Virology
- Clinical trials
- Histopathology (Stephen Pyecroft, Michelle Dennis, Roger Chong, Fran Stephens, John Humphrey)
- Feed analysis
- Clinical pathology
- Collaboration with staff, diagnosticians and national and international researchers

Histological findings- 2011 year class

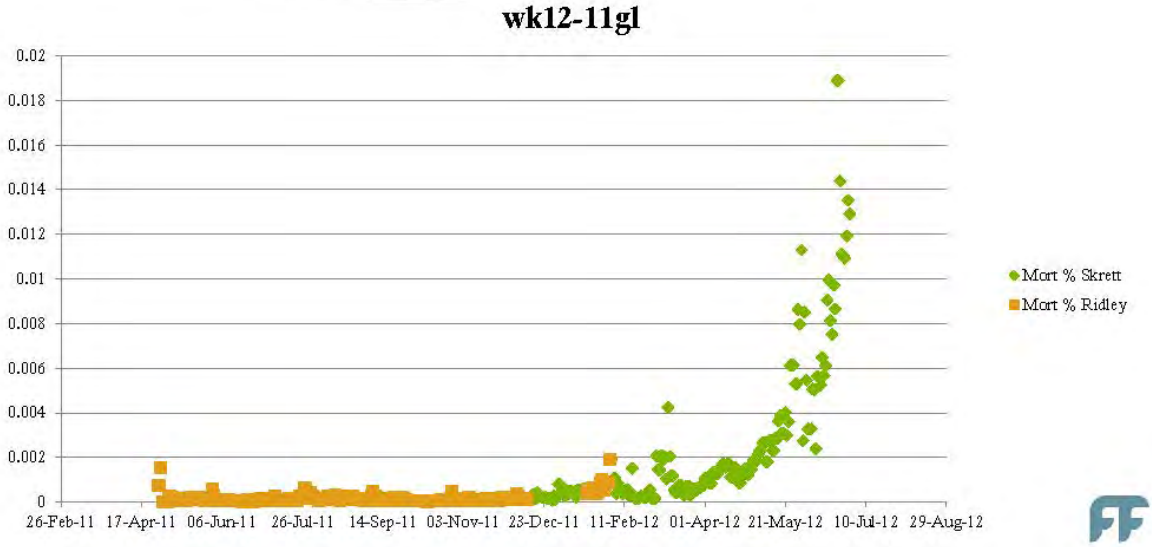
1. Lymphocytic Enteritis commonly associated with coccidia-like and myxidia-like infestations
2. Fluctuating gill and skin fluke infestation
3. Periodic epitheliocystis gill infection
4. Renal myxosporidiosis, tubular proteinosis
5. Photobacterium septicaemia
6. Mycobacteriosis (Whyalla only- resolved after shift to Arno)
7. Disseminated protozoan infection (possibly coccidia/myxidia)
8. Blood fluke (whyalla fish only, not been prominent at Arno)
9. Severe lymphoid depletion
10. Fibrosing pancreatitis
11. Hepatic necrosis



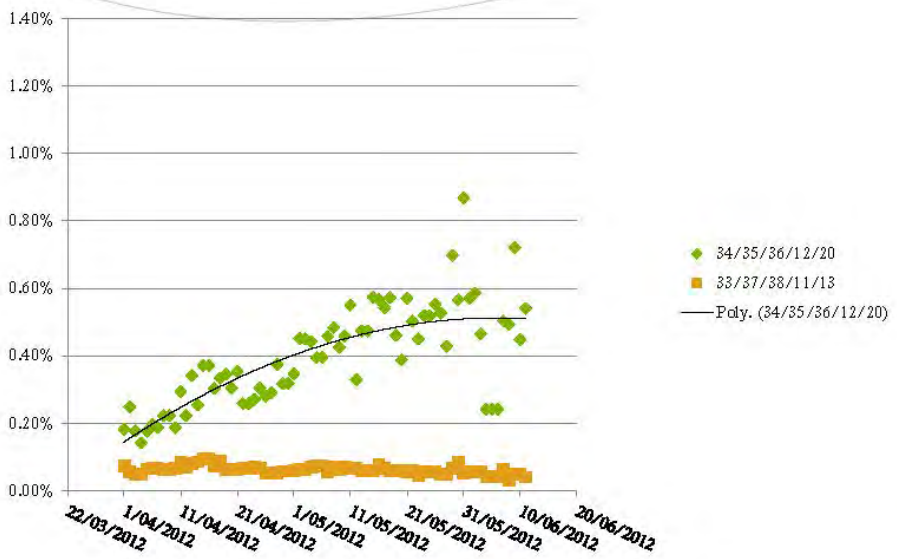
Mortality patterns



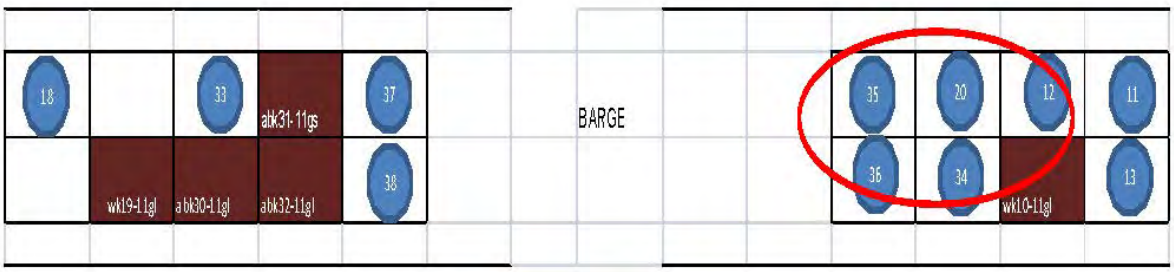
Mortality patterns



Aggregate mortality patterns



Cage locations on Arno grid



Treatment trials

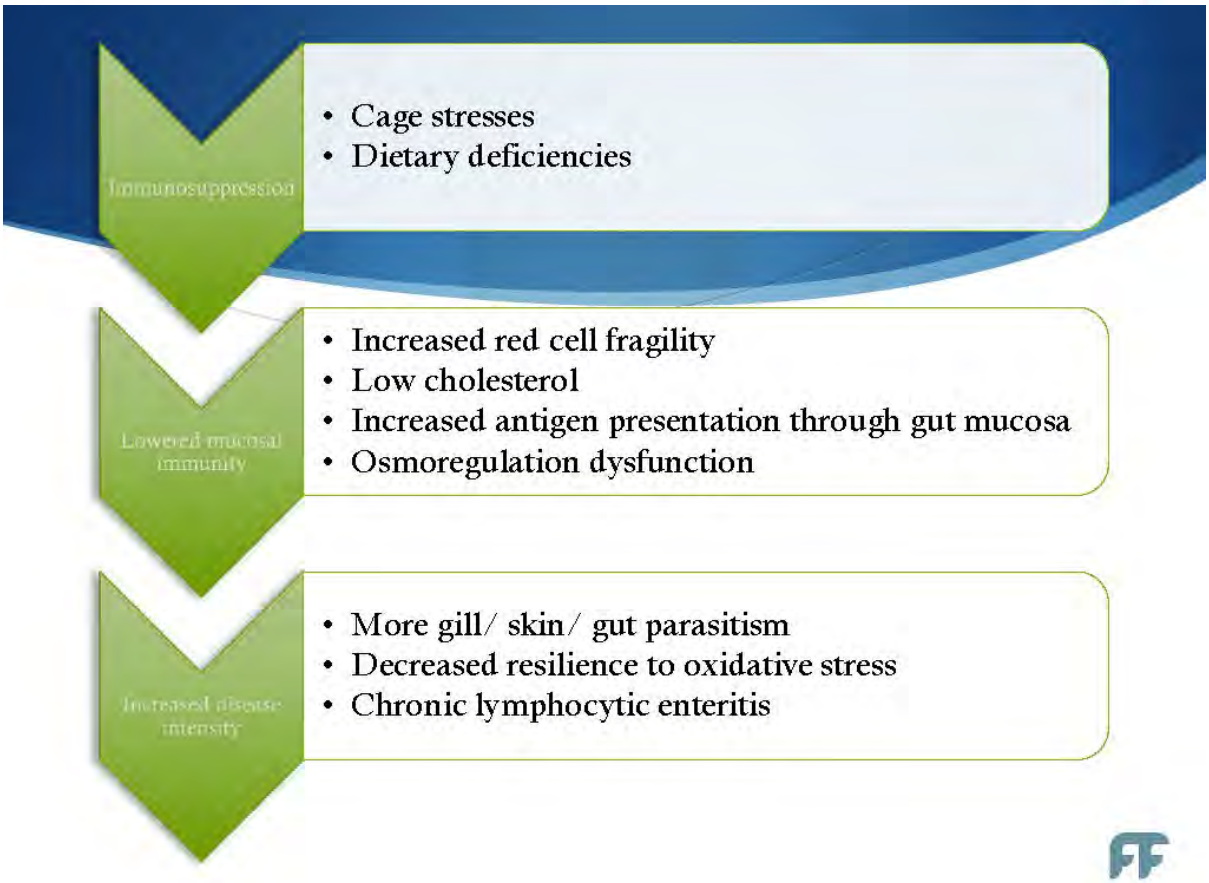
1. Oxytetracycline- controlled Photobacterium sepsis (autogenous vaccine created)
2. Trimethoprim sulphonimide- negligible effect on coccidia
3. Amprolium- negligible response of renal myxosporean
4. Amprolium/salinomycin- possible response in coccidia/myxidia
5. Diet trial: Skretting vs Ridley from mid December 2011 to June 6, 2012- statistically significant difference

Key diet differences-9mm

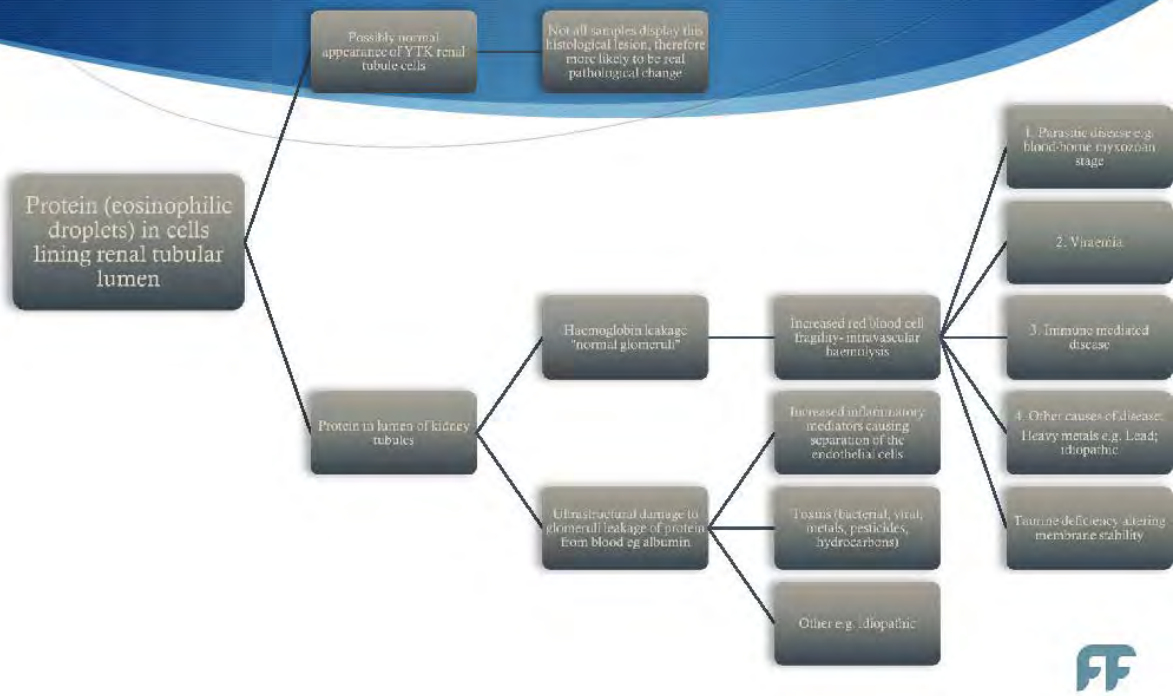
1. Crude protein: Ridley 47%> Skretting 40%
2. Crude lipid: Ridley 19% < Skretting 23%
3. Fish meal inclusion- confidential
4. Fish oil inclusion- confidential
5. Taurine level- to be tested, suspect deficient both diets
6. n3:n6 long chain fatty acid ratio- to be tested, suspect insufficient both diets

Treatment trials

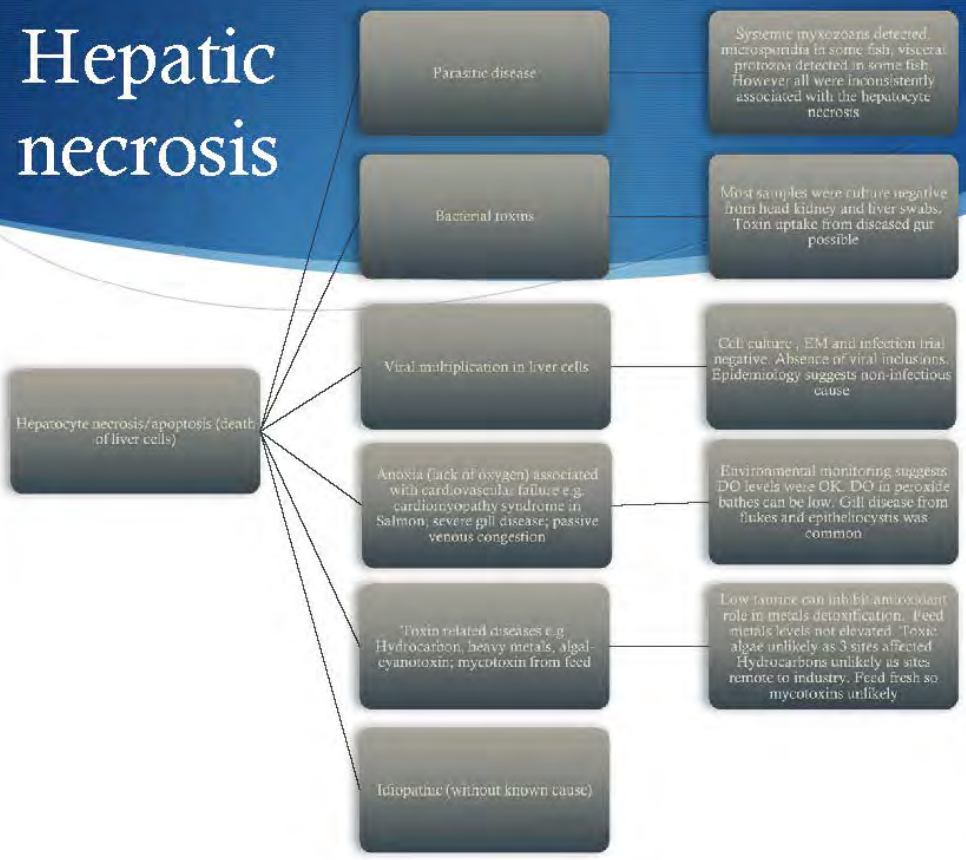
1. Oxytetracycline- controlled Photobacterium sepsis (autogenous vaccine created)
2. Trimethoprim sulphonimide- negligible effect on coccidia
3. Amprolium- negligible response of renal myxosporean
4. Amprolium/salinomycin- possible response in coccidia/myxidia
5. Diet trial: Skretting vs Ridley from mid December 2011 to June 6, 2012- statistically significant difference



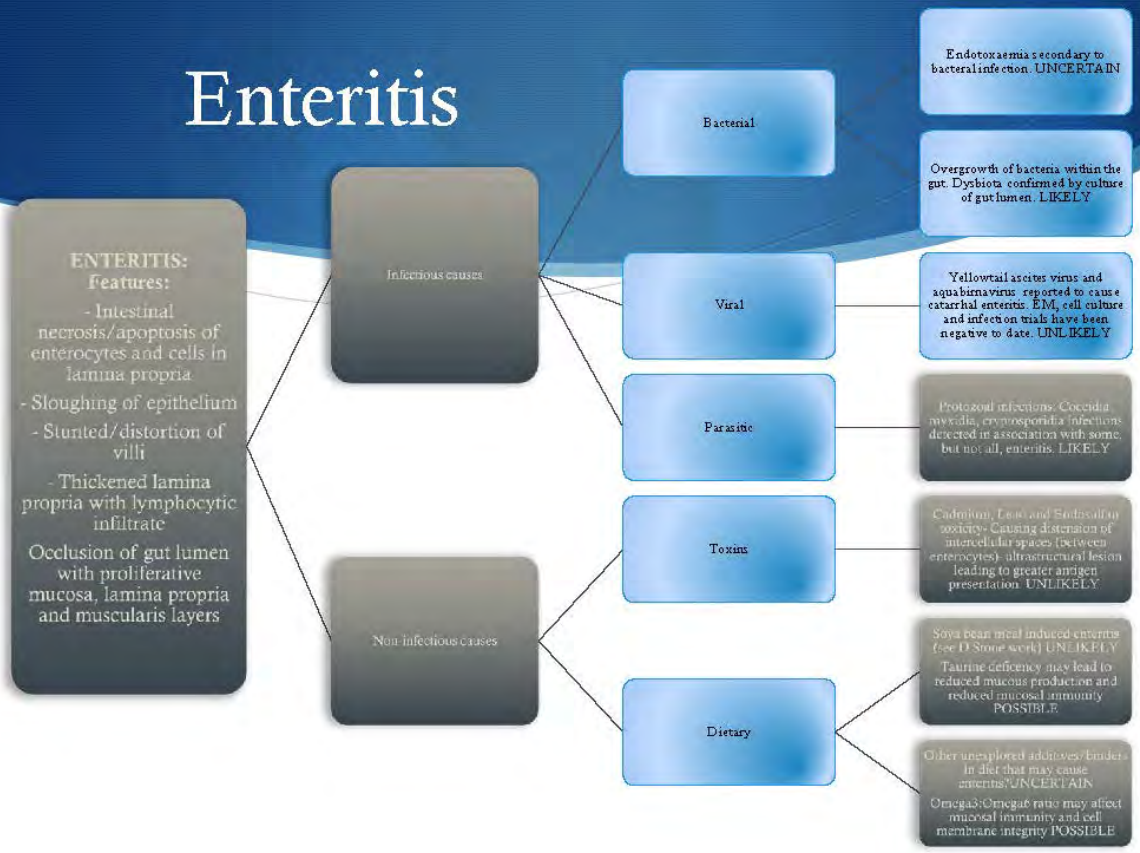
Renal proteinosis



Hepatic necrosis



Enteritis



Evidence of taurine deficiency

- Feed test results
- Confirmed anaemia- inc. MMC's in spleen
- Low blood cholesterol
- Suspect immunosuppression
- YTK cannot make enough endogenously
- Green livers in starved cages 12-11, 11-11
- Liver levels- to be confirmed, with response trial

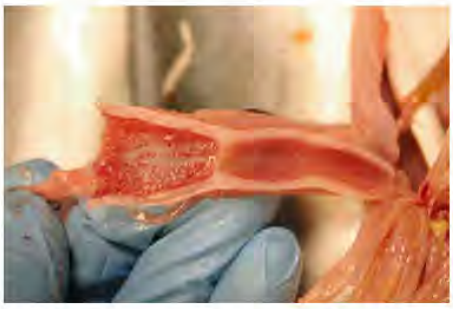
Complicating n3 deficiency

- Feed test results- levels low in some diets (reduced with fish oil decrease
- Low taurine= low bile salt= low lipid digestion/absorption
- Low temperatures decrease pancreatic lipase activity
- High lipid diet drives increased bile salt demand
- Low taurine= low cholesterol= impact on mucous production and cell membranes
- Low n3 uptake further impacts cell membrane stability
- Low taurine= osmoregulatory impairment

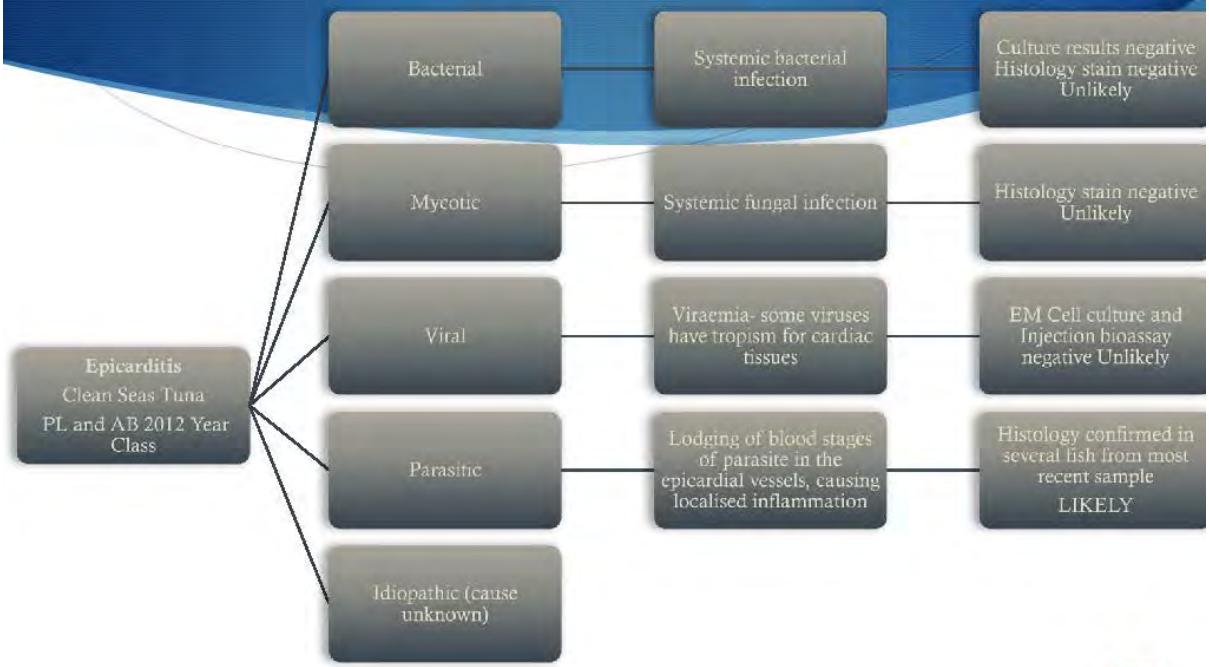
Histological findings- 2012 year class

1. Lymphocytic Enteritis rarely associated with coccidia-like and myxidia-like infestations. Bacteria overgrowth
2. Absence of gill fluke initially
3. Epitheliocystis gill infection
4. Severe renal myxosporidiosis, tubular proteinosis
5. Hepatic necrosis
6. Epicarditis

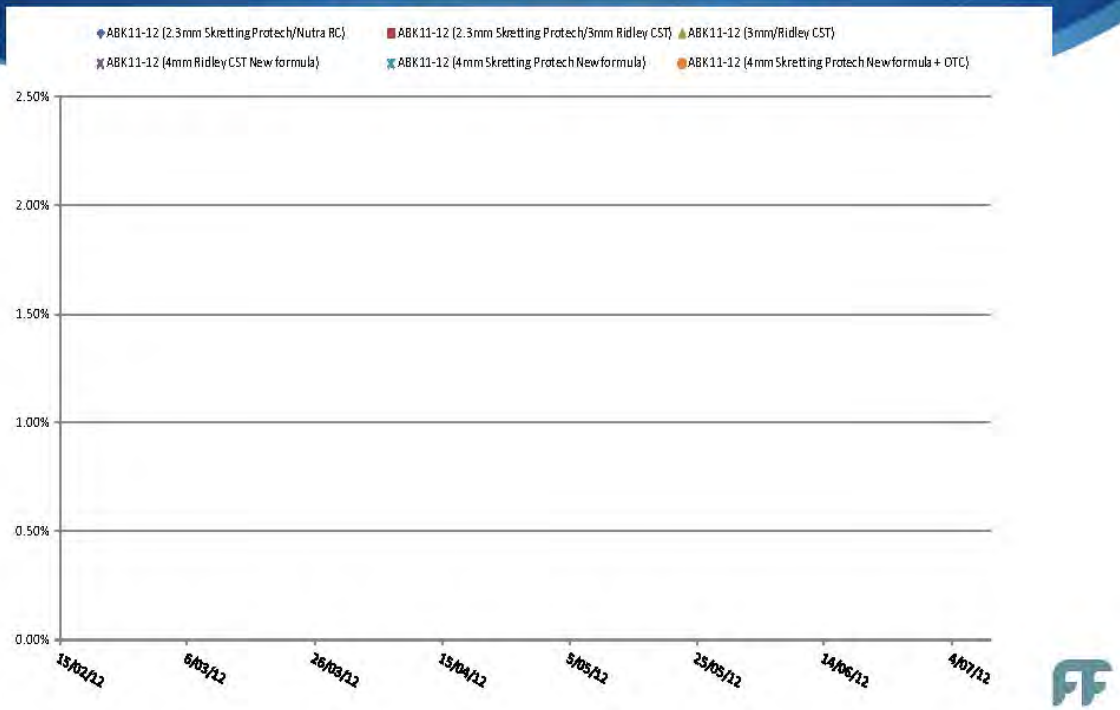
8. Severe lymphoid depletion
9. Splenic erythrophagia/ high MMC's



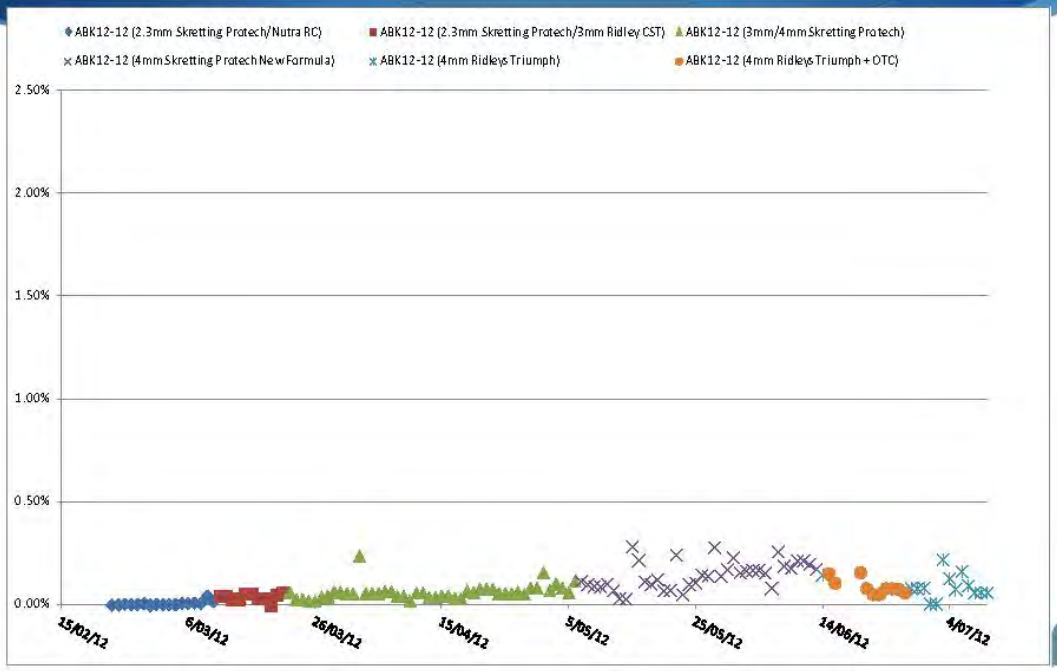
Epicarditis



Port Lincoln 11-12



Port Lincoln 12-12



Treatment trials

1. After high mortalities in Outer Arno 2012 stockings decided to place 2 cages in port lincoln in sheltered site to facilitate feed supply, and minimise exposure to rough sea conditions
2. Cages were next to each other- sampled weekly for histology
3. Both developed full range of pathology lesions
4. Intensity of mortality on Ridley 3mm >> Skretting 3mm
5. Oxytetracycline 100mg/kg on feed
6. Diet change alone on high mortality cage coincident with lowering of mortality

Key diet differences-3mm

1. Crude protein: Ridley 54%> Skretting 50%
2. Crude lipid: Ridley 17% < Skretting 18%
3. Fish meal inclusion- confidential
4. Fish oil inclusion- confidential
5. Other protein/oils confidential- unknown??
6. Taurine level- tested: deficient both diets
7. n3:n6 long chain fatty acid ratio- tested, insufficient in both diets

Diet response

- Diet changes were:
  - Increased fish meal- confidential
  - Increased fish oil- confidential
- Did fish respond to :
  - Increased taurine from extra fish meal?
  - Increased n3 from increased fatty acid supply?
  - Other factors?

Resolving the issues

- Full open formulation
- Raw material QA on each feed run
- Post milling QA to determine losses
- Monitoring uptake in fish
  - Bloods: PCV; MCV; cholesterol; bilirubin
  - Tissue levels of taurine- liver
- Longer feed trials- 5 weeks inadequate
- Do assumptions of other unessential amino acids hold in YTK?
- Sea cage based trials- as cage stressors likely alter (increase) dietary requirements of key amino acids and long chain fatty acids

Gut biota- culture study

- Intestinal swabs cultured on range of media
- Very low biodiversity
- Virtual monocultures of Vibrio scopthalmi
  - Why?
  - Are sanitisers in feed? Disclosure?
  - Do they affect gut microbial population

Questions for discussion

1. What level of fish meal origin taurine is sufficient in diets for fish of different sizes in different water temperatures ?
2. What level of supplementation of crystalline taurine is therefore required ?
3. Is the level of taurine supplementation required altered by other dietary components- eg SPC affecting bioavailability?
4. What is the optimal DP level in diets for YTK of different sizes, in different water temperatures ?
5. What is the optimal DE level in diets for fish of different sizes ?
6. Changes to diets were requested to increase FM/FO inclusion for the Port Lincoln 2012 fish. Why have the analysis results not reflected an increase in n-3 fatty acids and taurine increase, compared to previous formulations?
7. How can we modify n3:n6 ratio cost effectively in diets?
8. Can meat meal be used to boost taurine levels?

Questions for discussion

9. What is the best method for identification of the gut protozoa which have been labelled coccidian and myxidia based on histological appearance?
10. Is it feasible to undertake infection trials with these organisms on fish which are on a range of diets, to explore the role of diet in assisting infection?
11. Are treatment trials for these organisms able to be organised/ warranted and commercially viable?
12. Are feed sanitisers used in diets? If so which diets, and are they capable of modifying gut flora?
13. Can a rapid testing methodology to assess osmoregulatory status/stress be developed?

Alternative explanations?

- Are there any?
- Does the gathered evidence fit them?

Acknowledgements

- Mike Thompson
- Trent D'Antignana
- Damian Critchley
- Tony Barton
- Sam Feige
- Craig Foster
- Pathologists
- Microbiologists
- Parasitologists
- Virologists
- Dr Masashi Maita
- Dr Mark Sheppard

Appendix 9.6c: A Japanese perspective on yellowtail health (Masashi Maita)

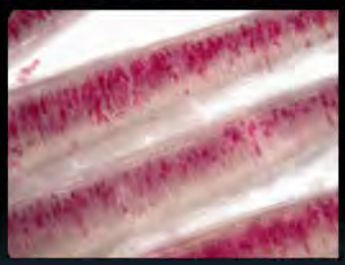
A JAPANESE PERSPECTIVE ON YELLOWTAIL HEALTH

-The problem of Taurine deficiency-

Masashi MAITA, Ph.D
Tokyo University of Marine Science & Technology

Recent mass mortality case in farmed YT kingfish in Japan

Epitheliocystis disease



Fat disease

Fat disease

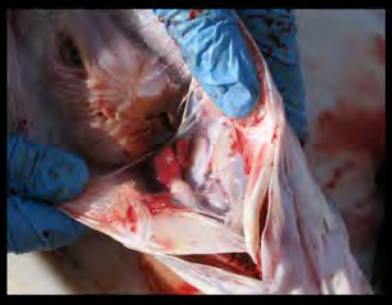
Excess load of dietary fat

Disturbance of circulation by triglyceride drop formed in artery of Gill

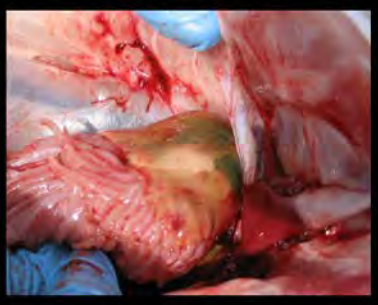
Fish death

Common symptoms of kingfish in PL

Peritoneum adhesion

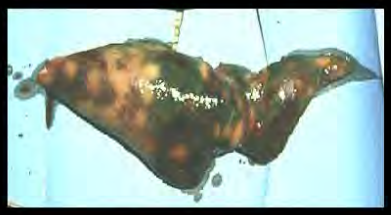


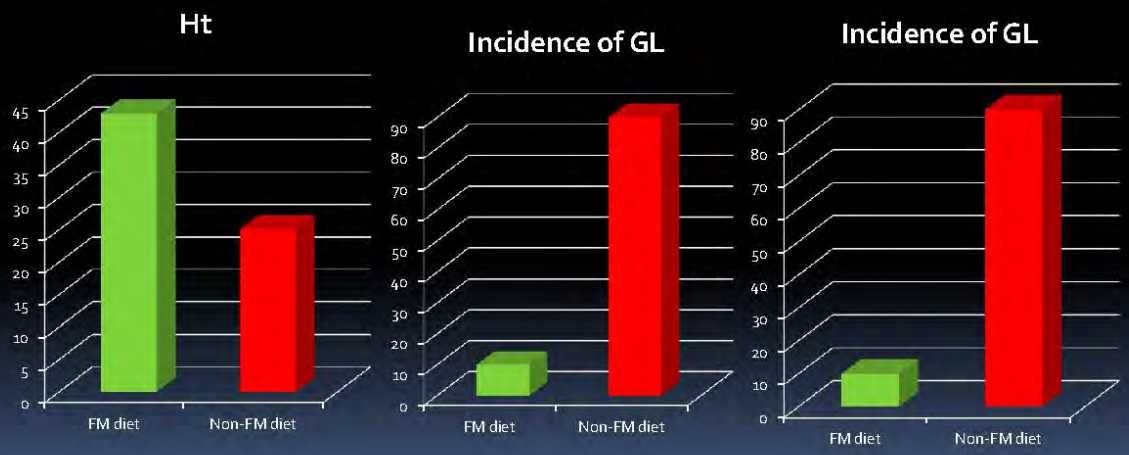
Green liver



High incidence of GL relate to taurine deficiency

Accumulation of visceral fat





Proximate composition* of the experimental diets

	NTS	LTS	HTS	FM
Crude protein	469	487	499	444
Crude fat	137	109	105	141
Crude sugar	200	214	207	165
Crude ash	64	59	57	117
Digestive energy (kcal/kg)	3770	3660	3670	3590
Taurine (g/kg)	0.73	2.50	21.4	4.08

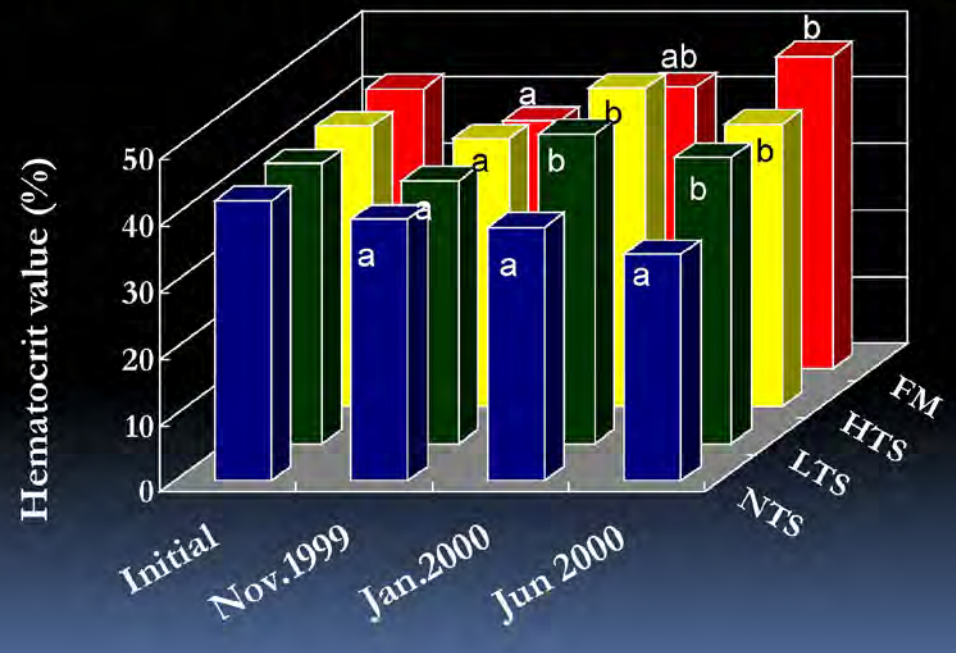
* (g/kg on dry basis)

(Murata et al)

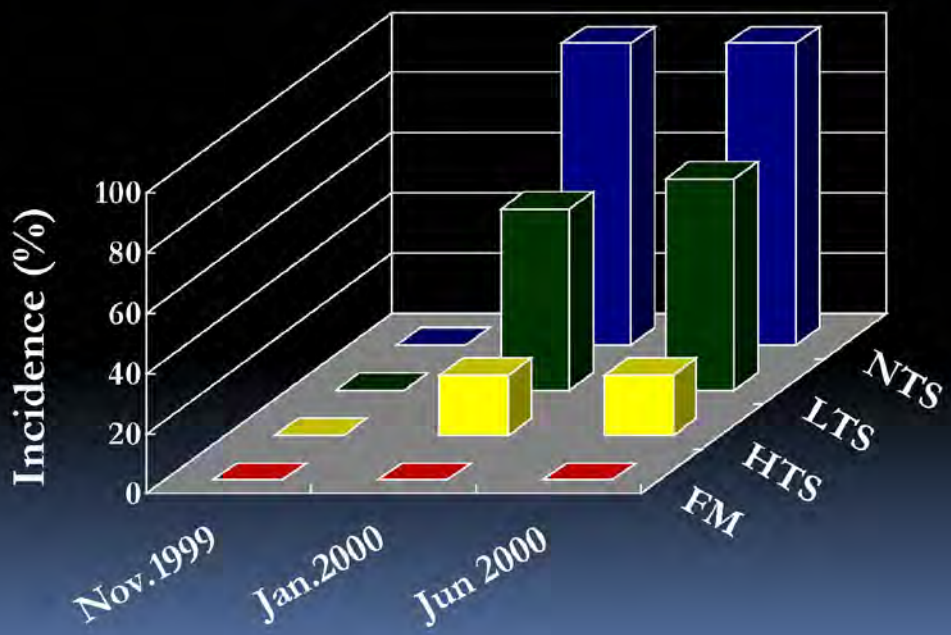
Experimental condition

Fish	Juvenile yellowtail
Average body weight	240 g
Number of fish	95 fish/net cage (3x3x3 m)
Feeding	Satiation 5 days per week
Duration	41 weeks (Sep. 99 - Jun. 00)
Water temperature	12.8-27.4 ℃

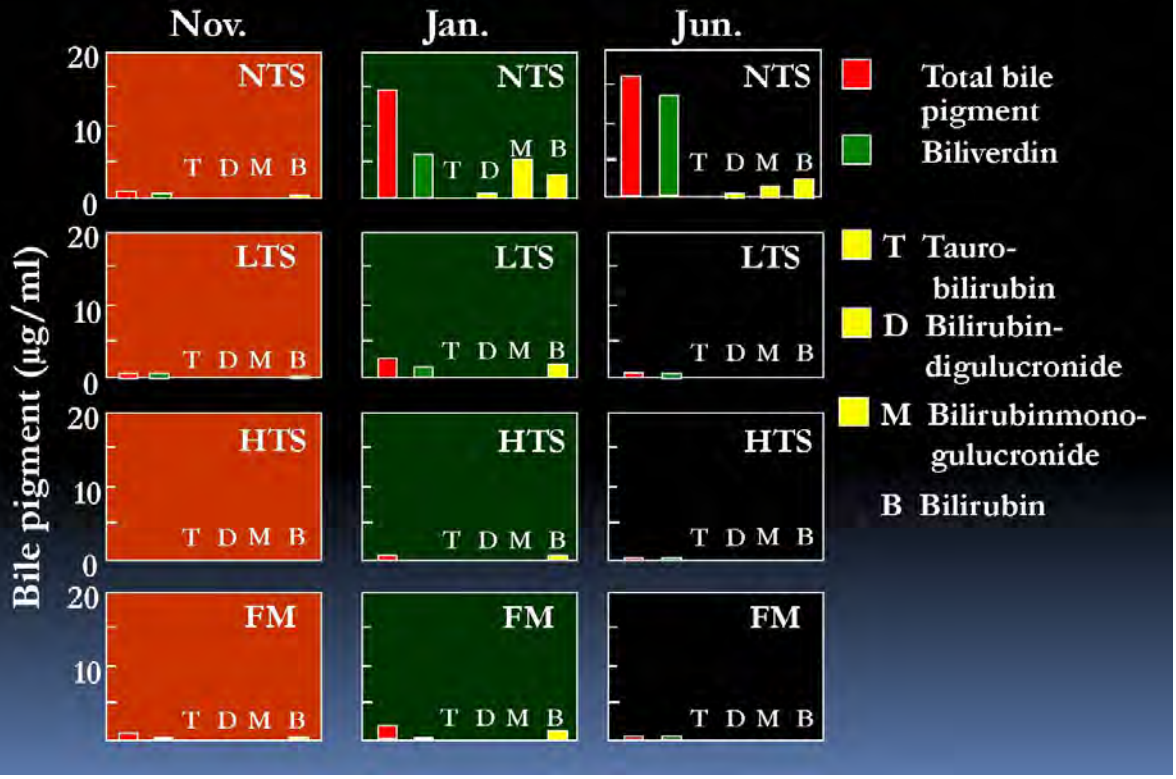
Changes of hematocrit values in yellowtail fed the experimental diets



Incidence of green liver in yellowtail after feeding the experimental diets



Bile pigment contents in the liver of yellowtail



The chain of events leading to the appearance of green liver in yellowtail fed non-FM diet may be as follows:

(1) Bilirubin cannot be excreted adequately into the bile

(2) Lack of taurine leads to greater hemolysis

(3) These events result in biliverdin accumulation in liver causing the green color

(4) Inadequate taurine biosynthesis by the fish

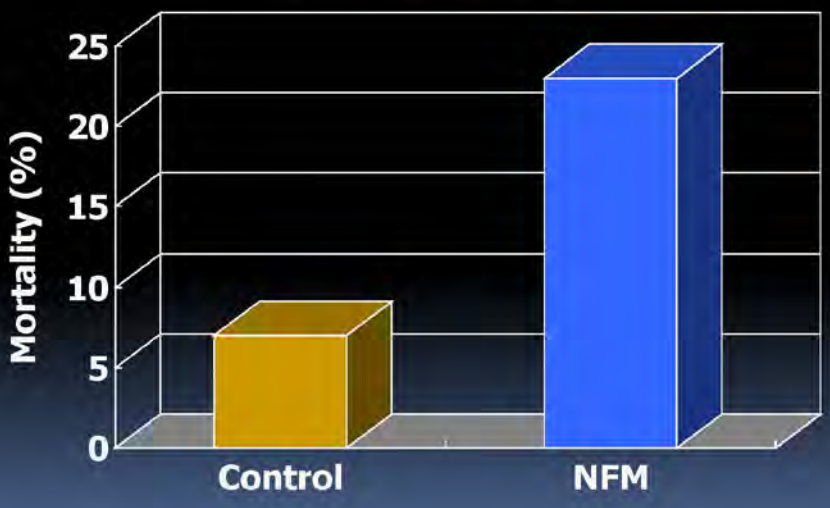
Hematological parameters in yellowtail fed non-fish meal diet (30 days of feeding)

		Control	Experimental
Hematocrit value	(%)	37.8±2.5	24.7±2.9**
Total cholesterol	(mg/dl)	245±35	143±25**
Phospholipid	(mg/dl)	593±79	354±56**
Triglyceride	(mg/dl)	124±33	120±97
Urea nitrogen	(mg/dl)	18.8±3.2	11.1±3.7**
Glucose	(mg/dl)	163±37	162±33
Total protein	(g/dl)	3.0±0.3	2.9±0.2

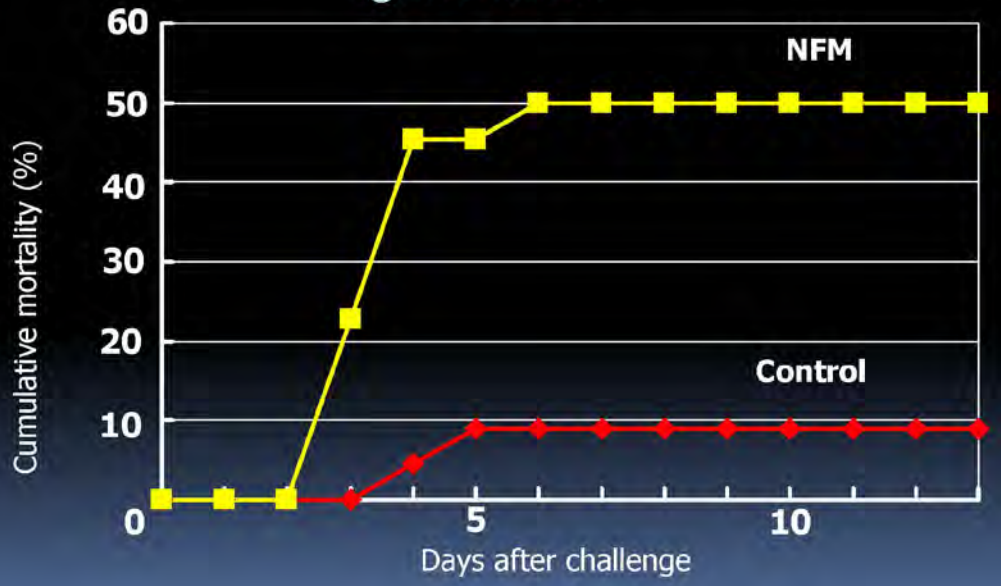
Hematological parameters in yellowtail fed non-fish meal diet (60 days of feeding)

		Control	Experimental
Hematocrit value	(%)	43.3±2.8	34.5±2.9**
Total cholesterol	(mg/dl)	280±10	140±23**
Phospholipid	(mg/dl)	552±20	447±41**
Triglyceride	(mg/dl)	145±19	138±32
Urea nitrogen	(mg/dl)	21.6±1.9	27.8±21.7**
Glucose	(mg/dl)	132±13	131± 12
Total protein	(g/dl)	2.9±0.2	2.7±0.2

Natural mortality among yellowtail caused by an infection with *P. piscicida*



Mortality of yellowtail resulting from artificial infection with *L. garvieae*



Changes in the condition of yellowtail fed on non-fish meal diet

- Fish appeared healthy (active feeding)

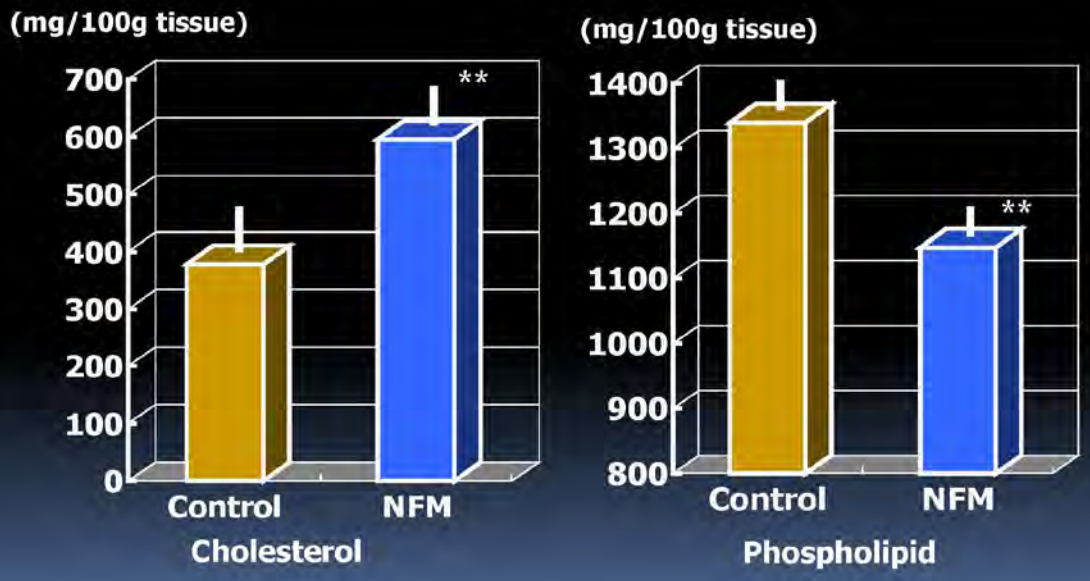
	30 days	60day
•Ht value	↓	↓
•TC/PL	↓	↓
•Urea nitrogen	↓	↑
•Disease resistance	↓	↓

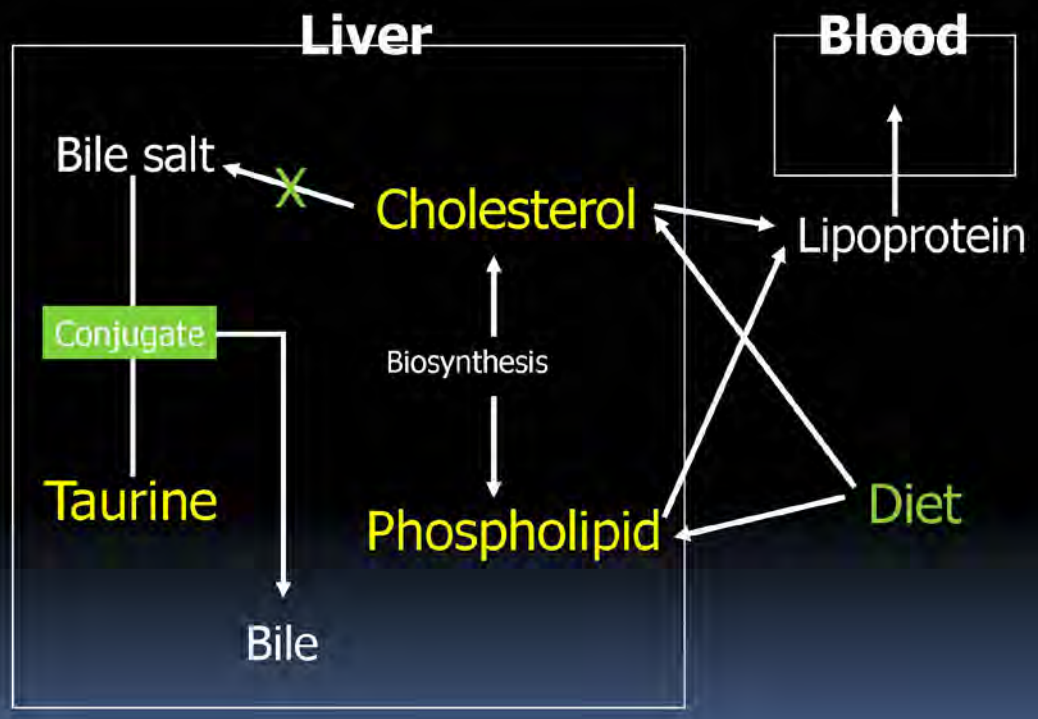
Derangement of lipid metabolism

Anemia

Disease resistance

Lipid contents in liver of yellowtail (60 days of feeding)



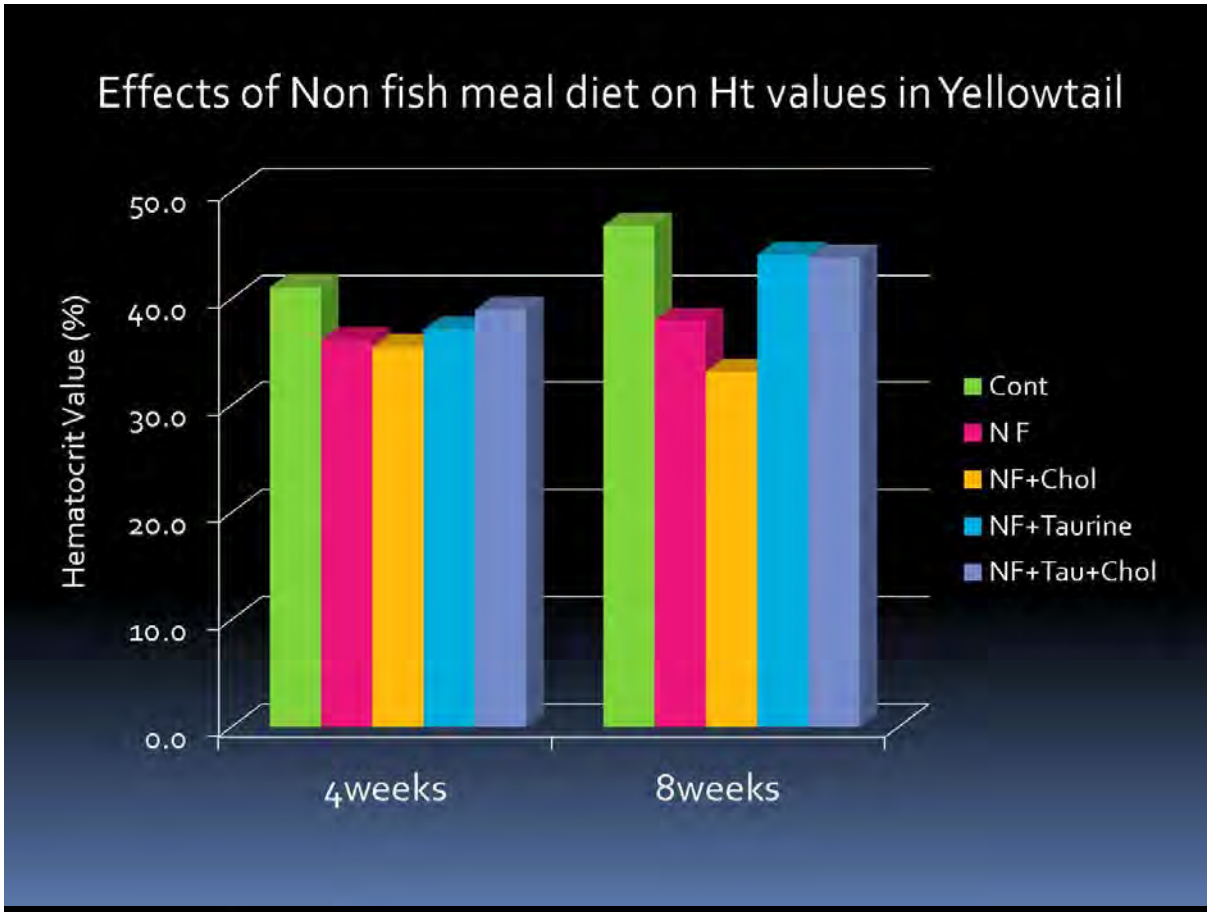


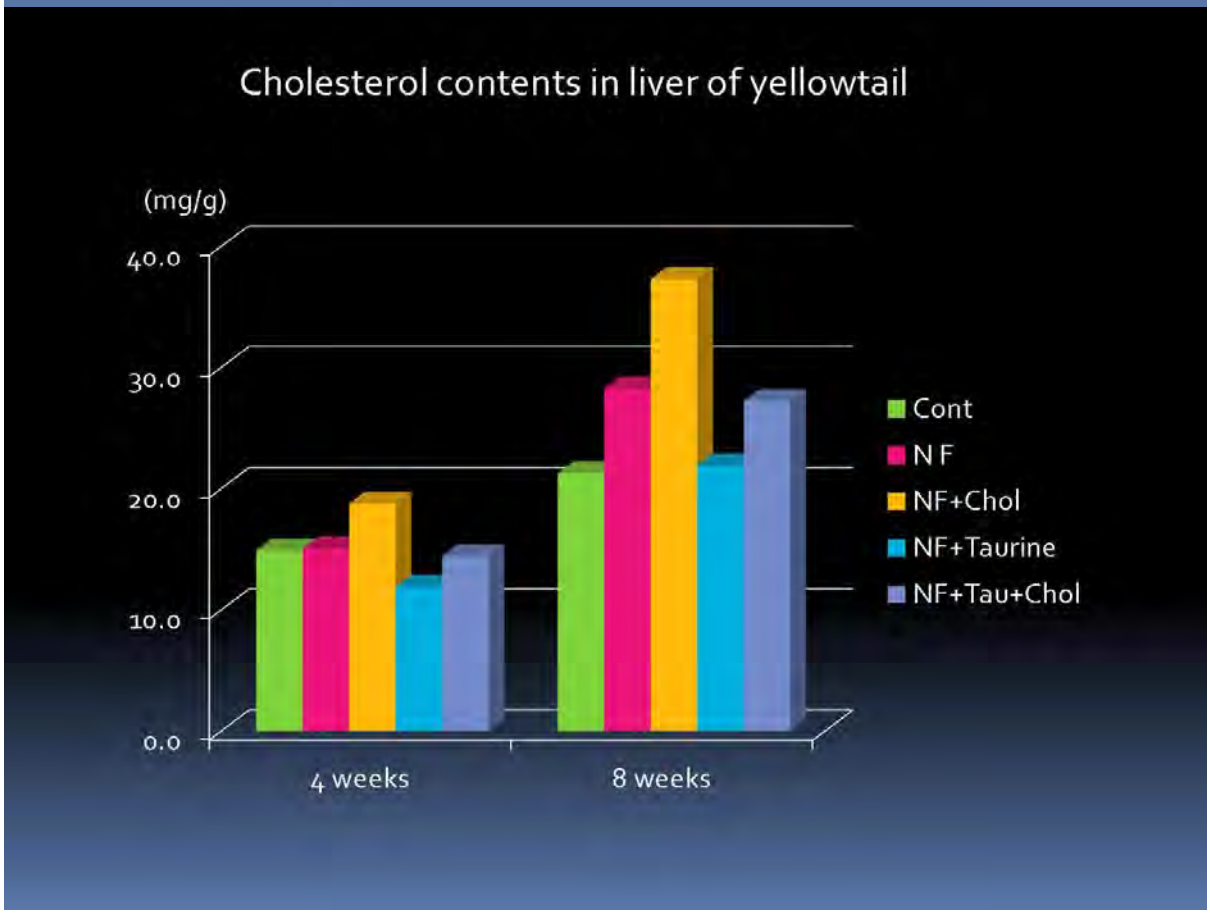
Proximate composition of the experimental diets

	Cont	N F	NF+Chol	NF+Taurine	NF+Tau+Chol
Moisture (%)	6	6	6	6	6
Crude Protein (%)	45	43	45	44	45
Crude fat (%)	23	24	22	23	23
Crude ash (%)	9	8	9	8	8
Cholesterol (mg/kg)	2850	1800	10700	1490	8600
Taurine (g/kg)	3.216	070.7	1.164	7.97	7.62

Formulation of the experimental diets

(g/kg)	Cont	N F	NF+Chol	NF+Taurine	NF+Tau+Chol
Fish meal	480	0	0	0	0
SPC	0	200	200	200	200
SBM	90	150	150	150	150
CGM	0	100	100	100	100
Wheat flour	110	54	54	44	44
Taurine	0	0	0	10	10
High chol fish oil	0	0	140	0	140
Fish oil	150	170	30	170	30
Others	170	326	326	326	326


Effects of Non fish meal diet on Ht values in Yellowtail
Hematocrit Value (%)
50.0
40.0
30.0
20.0
10.0
0.0
4weeks
8weeks
Cont
N F
NF+Chol
NF+Taurine
NF+Tau+Chol


Cholesterol contents in liver of yellowtail
(mg/g)
40.0
30.0
20.0
10.0
0.0
4 weeks
8 weeks
Cont
N F
NF+Chol
NF+Taurine
NF+Tau+Chol

Mortality of yellowtail resulting from artificial infection with *L. garvieae*

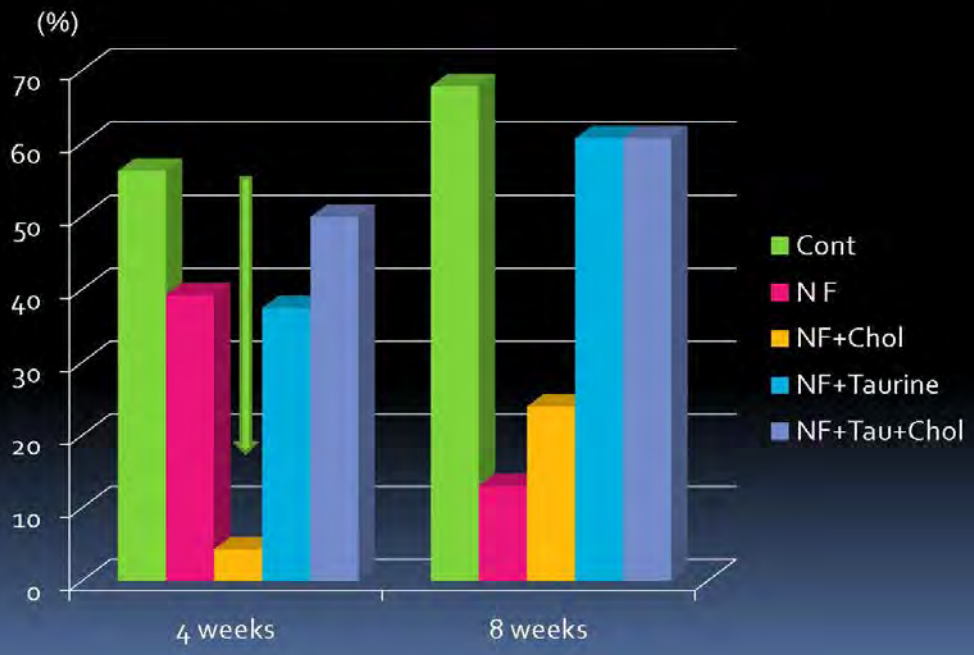
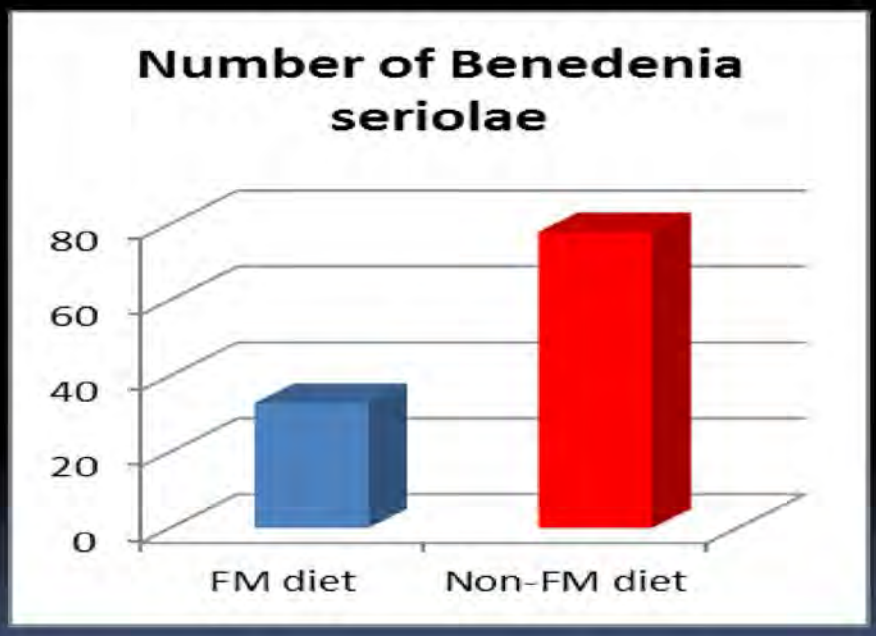


Table3. Mortality in the first day of the challenge test.

	F	N	C*	T	CT
Dead	0	4	9	0	0
Alive	32	28	23	32	32

* significant difference subjected by Fisher's exact test($p<0.01$) from F, T and CT. There is no significant difference between C and N, and also F and N.



Critical level of fishmeal in YT diet?

Table 1. Composition of the experimental low or non-fish meal diets for yellowtail and red sea bream

Ingredient(%)	Diet no. 1	2	3	4	5	6
Fish meal	65	40	30	20	10	0
Krill meal	0	0	0	0	0	10
Defatted soybean meal	5	8	9	10	10	10
Corn gluten meal	0	10	10	10	10	10
Meat meal	0	10	10	10	10	10
Meat and bone meal	0	0	10	8	10	9
Poultry feather meal	0	0	0	4	8	8
Blood meal	0	0	0	5	8	8
Wheat flour	10	9	8	8	8	8
Mineral mixture	5	5	5	5	5	5
Vitamin mixture	4	2	2	2	2	2
Feed oil	11	16	16	18	19	20
Nutrient contents determined : As is basis(%)						
Crude protein	47.2	47.4	47.9	46.8	46.7	45.1
Crude lipid	23.7	19.5	19.8	19.1	18.4	20.5
Crude ash	9.0	11.3	11.6	11.0	11.1	10.7
Moisture	5.1	8.2	7.0	7.0	5.5	6.5
Gross energy (kcal / g)	5.6	5.0	5.1	5.1	5.1	5.2
Dry matter basis(%)						
Crude protein	49.7	51.6	51.5	50.3	49.4	48.2
Crude lipid	25.0	21.2	21.3	20.5	19.5	21.9
Crude ash	9.5	12.3	12.5	11.8	11.7	11.4

Table 3. Growth and feed performances of yellowtail fed the experimental low or non-fish meal diets

Diet no.	Av.body wt. (g) Initial	Av.body wt. (g) Final	Growth rate (%)	Feed gain ratio*1	Daily feed intake*2	Protein efficiency ratio*3	Mortality (%)
Expt. I (Mie prefecture)							
1 FM diet	142.9	598.6	318.9	1.59	2.71	1.33	8.0
2 40% FM	142.9	546.8	282.6	1.81	2.98	1.17	2.3
3 30% FM	141.4	513.2	262.9	1.94	3.06	1.08	4.0
4 20% FM	142.6	464.0	225.4	2.20	3.23	0.97	8.0
5 10% FM	142.0	355.0	150.0	2.72	3.27	0.79	7.7
6 0% FM	141.4	402.6	184.7	2.34	3.09	0.95	10.3
Expt. II (Oita prefecture)							
1 FM diet	102.0	621.6	509.4	1.64	2.36	1.29	6.0
2 40% FM	106.5	589.1	453.1	2.07	2.60	1.02	5.4
3 30% FM	107.0	590.2	451.6	1.95	2.69	1.07	16.7
4 20% FM	106.6	527.9	395.2	2.21	2.90	0.97	20.7
5 10% FM	104.9	342.6	226.6	3.10	3.17	0.69	66.0
6 0% FM	103.2	336.3	225.9	3.49	3.42	0.64	79.3

*1 g feed / g gain.
*2 g/100g body wt.
*3 g gain / g protein intake.

- ✓Mortality was caused by Pseudotuberculosis.
- ✓Effects of non-fish meal diet was different depend on the rearing condition.

Table 5. Results of hemochemical examination in yellowtail fed the experimental low or non-fish meal diets *1

		Diet no. 1	2	3	4	5	6
Expt. I							
Ht	(%)	41.0±4.9^{a}	40.1±1.6^{a}	44.4±2.8^{a}	39.1±2.5^{ab}	32.7±4.4^{b}	33.7±4.8^{b}
ALP	(IU / l)	153±22^{b}	137±12^{b}	172±15^{ab}	162±36^{b}	212±37^{a}	143±19^{b}
GLU	(mg / 100ml)	120±5	116±12	123±19	117±14	126±26	117±23
TG	(mg / 100ml)	196±54	126±26	182±24	146±26	167±57	150±19
PL	(mg / 100ml)	847±45^{a}	751±23^{b}	859±34^{a}	695±64^{bc}	621±81^{c}	575±91^{c}
TCHO	(mg / 100ml)	292±22^{a}	285±6^{a}	308±11^{a}	251±24^{b}	202±23^{c}	202±32^{c}
FCHO	(mg / 100ml)	123±6^{ab}	111±4^{b}	127±3^{a}	101±10^{bc}	92±147^{c}	85±11^{c}
Ester ratio	(%)	57.7±3.0^{a}	61.2±2.1^{a}	58.9±1.0^{ab}	59.8±0.9^{ab}	54.6±2.1^{b}	57.4±2.3^{b}
BUN	(mg / 100ml)	18.2±2.0^{ab}	20.9±3.1^{a}	20.4 ±1.8^{ab}	19.9±2.8^{ab}	16.2±2.6^{b}	12.2±4.2^{b}
CRE	(mg / 100ml)	1.3±0.3^{ab}	1.1±0.1^{ab}	1.0 ±0.1^{b}	1.3±0.3^{a}	1.0±0.1^{b}	0.9±0.1^{b}
TP	(g / 100ml)	3.5±0.2	3.6±0.1	3. 8±0.1	3.5±0.3	3.6±0.4	3.3±0.4
Condition factor		16.9±1.3^{a}	16.2±0.4^{a}	16.1±0.6^{a}	15.3±0.5^{b}	13.9±0.3^{c}	13.7±0.6^{c}
HSI*2		1.48±0.24	1.39±0.07	1.41±0.15	1.48±0.15	1.39±0.22	1.42±0.22
Expt. II							
Ht	(%)	41.9±1.8^{a}	42.3±2.0^{a}	41.4±1.9^{a}	39.9±2.5^{a}	30.1±5.8^{b}	24.0±5.2^{c}
ALP	(IU / l)	152±24	153 ±26	134 ±23	144±25	141±16	141±36
GLU	(mg / 100ml)	87±5^{b}	88 ±4^{b}	99±6^{ab}	92±8^{b}	102±10^{a}	97±5^{ab}
TG	(mg / 100ml)	200±36^{a}	184±62^{ab}	139±58^{bc}	138±35^{bc}	111±31^{c}	111±21^{c}
PL	(mg / 100ml)	769±64^{a}	713±51^{ab}	711±60^{ab}	631±102^{b}	502±77^{c}	513±83^{c}
TCHO	(mg / 100ml)	301±25^{a}	271±20^{ab}	306±74^{a}	202±41^{b}	171±19^{c}	165±15^{c}
FCHO	(mg / 100ml)	124±17^{a}	106±9^{b}	112±11^{ab}	98±24^{bc}	75±10^{c}	81±5^{c}
Ester ratio	(%)	59.1±2.2^{a}	61.0±2.4^{a}	61.4±9.2^{a}	55.9±3.7^{ab}	56.2±1.7^{ab}	50.8±2.1^{b}
BUN	(mg / 100ml)	11.6±1.8^{b}	13.3±0.7^{b}	15.4±2.8^{ab}	16.1±2.3^{a}	15.5±1.9^{ab}	16.2±1.6^{a}
CRE	(mg / 100ml)	1.2±0.4^{a}	0.8±0.1^{b}	0.8±0.0^{b}	0.9±0.1^{b}	0.8±0.0^{b}	0.9±0.1^{b}
TP	(g / 100ml)	3.1±0.1^{ab}	3.3±0.1^{a}	3.2±0.2^{a}	3.3±0.3^{a}	3.1±0.2^{a}	2.8±0.1^{b}

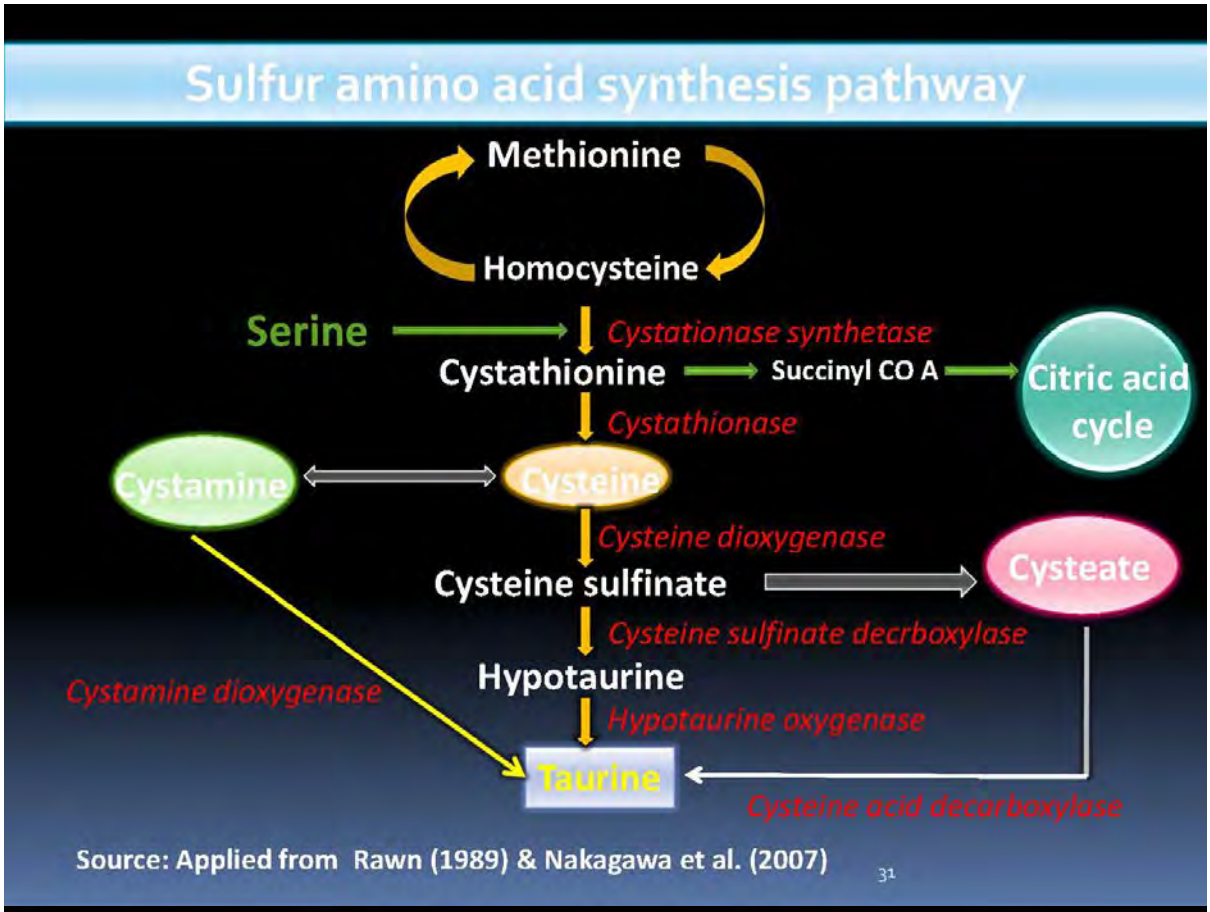
*1 Mean ± standard deviation (n=5). Figures in a row with different superscripts are significantly different from each other ($p < 0.05$) when analyzed using Duncan's multiple range test.
*2 Hepatosomatic index.

- Critical level of fish meal in YT diet would be 30-40 %.
- Less than 30 % of fish meal diet may cause the decline of disease resistance.
- Fish were not killed by Taurine deficiency itself but cause higher mortality due to infection with secondary pathogens.
- Fish fed non-FM diet (Taurine deficient) showed low resistance to handling stress.

Mortality and plasma constituent levels of striped jack fed various quality of moist pellet

Crud fat	Contorl(13 %)	19 %	23 %
Mortality (%)[1]	0	2.4	2.4
Mortality (%)[2]	25	42	60
TC (mg/dl)	248±29	238±19	225±25
TG (mg/dl)	197±32	212±54	192±40
ALP (IU/L)	122±62	192±73*	183±59*
TP (g/dl)	3.5±0.4	3.4±0.2	3.4±0.1
GLU (mg/dl)	79± 8	84± 9	87±12
BUN (mg/dl)	6.0±1.7	5.5±1.4	5.0±0.9

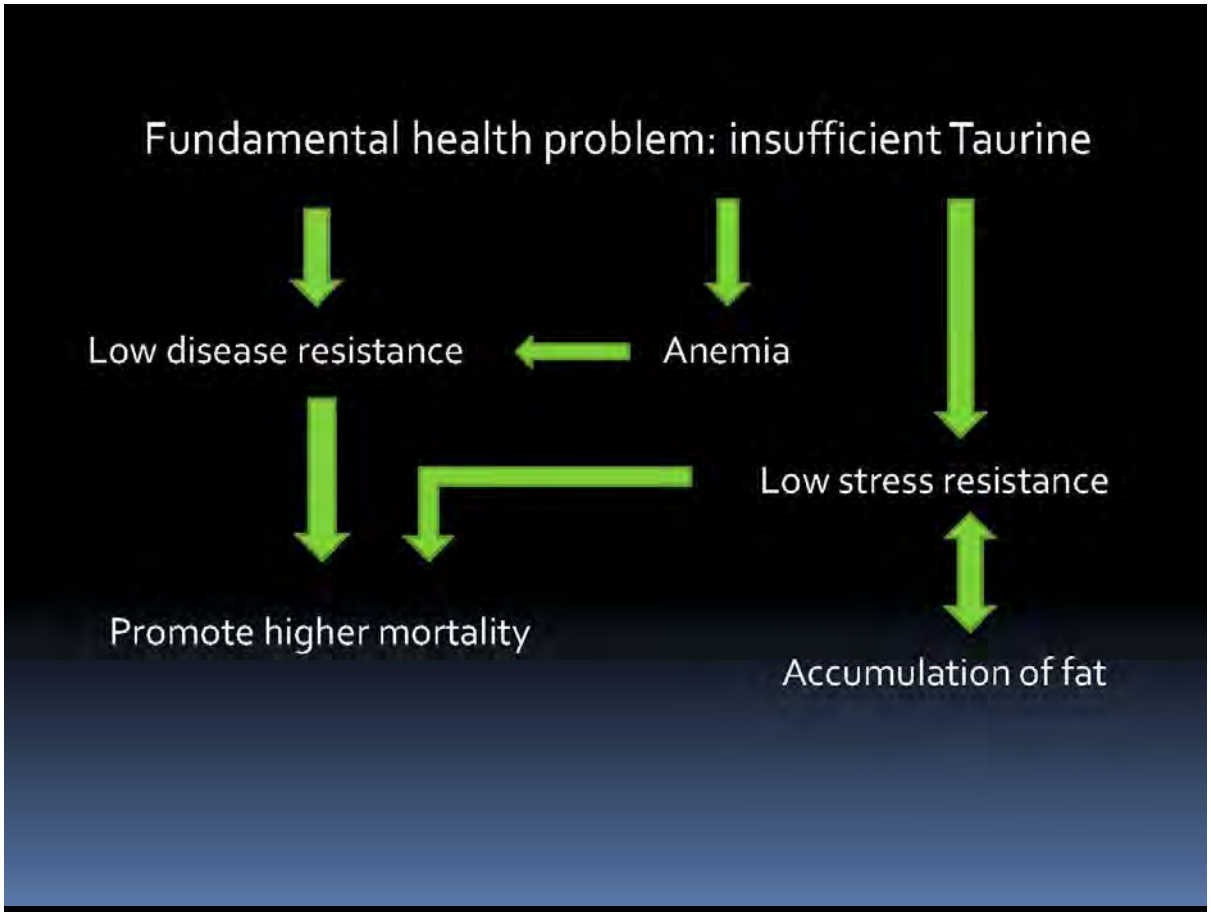
1. Natural infection with *L.garviae* 2. Artificial infection with *L.garviae*



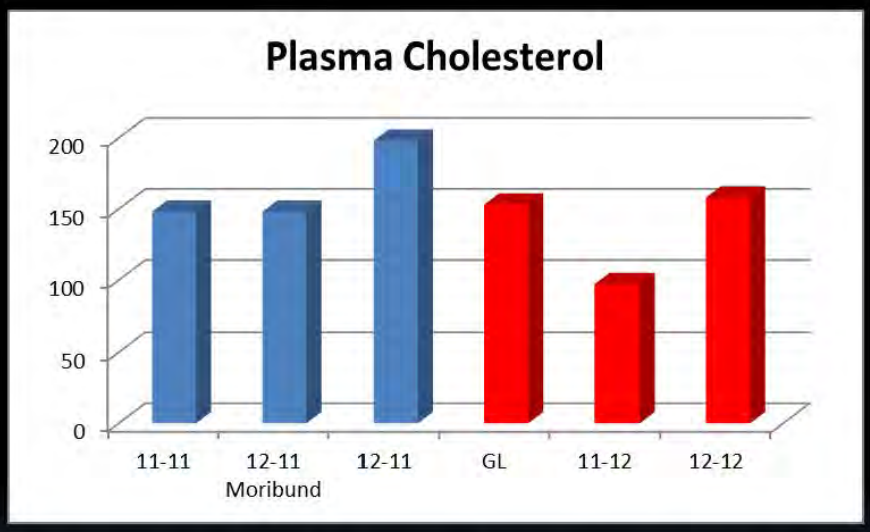
Ability of taurine biosynthesis in YT liver

	Biosynthesizing enzyme activity (nmol/min/mg protein)[1]			
Diet group →	NTS	LTS	HTS	F M
CSD [2]	tr [4]	0.05±0.11	tr	0.05±0.07
CAD [3]	0.20±0.07^{ab}	0.12±0.05^{a}	0.33±0.21^{b}	0.14±0.13^{ab}
Conversion of cysteate	0.01±0.03	0.06±0.04	0.12±0.08	tr

[1] Activities are expressed as taurine and hypotaurine formed.
[2] Cysteinsulfinate decarboxylase [3] Cysteamine dioxygenase
[4] Detected, but in trace.
Values (mean±sd, n=5)
Unlike superscripts in the same row indicate significant difference (p<0.05).
(Murata et al., 2004)

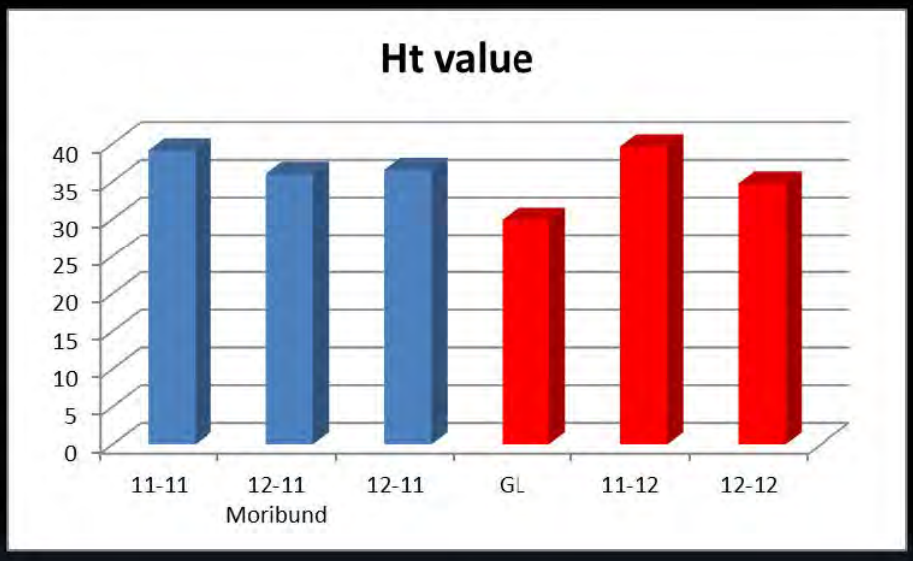


Hematological examination in CleanSeas



Normal plasma cholesterol level: 250-300 mg/100ml

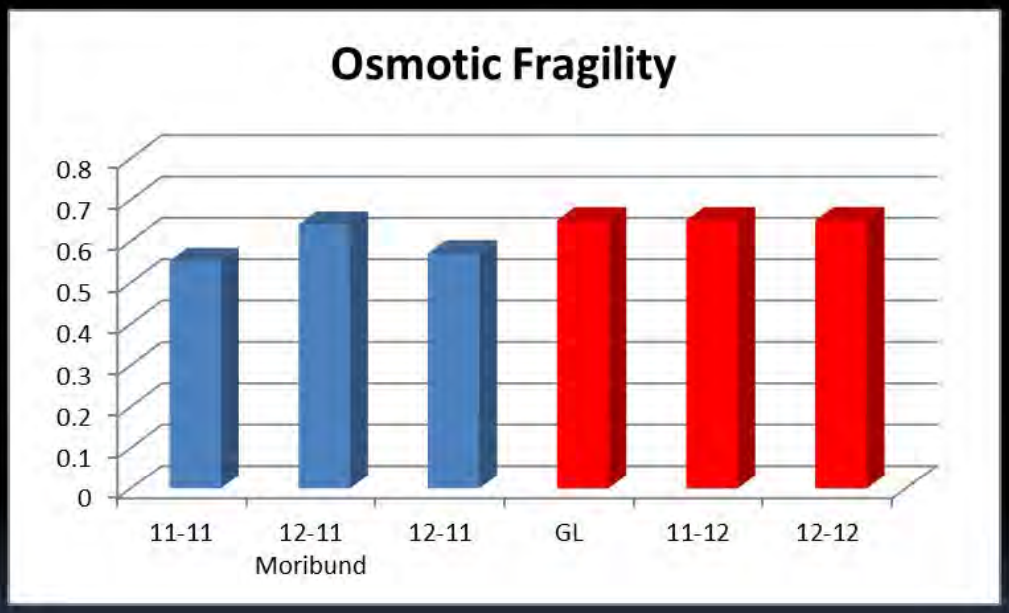
All the examined fish showed low plasma cholesterol levels.

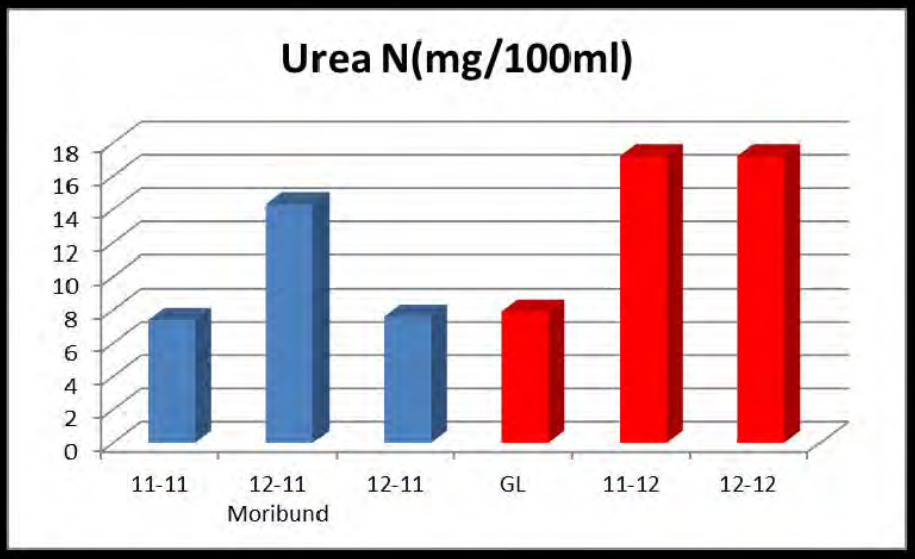


Normal levels of Ht values: 38-45 %

Ht values in 2 years old fish was lower than normal levels.

Many of fish showed slight anemia but it were not severe.





Normal plasma Urea N levels is changed by growing stage, WT : 12-17 mg/ml in adult fish, 20- 25 mg/ml in juvenile fish.

Low Urea N levels were observed in 2 years old fish except for Moribund.

Higher Urea N levels were observed in juveniles. But in normal range.

Appendix 9.6d:Yellowtail Kingfish lesions seen in sea caged fish from SA as well as WA (Fran Stephens)

Yellowtail kingfish lesions seen in sea caged fish from SA as well as WA

Fran Stephens

Government of Western Australia
Department of Fisheries

Fish for the future

No commercial sea cages
Small scale trials:

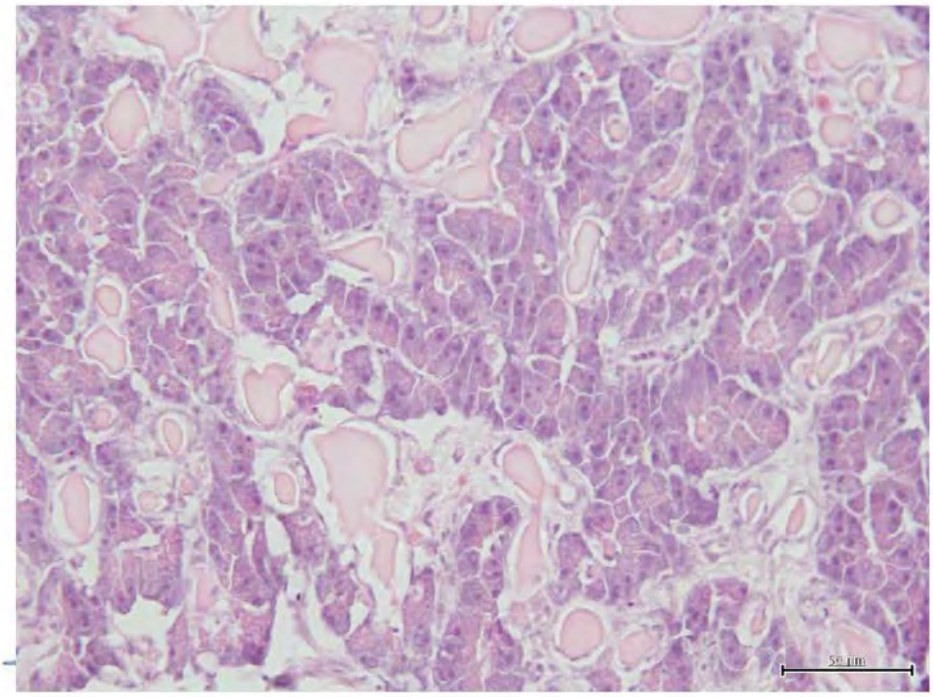
1. Jurien Bay 2008
   Commercial diet
2. Geraldton 2010-2011
   commercial diet then
   FM/FO only
3. Geraldton 2011-2012
   FM/FO diet only

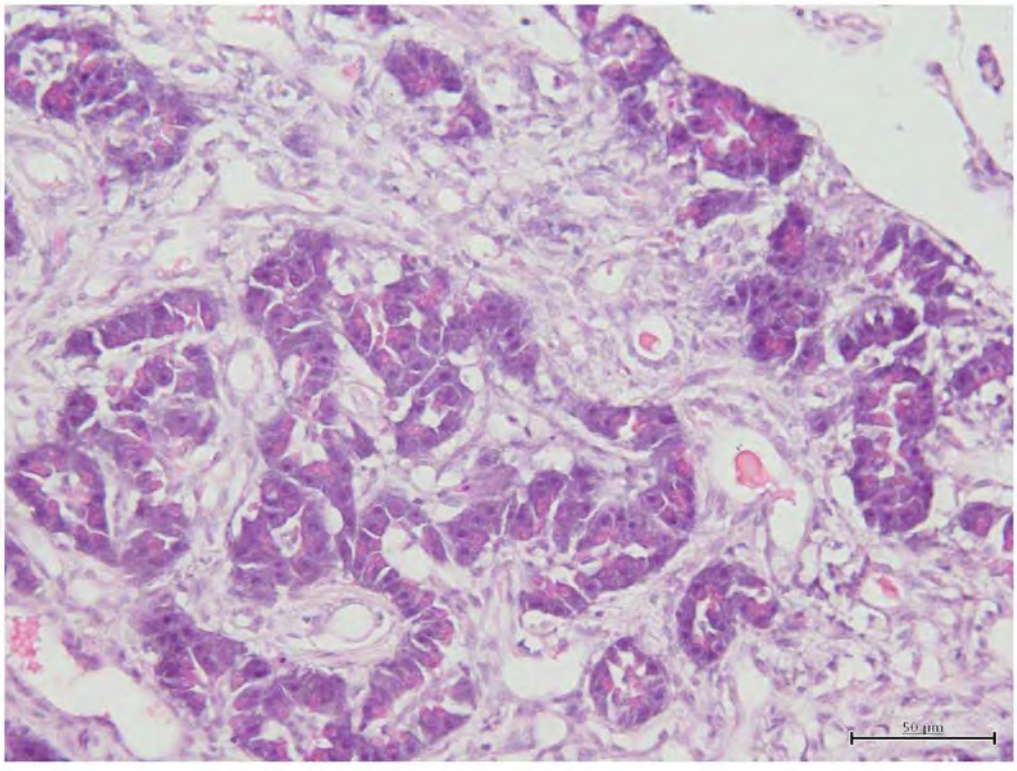
Water 18 -28°C
Windy, rough seas
Depth 6-13 m

Government of Western Australia
Department of Fisheries

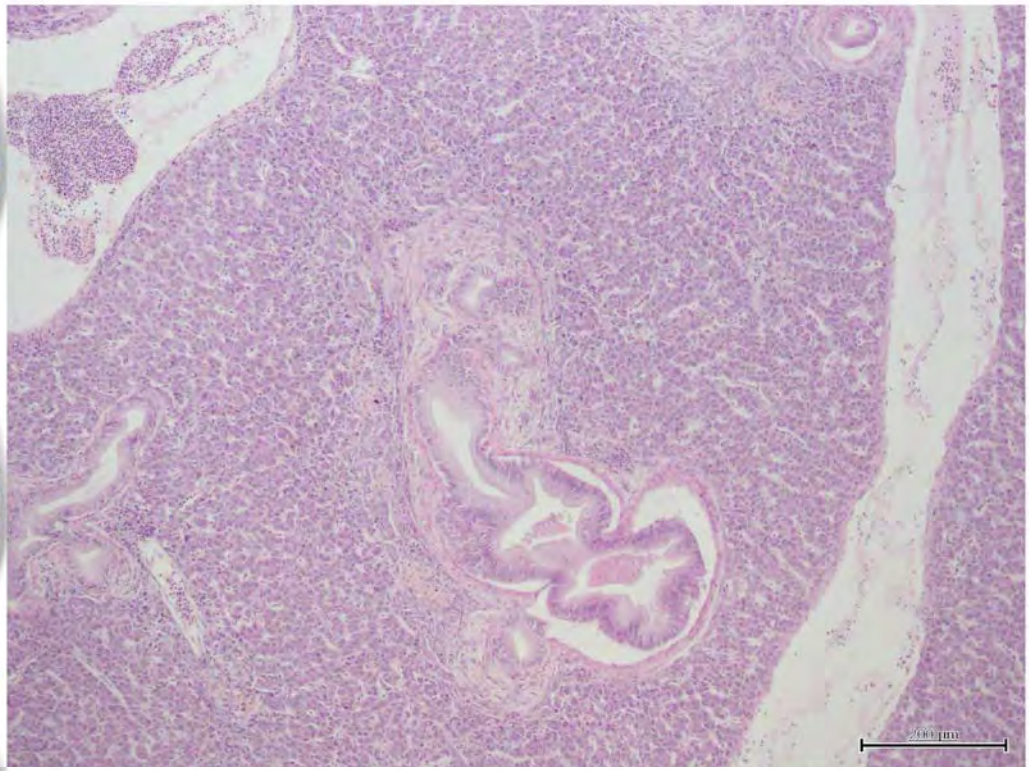
Fish for the future

Cystic and fibrotic changes exocrine pancreas

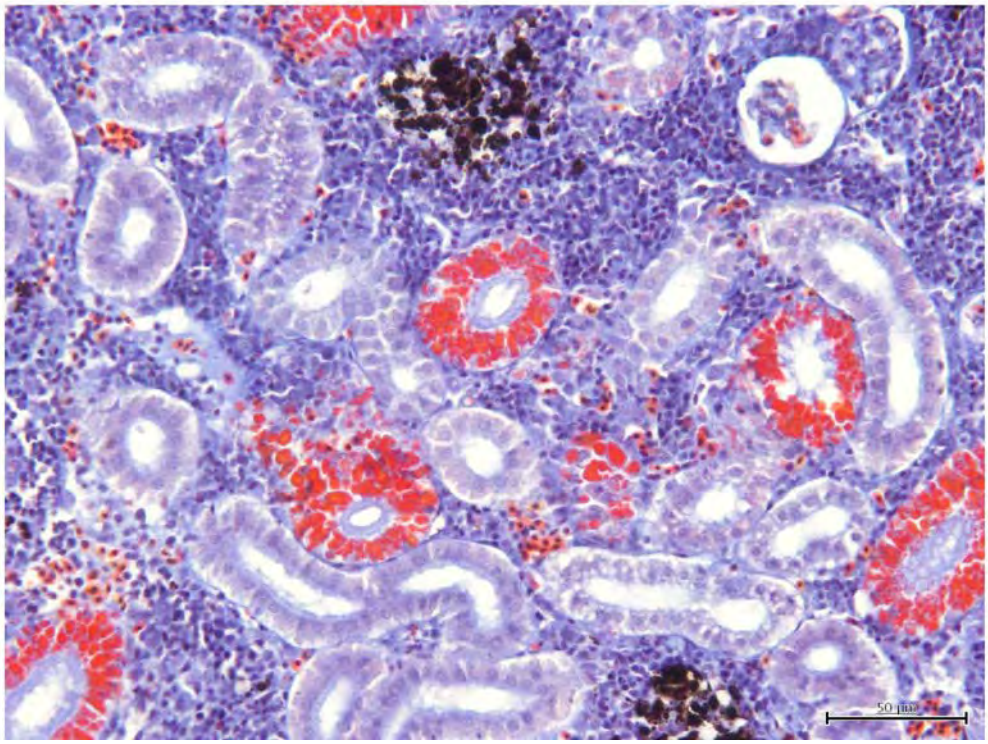




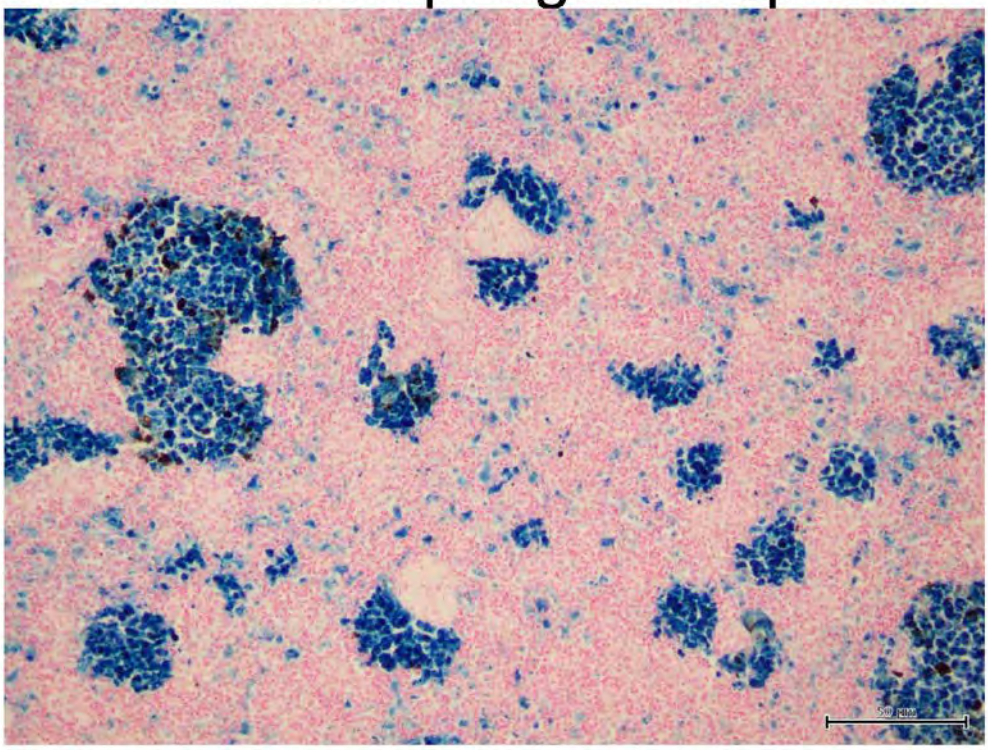
Cholangitis



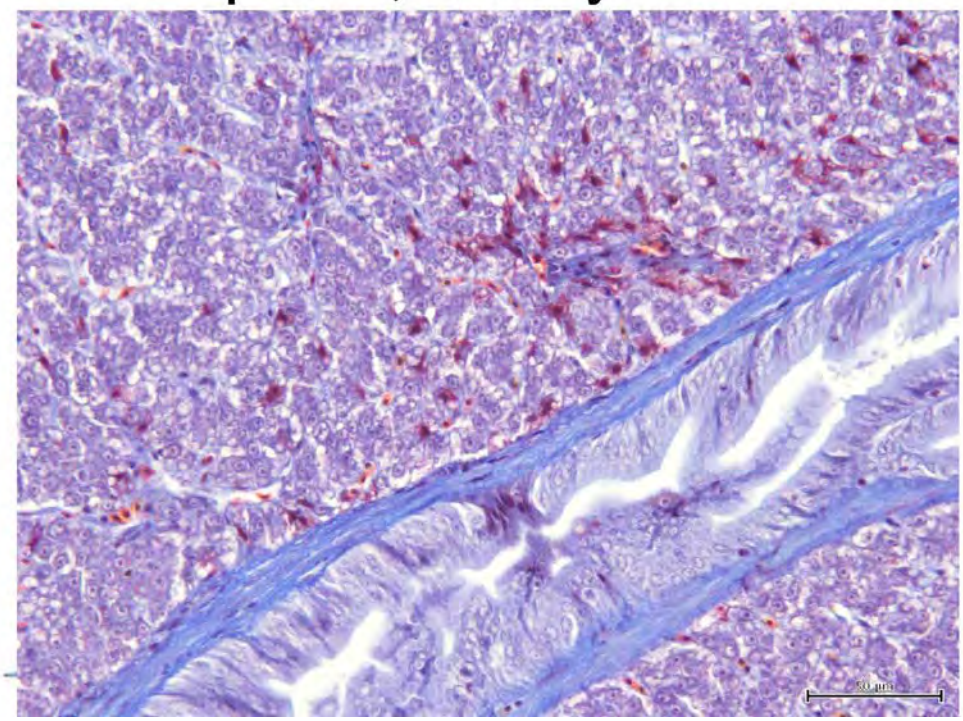
Protein? in kidney tubules (MSB stain)

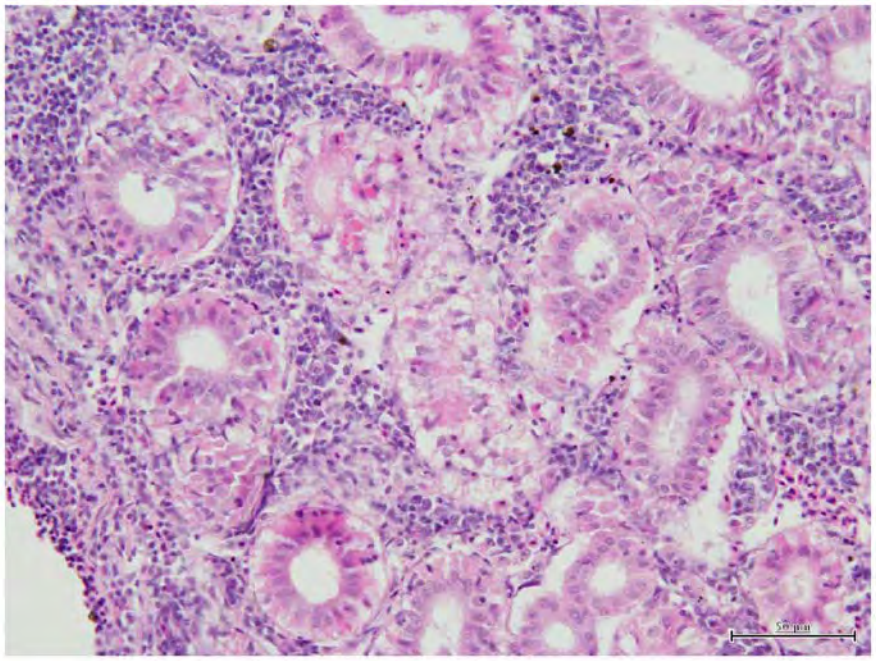


Perls positive melanomacrophages in spleen



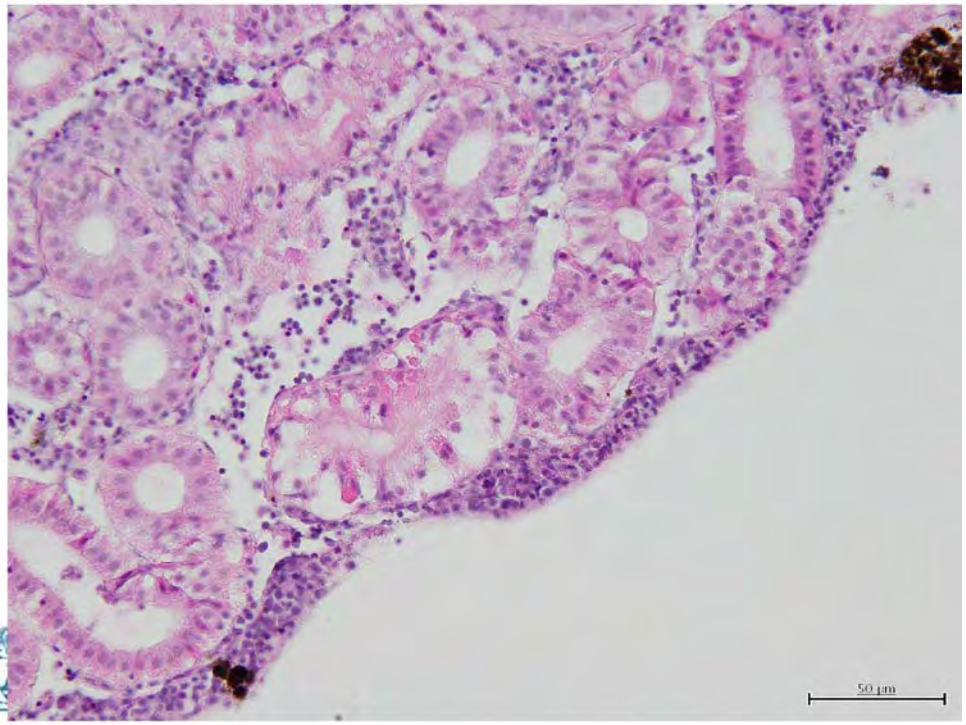
Yellow/black pigment in liver, spleen, kidney



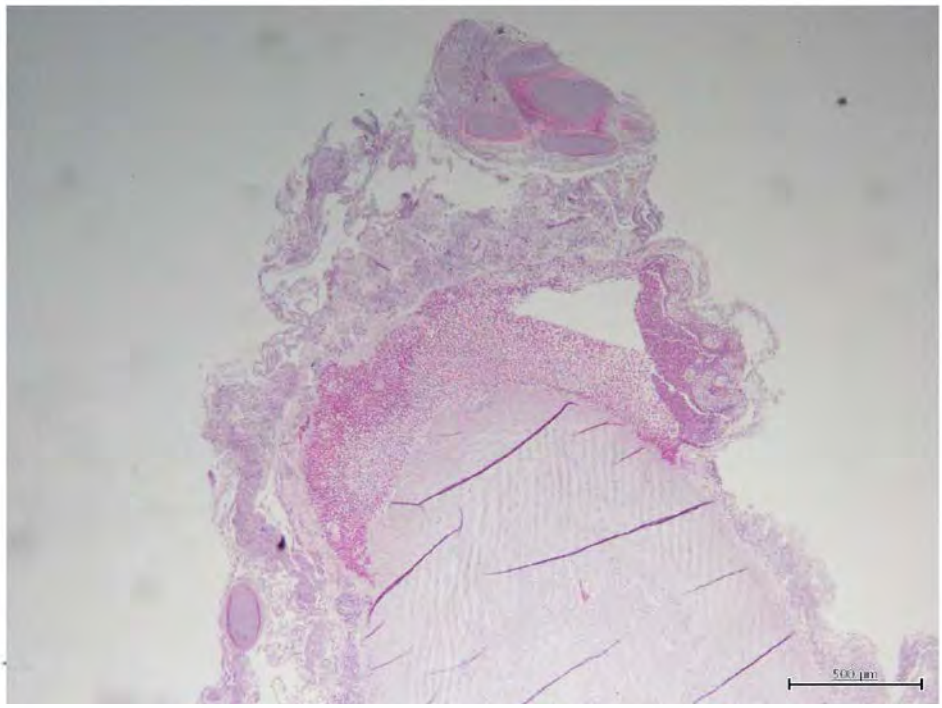


Government of **Western Australia**
Department of **Fisheries**

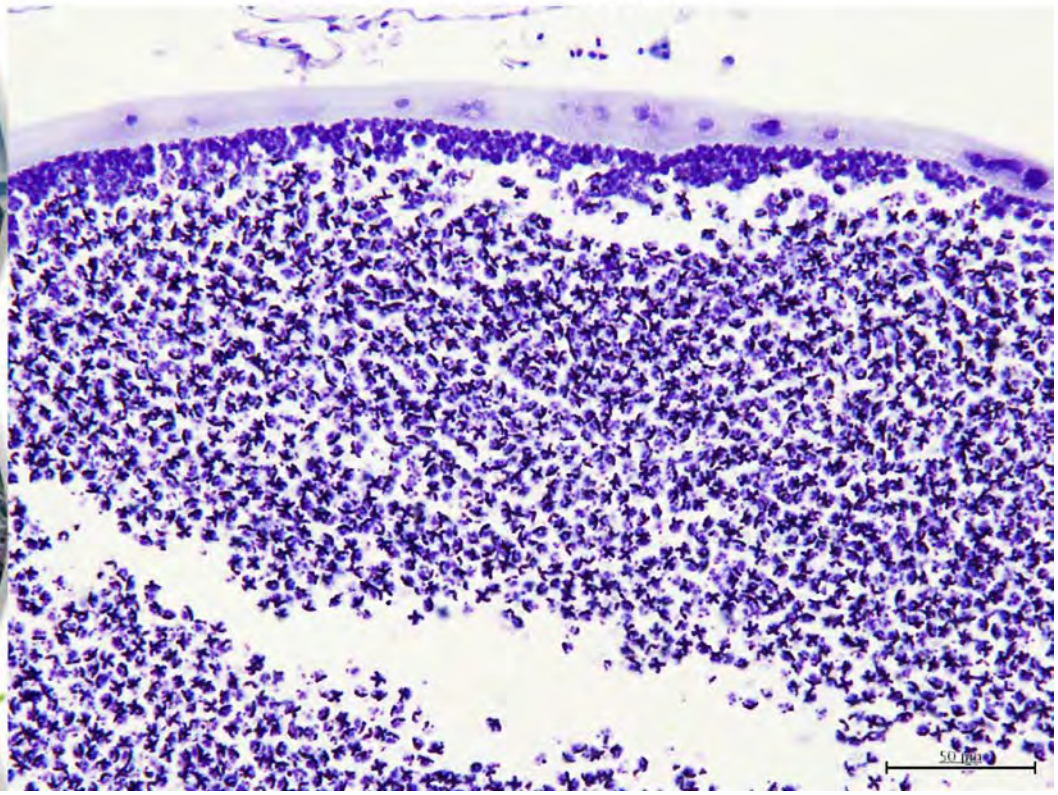
Fish for the future



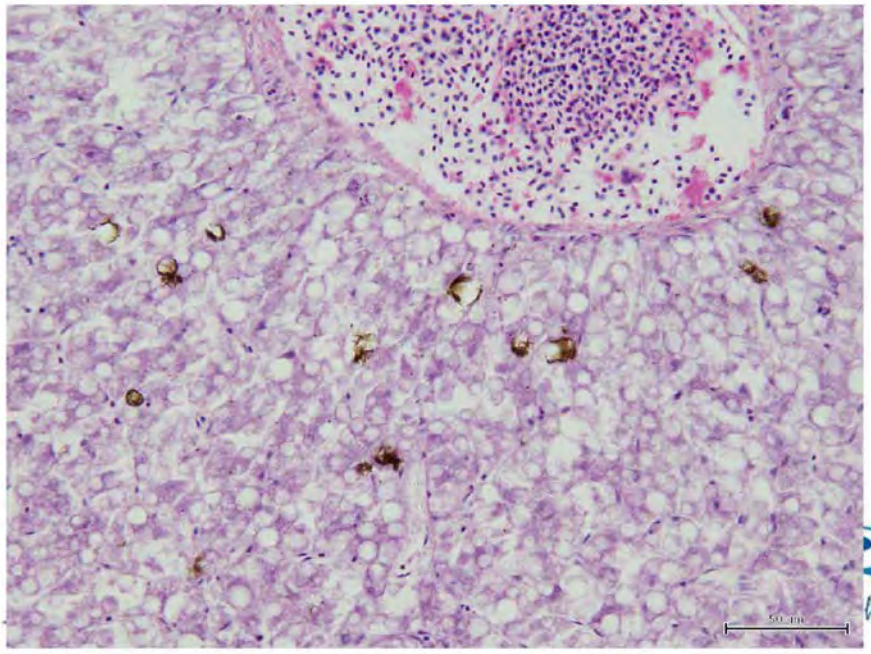
Kudoa-like myxozoan plasmodia on heart



Giemsa stain



Unusual pigments in liver, spleen, kidney- ceroid,lipofuscin-like



Comment on blood samples

- Problems with clotting/ gelatinisation
- Some fish with low PCV and Hb
- Same fish have low total plasma protein and albumin
- High GLDH

Government of Western Australia
Department of Fisheries

Fish for the future

Appendix 9.6e: Assessment of soybean enteritis like condition in juvenileYellowtail Kingfish Seriola lalandi held under different feed and temperature regimes (David Stone)

AUSTRALIAN SEAFOOD COOPERATIVE RESEARCH CENTRE

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.S.[2,3]

1. University of Tasmania, AMC, NCMCR, Launceston, Tasmania
2. Flinders University, School of Biological Sciences, Bedford Park, South Australia
3. SARDI Aquatic Science Centre, West Beach, South Australia
3.

AS CRC Projects
- Sustainable feeds and feed management for Yellowtail Kingfish (2009/728) D. Stone (PI), M. Booth, J. Qin, B. Chiera, L. Ward, J. Ciura, M. Thomson
- PhD. Project: Nutritional factors influencing the performance of Yellowtail Kingfish cultured at low temperatures.
Student, J. Bowyer, Supervisors, J. Qin & D. Stone

CST YTK workshop July 2012

Experiments

- **Presented here is a summary of the histological assessment of intestinal tissues obtained from yellowtail kingfish following four growth experiments** (growth data provided previously by J. Bowyer and D. Stone)
- **Briefly, the 4 pilot experiments investigated the effects of substituting fish meal and fish oil with terrestrial protein and lipid sources at optimal (22°C) and sub-optimal (18°C) water temperatures**
- **Experiments 1 and 2: Terrestrial protein sources investigated:**
  - **Solvent extracted soybean meal (SESBM) at 18 and 22°C**
  - **Soy protein concentrate (SPC) at 18 and 22°C**
- **Experiments 3 and 4: Terrestrial lipid sources investigated:**
  - **Poultry & canola oil at 22°C**
  - **Poultry & canola oil at 18°C**

Summary of experimental designs

Experiment	Feeds	Temps	Replicate fish for histology
Protein Exp. 1 Solvent extracted soybean meal	Substitution 0, 10, 20, 30%	18°C, 22°C	4 tanks per feed (2x 18°C tanks, 2x 22°C tanks) 7 fish per tank
Protein Exp. 2 Soy protein concentrate	Substitution 0, 20, 30, 40%	18°C, 22°C	4 tanks per feed (2x 18°C tanks, 2x 22°C tanks) 7 fish per tank
Lipid Exp. 1 dietary oils - 20% oil added FO = Fish oil; PO = Poultry oil; CO = Canola oil	Full FO, PO, CO FO & PO blend FO & PO blend	22°C	3 tanks per feed 3 fish per tank
Lipid Exp. 2 dietary oils 20% oil added FO = Fish oil; PO = Poultry oil; CO = Canola oil	Full FO, PO, CO FO & PO blend FO & PO blend	18°C	3 tanks per feed 3 fish per tank

Methods - Tissue collection and staining

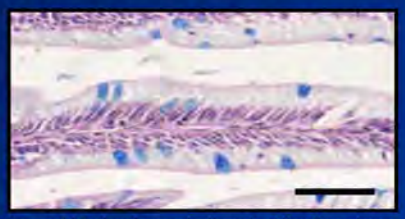
- Proximal anterior and distal intestine tissue samples were sampled as potential sites of dietary related cellular changes
- Tissue fixation (10% buffered formalin)
- Intestinal tissues were then stored in 70% ethanol and transferred to UTAS for processing
- All tissues were dehydrated prior to embedding in paraffin wax and 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 alcian blue (pH 2.5) which stains acidic mucopolysaccharides blue

Methods - Scoring SBM induced enteritis

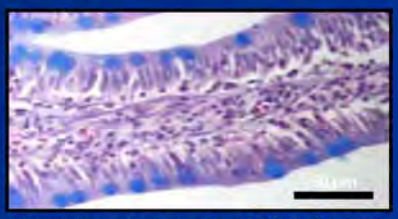
The morphology of the anterior and distal intestine sections were assessed according to the following criteria (used to classify conditions of SBM-induced enteritis in Atlantic salmon (Baeverfjord and Krogdahl, 1996; Uran 2008):

(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 leukocyte population in the lamina propria and submucosa
(5) Goblet cell proliferation

- Sections were allocated semi quantitative scores on a scale of 1-5 according to Uran, (2008); 1 represents normal tissue structure and 5 represents extensive structural changes



Normal hind gut morphology in Atlantic salmon

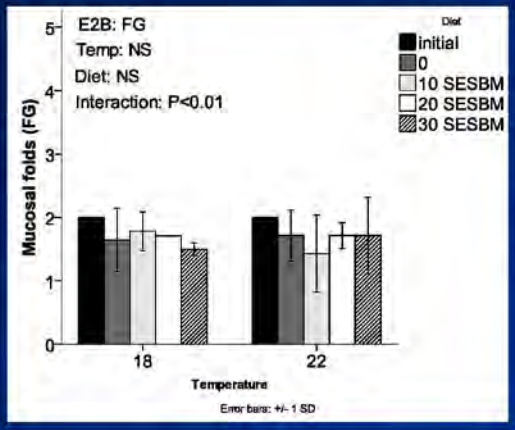


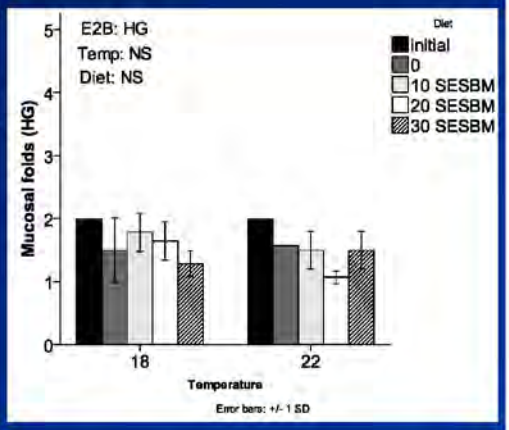
Typical signs of sub-acute enteritis in the hind gut of Atlantic salmon

Micrographs from Uran (2008)

Results
Exp. 1 SESBM

Results Exp 1 SBM-Mucosal folds

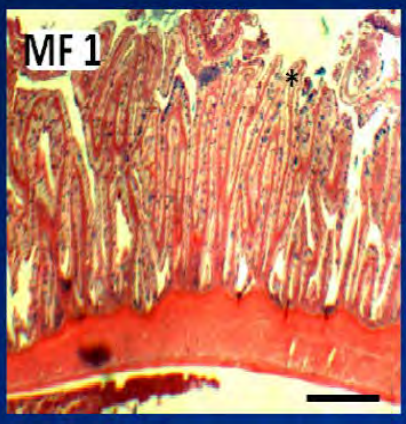




Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

Mucosal fold



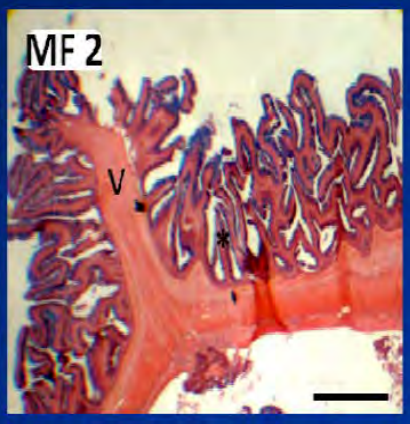
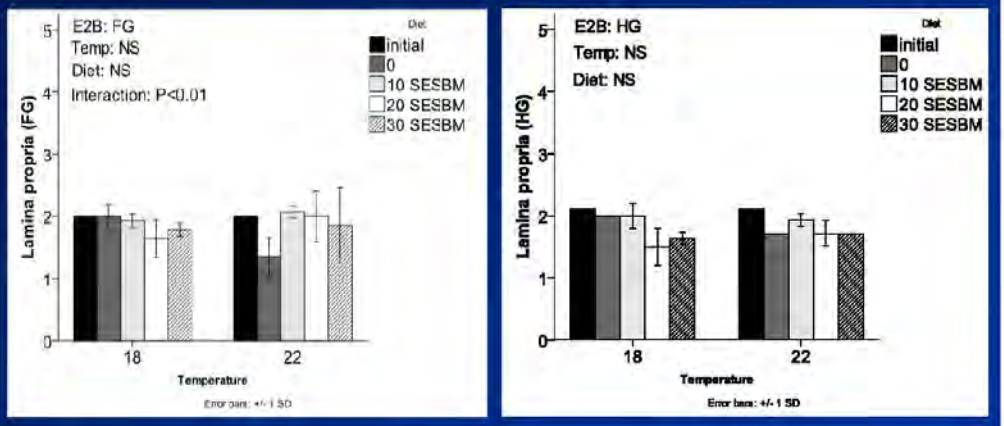


Figure. **MF1** shows the maximum mucosal fold (MF) height and individual mucosal folds are indicated by an asterisk (score 1 = normal). **MF2** shows thickened mucosal folds of reduced height (asterisk) lining the basal mucosa and ileal valve (v) (score 2 = slight change). Scale bar = 500 µm.

SESBM-Lamina propria



Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

Lamina propria

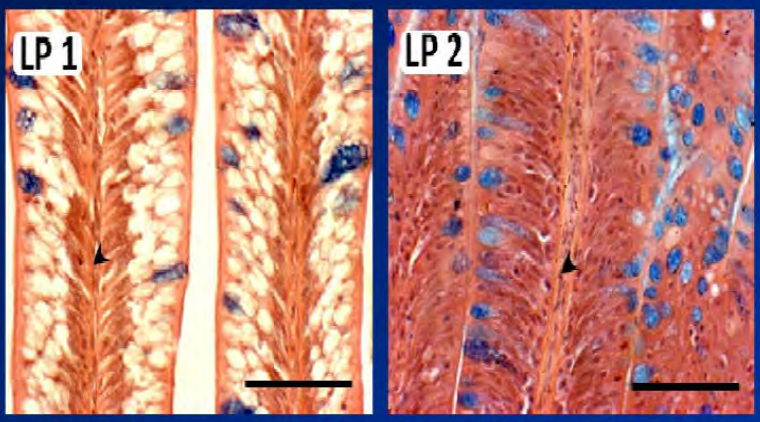
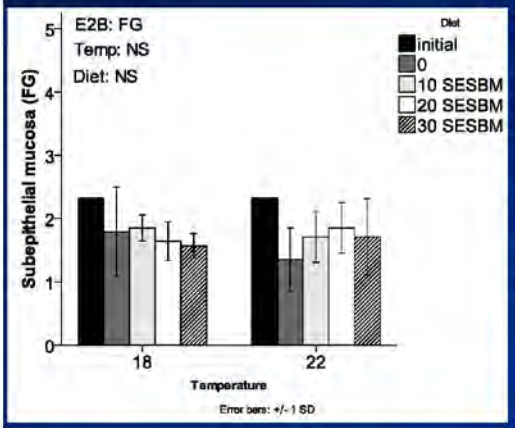
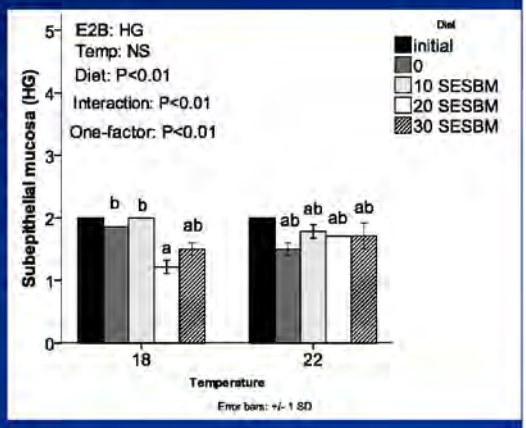


Figure. **LP 1** shows a normal villi with narrow lamina propria (LP) (arrow) containing few leucocytes (score 1), while **LP2** shows a score of 2 where some cellular infiltration has caused a slight widening of the lamina propria (arrow). Scale bar = 100 µm.

SESBM-Sub-epithelial mucosa





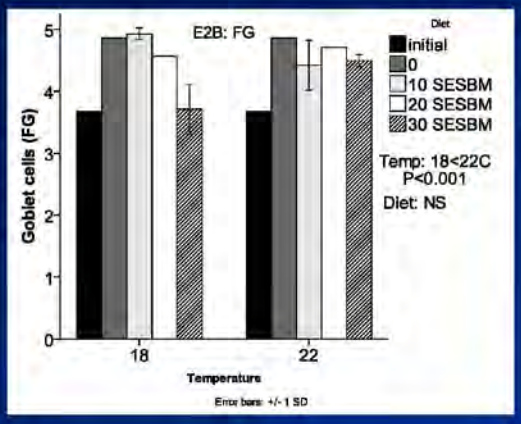
Foregut Hindgut

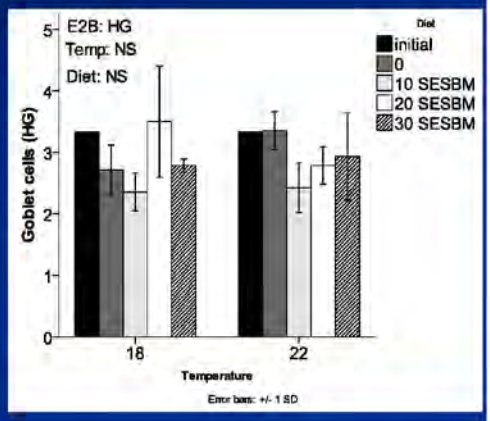
1 represents normal tissue structure and 5 represents extensive structural changes

Sub-epithelial mucosa

Figure. **SEM 1**; normal sub-epithelial mucosa appearance, minimal cellular infiltration below the intestinal epithelia, **SEM 2**; mild cellular infiltration below the intestinal mucosa (arrow). Scale bar = 100 µm.

SESBM-Goblet cell proliferation





Foregut

Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

Goblet proliferation

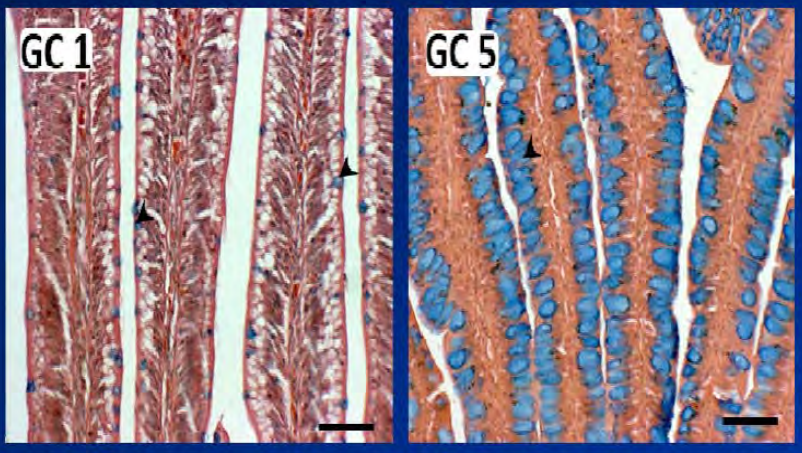
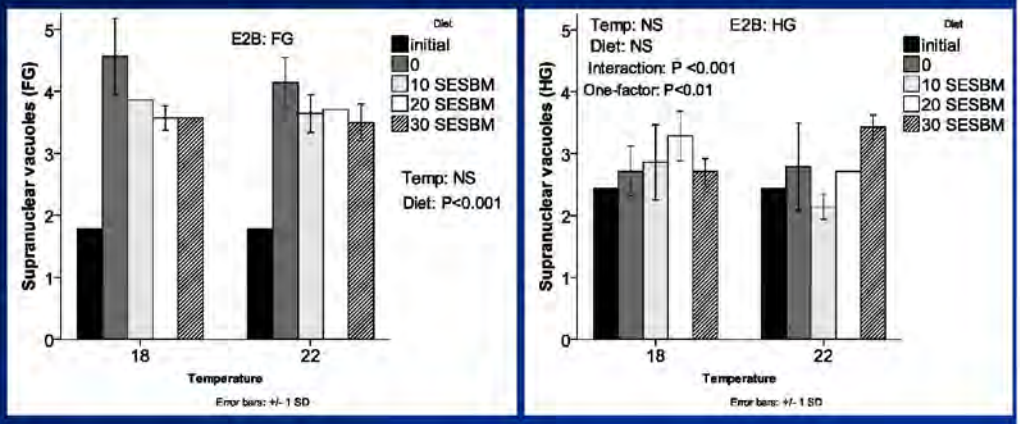


Figure. **GC1**; occasional goblet cells (GC) distributed at the proximal edge of the intestinal epithelium (stained blue with H&E alcian blue, pH 2.5), **GC5** prolific abundance of goblet cells along the entire margin of the intestinal epithelium. Scale bar = 100 µm.

SESBM-Supranuclear vacuoles



Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

Supranuclear vacuoles

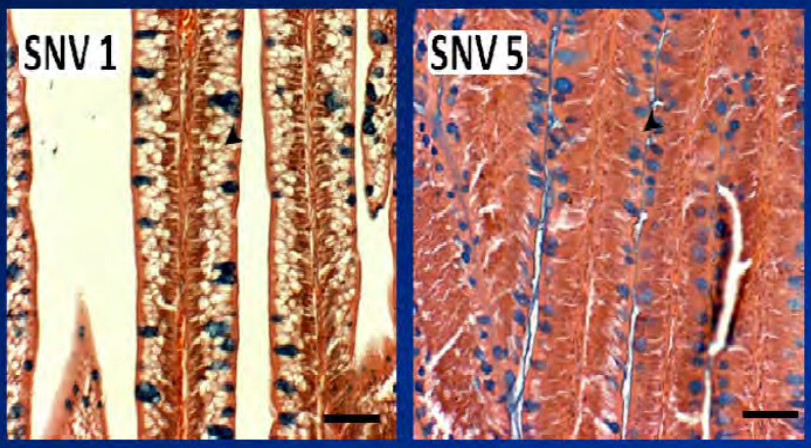


Figure. **SNV 1**; wide margin of supranuclear vacuoles (SNV) within enterocytes of the epithelium and uniformly arranged nuclei adjacent to the lamina propria, **SNV 5** extinction of all SNV along the epithelial margin and disorganisation of nuclei. Scale bar = 100 µm.

Summary of results of all experiments

Foregut score range

Experiment	MF	LP	SEM	GCP	SNV
SESBM 18°C	1.5 – 1.8	1.7 – 2.0	1.6 – 1.9	3.8 – 4.9	3.6 – 4.6
SESBM 22°C	1.5 – 1.8	1.3 – 2.0	1.5 – 1.9	4.5 – 4.9	3.5 – 4.1
SPC 18°C	1.5 – 1.8	1.1 – 1.7	1.5 – 1.9	4.5 – 4.9	3.2 – 4.0
SPC 22°C	1.5 – 1.7	1.3 – 1.5	1.3 – 1.5	4.4 – 4.9	3.0 – 3.8
Lipid 22°C	1.1 – 1.6	1.6 – 2.1	1.3 – 2.0	4.9 – 5.0	3.9 – 4.9
Lipid 18°C	1.3 – 1.8	1.7 – 2.1	1.5 – 1.7	3.9 – 5.0	4.4 – 5.0

Hindgut score range

Experiment	MF	LP	SEM	GCP	SNV
SESBM 18°C	1.3 – 1.8	1.5 – 2.0	1.3 – 2.0	2.3 – 3.5	2.7 – 3.3
SESBM 22°C	1.2 – 1.5	1.7 – 1.9	1.6 – 2.0	2.4 – 3.4	2.2 – 3.4
SPC 18°C	1.3 – 1.8	1.3 – 1.6	1.2 – 1.8	2.2 – 3.0	2.2 – 2.6
SPC 22°C	1.1 – 1.5	1.1 – 1.5	1.3 – 1.7	2.8 – 4.6	1.3 – 1.7
Lipid 22°C	1.2 – 1.6	1.1 – 1.8	1.3 – 1.6	4.2 – 4.7	2.9 – 4.8
Lipid 18°C	1.3 – 1.9	1.7 – 2.7	1.8 – 2.7	4.3 – 5.0	3.4 – 4.8

Generally the scores for LP, MF and SEM were very low and may indicate normal tissue structure for YTK.

MF = mucosal folding; LP = lamina propria; SEM = sub-epithelial mucosa; GCP = goblet cell proliferation; SNV = supranuclear vacuolation
Key to score: 1 represents normal tissue structure and 5 represents extensive structural changes

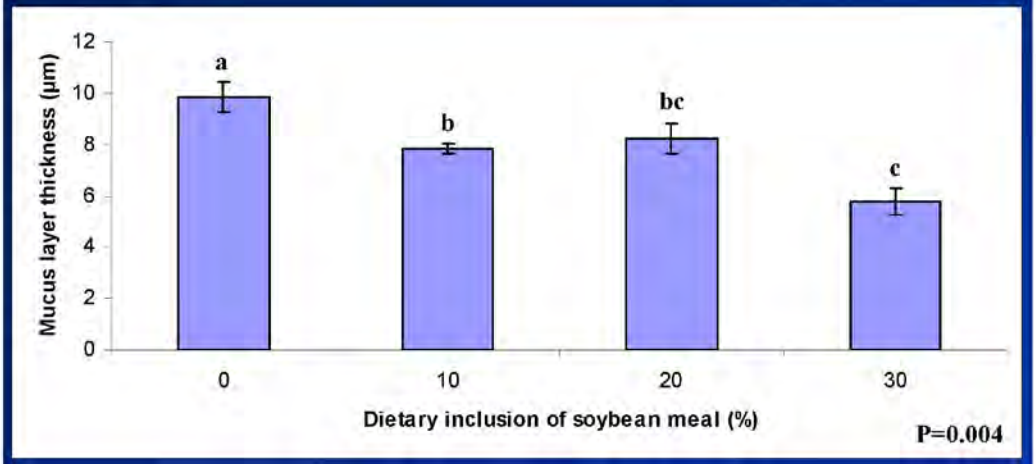
Conclusions

- The key criteria used to classify soybean enteritis in Atlantic salmon were measured and substantial changes in all parameters consistent with soybean enteritis were not observed in yellowtail kingfish
  - Extensive changes to Atlantic salmon intestinal structure occur at these SESBM inclusion levels
  - No major inflammatory responses observed in response to dietary treatments (i.e. responses typical of extensive leucocyte inflammation of connective tissues or drastic reduction in villi height indicative of soybean enteritis)
  - These were short term pilot studies of 5 weeks duration!
- Generally the scores for LP, MF and SEM in response to SESBM, SPC and oil substitution were very low and may indicate normal tissue structure for yellowtail kingfish.

Conclusions contd.

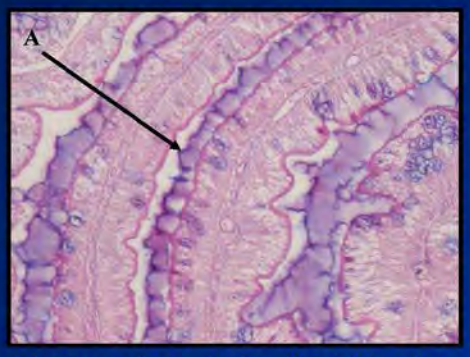
- Goblet cell proliferation and supranuclear vacuolation were high across all experiments and treatments
  - High in initial fish and fish fed control feeds
  - Reduced vacuolisation of absorptive enterocytes may have potential to reduced nutrient digestion
  - High goblet cell numbers may result in increased secretion of mucous
  - Both may impact on growth and health
- However, fish growth performance and survival did not correspond to the changes in intestinal structure (Bowyer et al. 2012 for lipid studies; Bowyer et al. unpublished data for the soybean studies)
- Closer examination revealed SESBM induced mucous layer erosion, altered mucous composition and increased goblet cell numbers (18°C) in digestive tract
- Growth, feed efficiency and digestive trypsin and lipases were significantly reduced by canola oil inclusion (Bower et al. unpublished data)
- Green liver was seen in YTK in response to canola oil and to a lesser extent poultry oil

Mucus layer thickness & SESBM

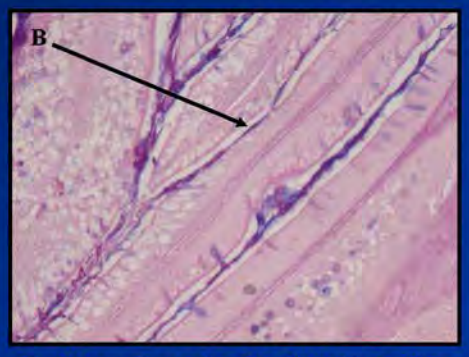


Mucus layer thickness in distal intestine of YTK fed increasing dietary levels of SE SBM (n = 4 replicate tanks; (Two Factor ANOVA: second factor, dietary inclusion level of SESBM)

Alternative protein studies: SESBM & the mucus layer in distal intestine of YTK

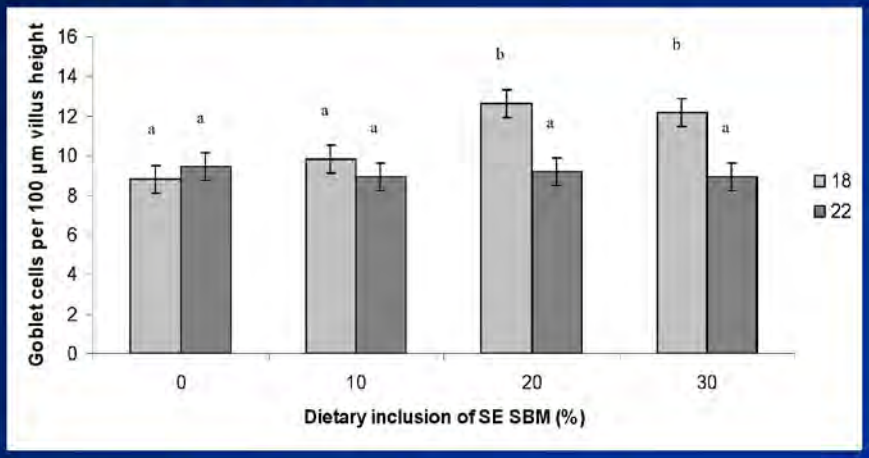


A) Mucus layer in the distal intestine of YTK fed 0% SESBM at 22°C (100X).



B) Mucus layer in the distal intestine of YTK fed 30% SESBM at 22°C (100X).

Alternative protein studies: SESBM & goblet cell number in in distal intestine of YTK

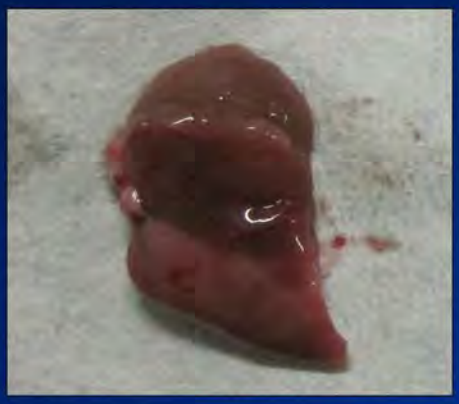


Number of goblet cells per millimetre of villus height in the distal intestine of YTK fed increasing dietary inclusion levels of SE SBM at 18°C and 22°C.

Means that have different superscript are significantly different (mean±S.E.M.; n=2 replicate tanks; One factor ANOVA, P<0.05)

Health and energy cost

Alternative lipid studies - green liver

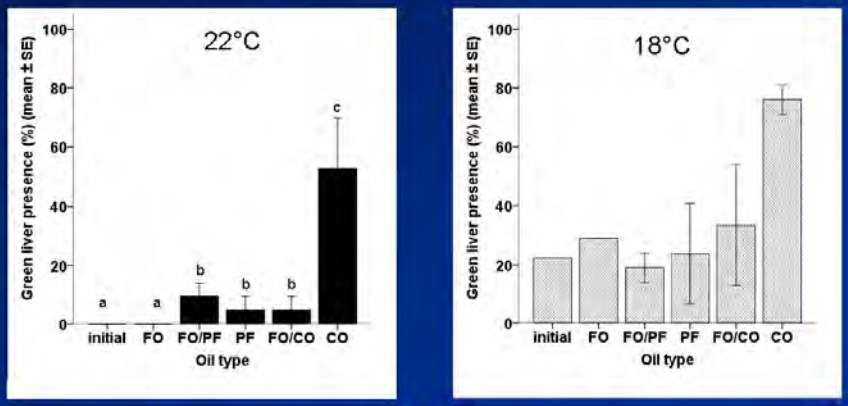


YTK liver fed Fish oil at 18°C showing healthy red appearance

YTK liver fed Canola oil at 18°C showing green liver presence

Green liver in fish fed non-FM diets is possibly due to impaired excretion of bile pigments from liver into bile, and haemolytic biliverdin overproduction linked to a dietary taurine deficiency (Shusaku et al 2005)

Alternative lipid studies - Green liver
(One-factor ANOVA)



Values are means ± SE for dietary treatment (n = 3); Values that share the same lower case superscript are not significantly different (One-Factor ANOVA, SNK, *P*>0.05).

Recommendations

- The cause of GCP and high levels of SNV warrants further investigation
- Initial, control fish were intended to provide the baseline reference in these experiments
  - The observed high GCP and SNV scores may be indicative of a pre-existing condition
- Confirmation of normal (naïve to formulated feeds) tissue morphology is required
- May provide a basis for developing a scoring system specific for YTK, rather than assuming equivalence to Atlantic salmon
- Differences in pre-feeding history and culture conditions may have influenced GC and SNV levels in fish prior to the studies
  - More research is needed

Pre-feeding Foregut scores

Experiment	MF	LP	SEM	GCP	SNV
SBM	2.0	2.0	2.3	3.8	1.8
SPC	2.0	1.8	2.0	3.7	2.5
Lipid 22°C	1.5	1.6	1.2	4.5	4.3
Lipid 18°C	1.8	1.5	1.3	4.3	4.0

Pre-feeding Hindgut scores

Experiment	MF	LP	SEM	GCP	SNV
SBM	2.0	2.1	2.0	3.3	2.4
SPC	1.8	1.6	1.9	3.3	2.4
Lipid 22°C	1.2	1.2	1.1	4.0	3.8
Lipid 18°C	1.2	1.4	1.2	3.8	3.4

Increased goblet cell proliferation (GCP) and the reduction in supranuclear vacuolisation (SNV) in initial fish prior to experiment were more severe in the larger fish (oil studies) than for smaller fish (soybean studies).

Fish pre-feeding history

Experiment	Source /size/date	Diet and feeding method	Stocking date/weight
Protein Exp. 1 SESBM	CST Port Augusta 5 – 10 g /fish 29/10/10	Skretting Nova (50% CP / 15%, 1.8mm) *ad libitum*	17th November 2010 ~22g/fish
Protein Exp. 2 SPC	CST Arno Bay 5 – 10 g fish 7/1/2011	Skretting Nova (50% CP / 15%, 1.8mm) *ad libitum*	24th January 2010. ~22g/fish
Lipid Exp. 1 at 22°C	CST Arno Bay 5 -10 g/fish 19/11/08	Ridley (50% CP/15 % CL, 2 mm) & Skretting Nova (45% CP / 20%, 3mm) *ad libitum*	23rd March 2009 ~96 g/fish
Lipid Exp. 2 at 18°C	CST Arno Bay 5 -10 g/fish 19/11/08	Ridley (50% CP/15 % CL, 2 mm) & Skretting Nova (45% CP / 20%, 3mm) *ad libitum*	22nd of July 2009 ~101 g/fish

Other data

Mucous layer thickness and SE SBM

Alternative protein studies

2 separate 5 week experiments
- Exp. 1: SESBM replaced 10, 20 or 30% FM at 18 & 22°C
- Exp. 2: SPC replaced 20, 30 or 40% FM at 18 and 22°C

- Test diets formulated to contain 50% CP & 15% CL
  – then balanced on an as fed basis to 41.5% DP & 14.5% DL

- Initial fish weight 22 g in each study

- 2 replicate 700-L tanks/diet (24 fish/tank)

- Fish fed twice daily to apparent satiation

Report on:
- Digestive tract histology (soybean meal induced enteritis in proximal and distal intestine)

- Previously reported on

- Weight gain (SESBM & SPC)

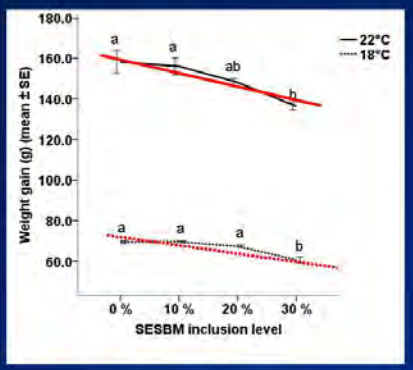
- Apparent feed conversion ratios (FCR) (SESBM & SPC)

- Mucous layer thickness and goblet cell numbers from distal section of digestive tract from 3 fish/tank (SESBM)

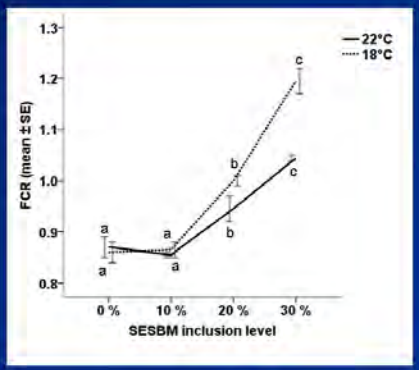
700-L tanks in re-circulating system in the Nutrition Laboratory at SARDI Aquatic Science Centre, West Beach

Tissue collection site for histological examination

Results: Solvent extracted soybean meal



Weight gain (g/fish)

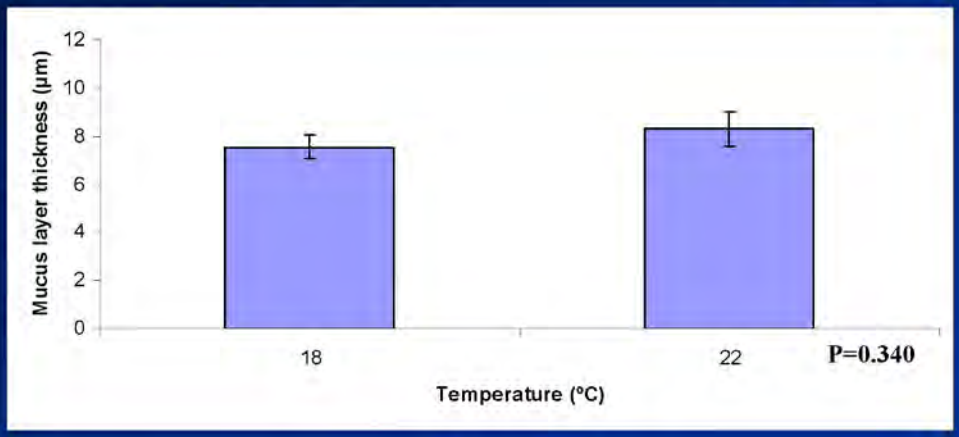


Apparent FCR

Values are means ± SE for dietary treatment (n = 2); Values that share the same lower case superscript are not significantly different (One-Factor ANOVA, SNK, $P<0.05$). Initial fish weight 22.6 g/fish.

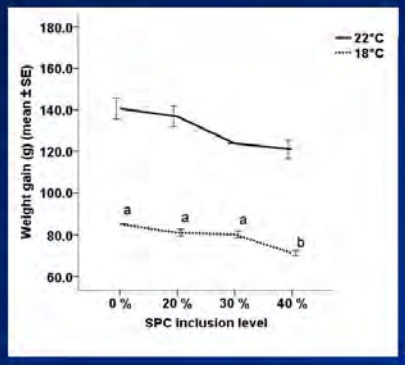
Add SESBM and reduce growth and feed efficiency and health

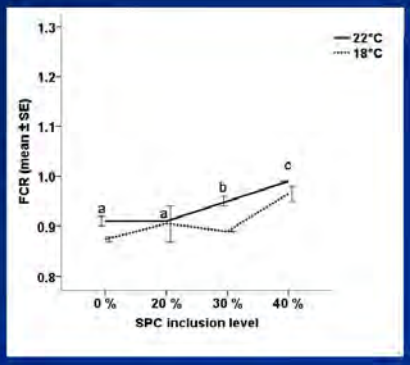
Alternative protein studies: Mucus layer thickness and SESBM (Bansemer et al.)



Mucus layer thickness in distal intestine of YTK subjected to 18°C & 22°C water temperatures (n = 8 replicate tanks; Two Factor ANOVA: first factor, temperature)

Alternative protein studies - Soy protein concentrate





Weight gain (g/fish) Apparent FCR

Values are means ± SE for dietary treatment (n = 2); Values that share the same lower case superscript are not significantly different (One-Factor ANOVA, SNK, *P*>0.05).

Add more than 20% SPC at 22°C and reduce growth and feed efficiency

Conclusion & Recommendations
Alternative proteins studies

- **Solvent extracted soybean meal (SESBM)**
  - Inclusion decreased growth & feed efficiency
  - Mucus layer decrease in thickness
  - Goblet cell numbers increase at 18°C & at >10% inclusion
  - Mucus types changed (not shown)
  - Digestive tract health concerns
    - Reduction in nutrient uptake?
    - Increased risk of disease?

- **Based on these results, and as a precautionary measure, CST removed all soybean products from their YTK production diets**

- **Soy protein concentrate (SPC)**
  - Can be fed at both water temps at 20% inclusion without significant effects on growth or feed efficiency (Health?)
  - However, pilot scale study – need to test for longer period

Aim: Terrestrial Lipid Studies

Investigate the effects of partial or total replacement of fish oil with poultry fat and canola oil at optimal (22°C) and sub-optimal (18°C) water temperatures on the growth performance and feed efficiency of YTK

AS CRC Student Projects

Jenna Bowyer (AS CRC PhD student, FUSA) Supervisors: J. Qin, D. Stone

Geoff Collins (AS CRC Honours 1st Class, FUSA) Supervisors: A. Ball, J. Qin, D. Stone

Nathan Rout-Pitt (AS CRC Honours 2nd Class Level A, FUSA) Supervisors: K. Schuller, D. Stone

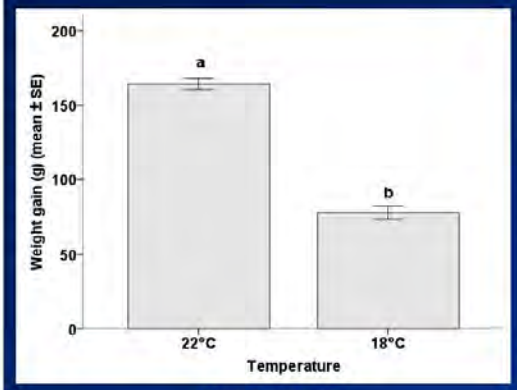
Methods: Alternative lipid studies

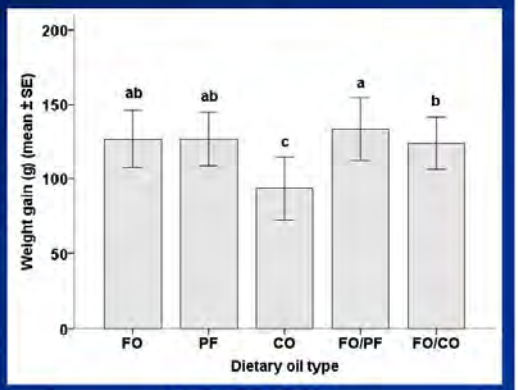
- 2 separate 5 week experiments
  - Experiment 3 at 18°C: Initial fish weight = 95 ± 0.1 g
  - Experiment 4 at 22°C: Initial fish weight = 101 ± 0.1 g
  - YTK separate cohorts (wts not sig. as a covariate)
- Test diets: 5 dietary oil combinations
  - Diet 1: Control diet, 20% fish oil (FO)
  - Diet 2: 20% poultry fat (PF)
  - Diet 3: 20% canola oil (CO)
  - Diet 4: 10% fish oil + 10% poultry fat (FO/PF)
  - Diet 5: 10% fish oil + 10% canola oil (FO/CO)
    - Diets formulated to contain 50% CP & 25% CL
    - Diets contained 5% residual fish oil
- 3 replicate 700-L tanks/diet (14 fish/tank)
- Fed twice daily to apparent satiation
- Report on:
  - Weight gain
  - Feed intake
  - Apparent feed conversion ratios (FCR)
  - Incidence of green liver
  - Digestive tract histology

Making diets at the SARDI Australasian Experimental Stockfeed Extrusion Centre

Sample collection in the Nutrition Laboratory at the SARDI Aquatic Science Centre

Results: Alternative lipid studies - Weight gain (g)





Temperature

(Two-factor ANOVA; $P < 0.001$; n = 15)

Dietary oil type

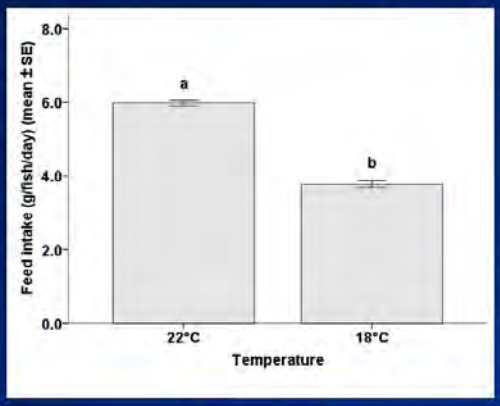
(Two-factor ANOVA; $P < 0.001$; n = 6)

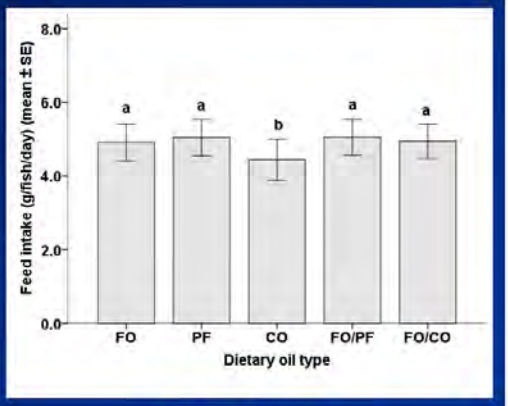
No significant interaction ($P > 0.05$)

Values that share the same superscript are not significantly different (Tukey's HSD; $P < 0.05$)

100% Canola oil significantly effected growth

Alternative lipid studies - Feed intake





Temperature
(Two-factor ANOVA; $P < 0.001$; n = 15)

Dietary oil type
(Two-factor ANOVA; $P = 0.003$; n = 6)

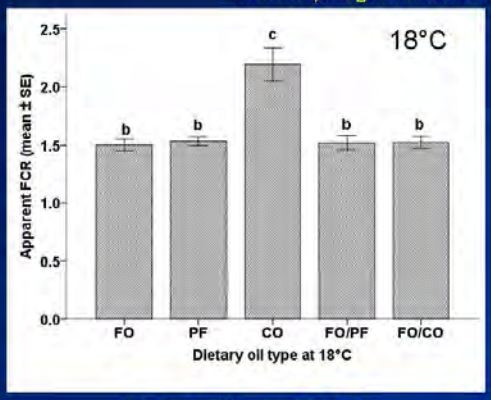
No significant interaction ($P > 0.72$)

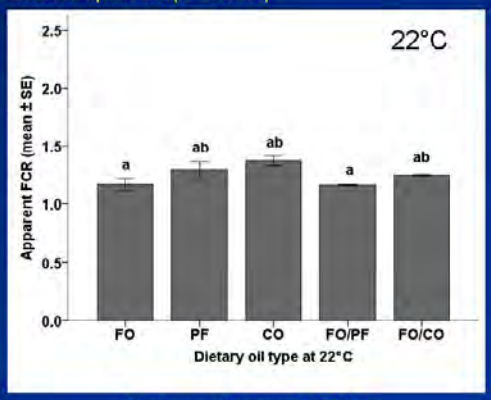
Values that share the same superscript are not significantly different (Tukey's HSD; $P < 0.05$)

100% Canola oil significantly effected feed efficiency

Alternative lipid studies - Apparent FCR

Two-factor ANOVA: Temperature ($P < 0.001$)
Dietary oil type ($P < 0.001$)
However, a significant interaction was present ($P = 0.001$)





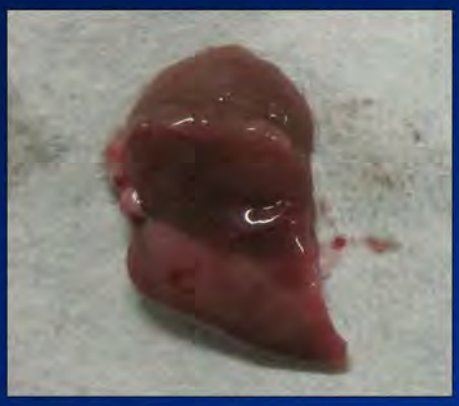
Dietary oil type @ 18°C

Dietary oil type @ 22°C

(One-factor ANOVA; $P < 0.001$; n = 3)

Values that share the same superscript ACROSS BOTH FIGURES are not sig. different (Tukey's HSD; $P < 0.05$).

Alternative lipid studies - green liver

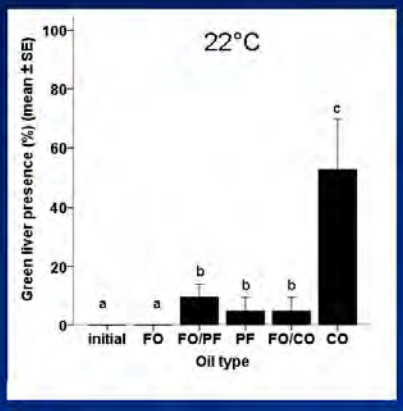


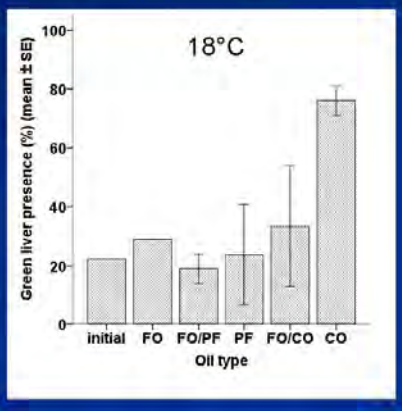
YTK liver fed Fish oil at 18°C
showing healthy red appearance

YTK liver fed Canola oil at 18°C
showing green liver presence

Green liver in fish fed non-FM diets is possibly due to impaired excretion of bile pigments from liver into bile, and haemolytic biliverdin overproduction linked to a dietary taurine deficiency (Shusaku et al 2005)

Alternative lipid studies - Green liver
(One-factor ANOVA)





Values are means ± SE for dietary treatment (n = 3); Values that share the same lower case superscript are not significantly different (One-Factor ANOVA, SNK, $P>0.05$).

Conclusions & Recommendations
Alternative lipid studies

POULTRY FAT

- **Substitution acceptable at both water temperatures**
  - **Must meet essential fatty acid requirement for EPA and DHA**
- **Improvement in FI:FO ratio!**
- **This was a 5 week pilot scale study – we need to do long term studies to investigate growth performance, feed efficiency, product quality and health**

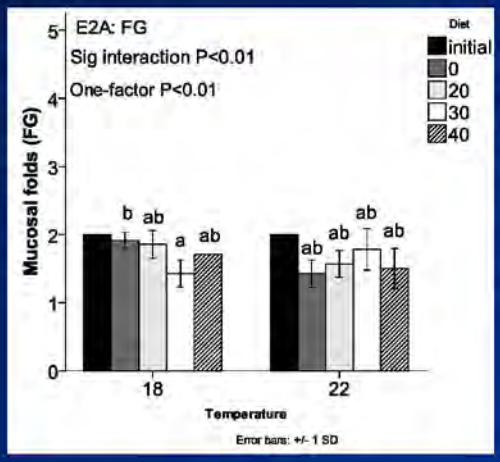
- **Based on this information CST modified their YTK diets**

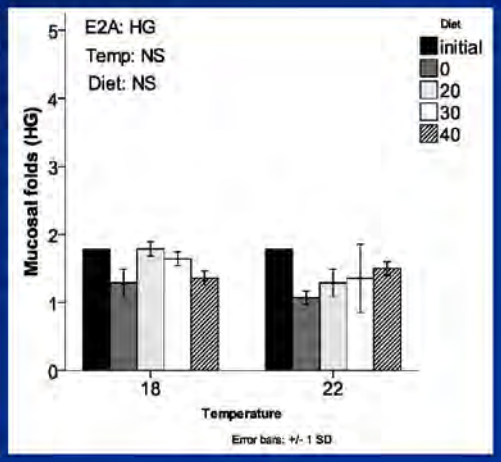
CANOLA OIL

- Substitution not recommended
- Health concerns
- **GREEN LIVER:** Previously only been linked to a taurine deficiency in YTK when replacing fish meal with soybean products
- The inclusion of 20% CO in diets vastly increased the occurrence of green liver
- Taurine has a metabolic effect on lipid metabolism in cats and rats (investigate with YTK)

- **Based on this information CST removed canola oil from their YTK diets**

Results Exp 2 SPC-Mucosal folds

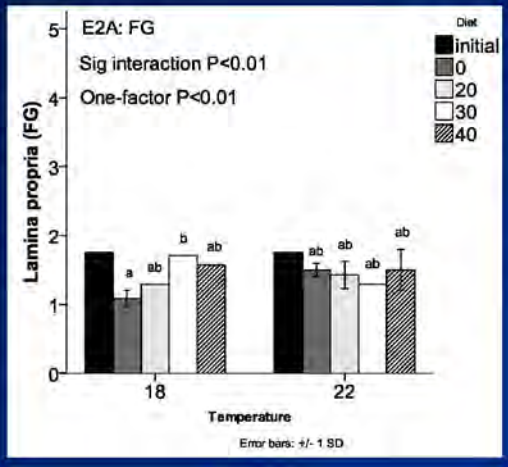


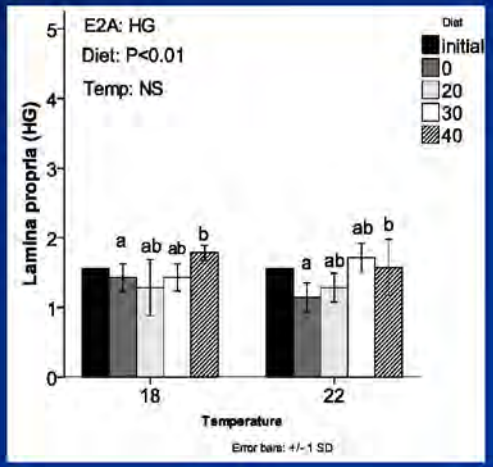


Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

SPC- Lamina propria

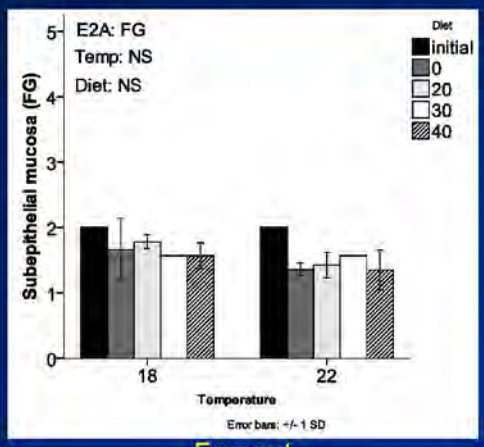




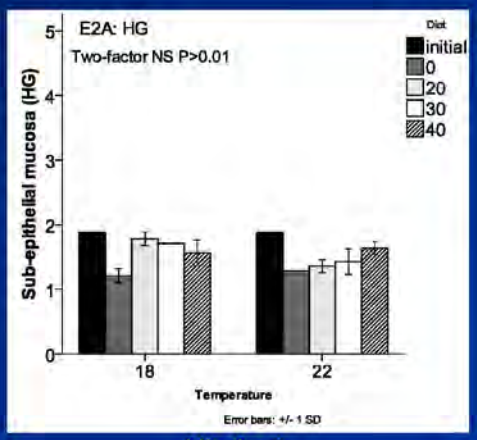
Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

SPC-Sub-epithelial mucosa



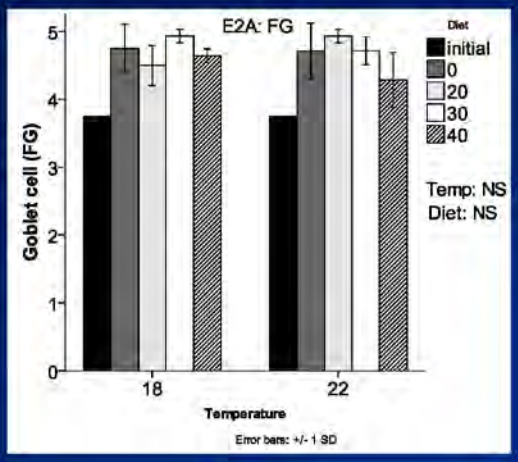
Foregut



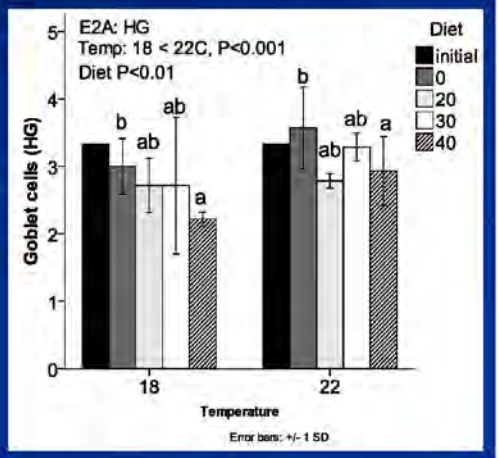
Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

SPC-goblet cell proliferation



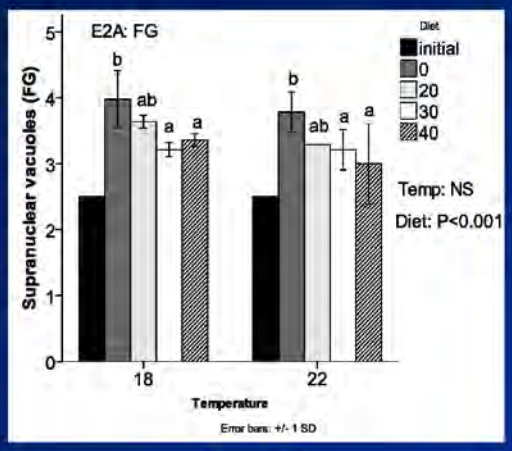
Foregut

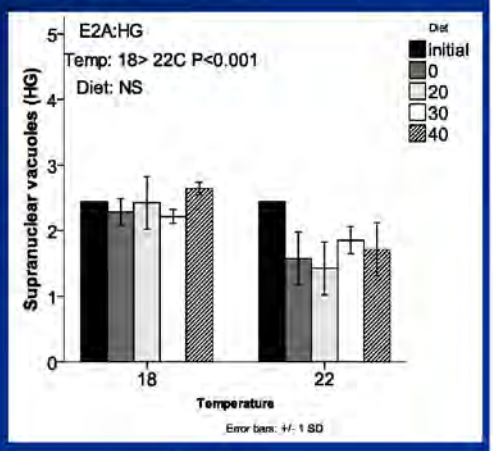


Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

SPC-Supranuclear vacuoles





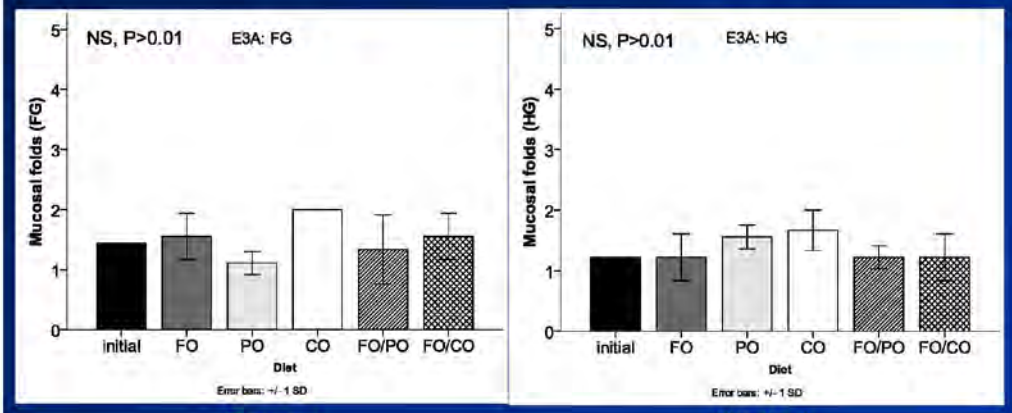
Foregut

Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

Results
Exp. 3 Lipid study at 22°C

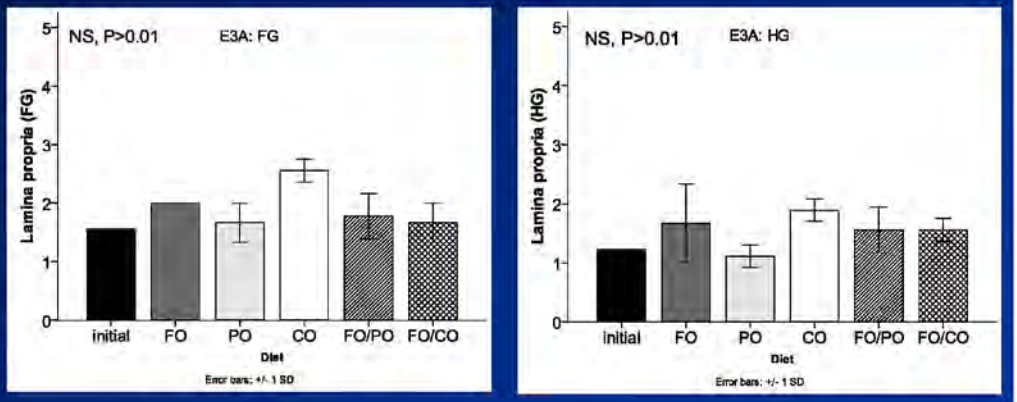
Results Lipid - 22°C Mucosal folds



Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

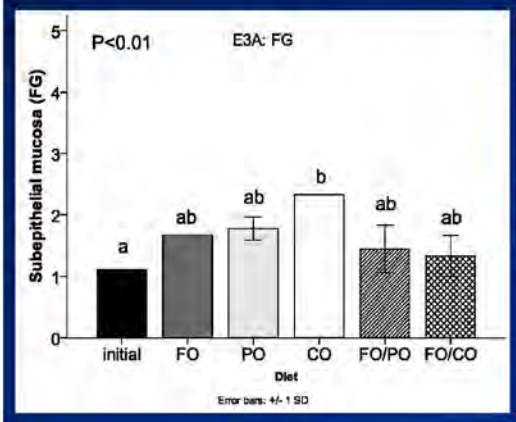
Lipids - 22°C Lamina propria

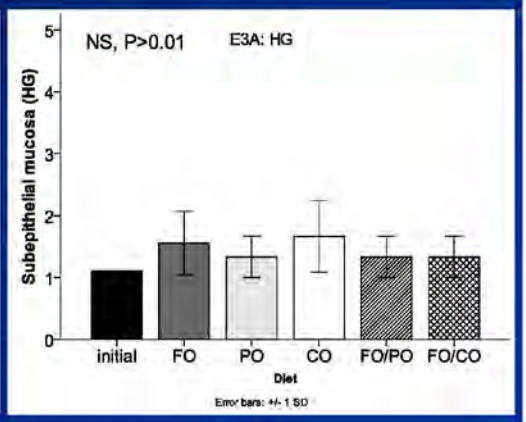


Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

Lipids-22°C Sub-epithelial mucosa

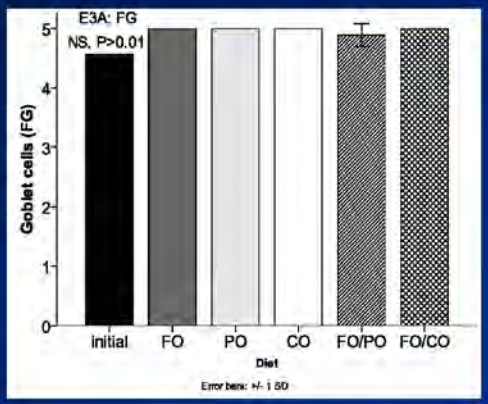


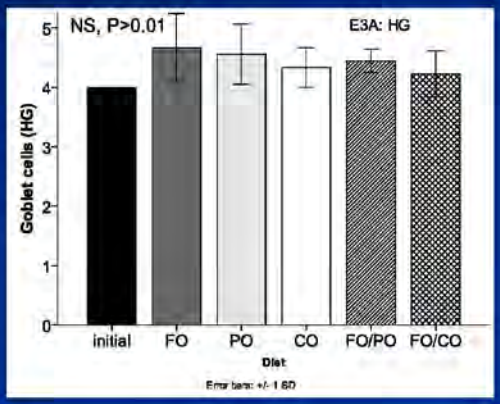


Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

Lipids - 22°C Goblet cell proliferation

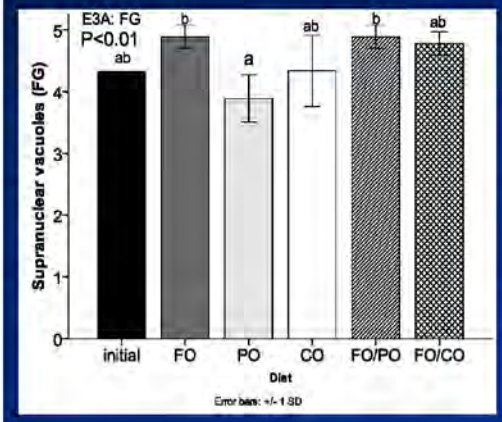


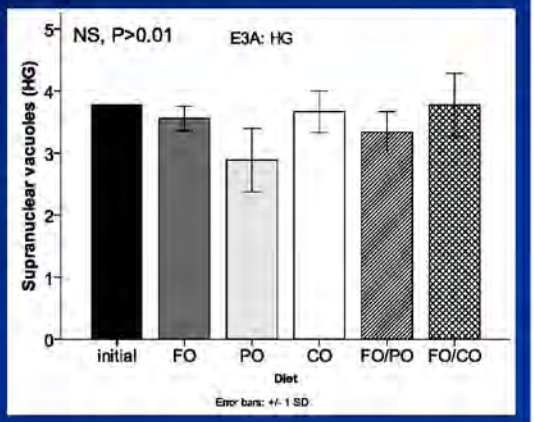


Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

Lipids-22°C Supranuclear vacuoles



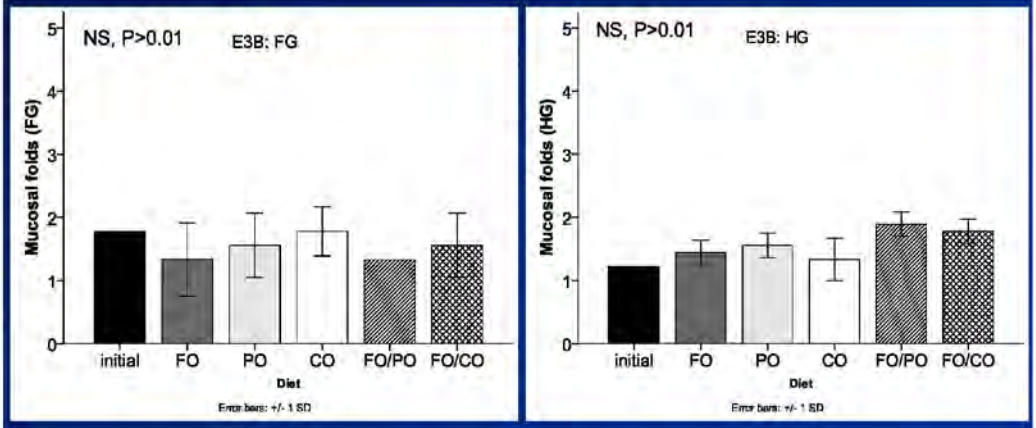


Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

Results
Exp. 3 Lipid study at 18°C

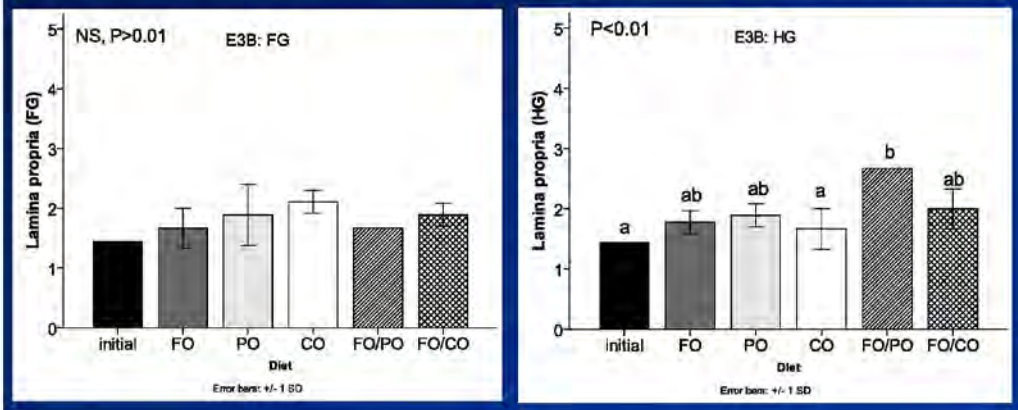
Lipid -18°C Mucosal folds



Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

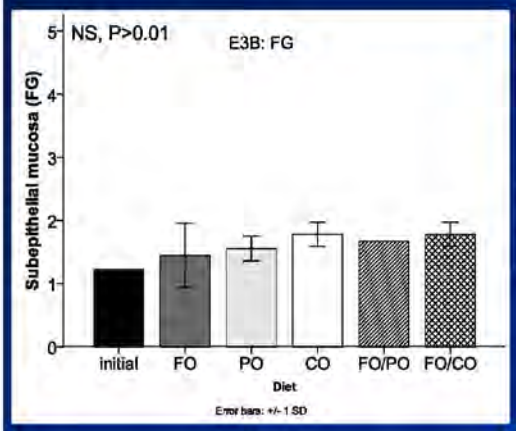
Lipids -18°C Lamina propria

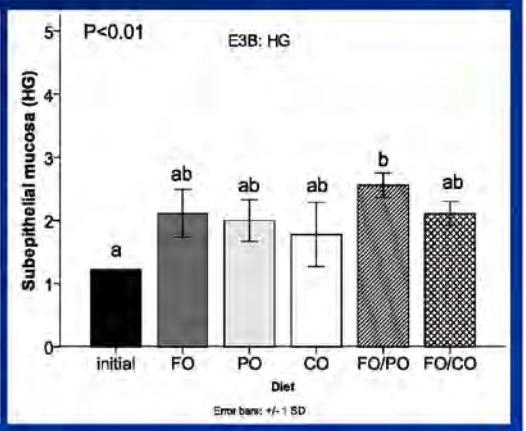


Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

Lipids -18°C Sup-epithelial mucosa

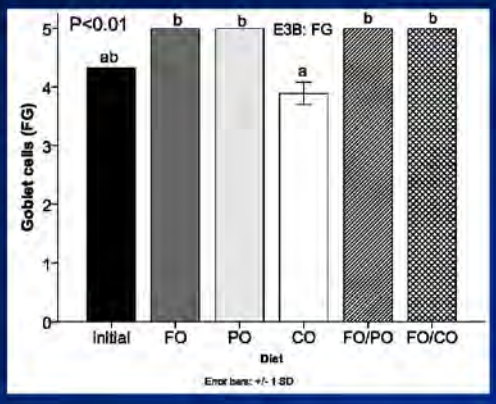


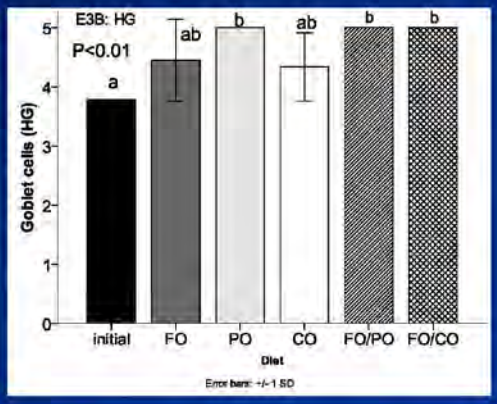


Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

Lipids -18°C Goblet cell proliferation

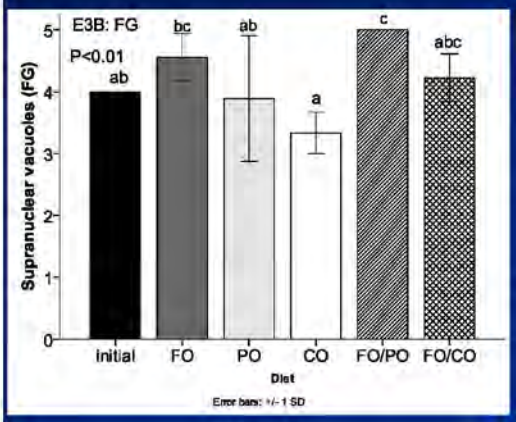


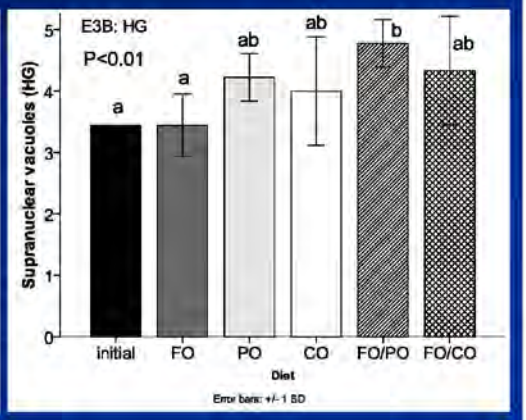


Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

Lipids -18°C Supranuclear vacuoles





Foregut Hindgut

1 represents normal tissue structure and 5 represents extensive structural changes

Background

- Soybean meal inclusion in salmon feeds cause san inflammatory response in the mucosal lining of the distal intestinal tract of Atlantic salmon, described as soybean enteritis (Van den Ingh et al., 1991).
  - The alcohol soluble fractions (soy saponins)are thought to be responsible.
- SBM induced enteritis described to a limited extent in rainbow trout, carp, snapper & Atlantic cod
  - although often does not progress to severe levels equivalent to those considered pathological in Atlantic salmon
- Potential to reverse the effects of enteritis with return to normal feeding
- Removal of the alcohol soluble fraction by processing to make soy protein fraction solves problem in Atlantic salmon
  - at additional cost
- The anterior distal intestine (hindgut) is the primary site in salmonids where enteritis-like changes are observed,
  - 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
- At the time of these studies SBM was being used in YTK diets
- Yellowtail kingfish possess two ileal valves, separating the foregut, midgut and hindgut,
- Fish oil substitution may also lead to digestive tract alterations

Methods – Statistics

- Data were assessed for normality and homogeneity of variance
- 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 not independent therefore were statistically analysed separately.
- Lipid experiments were analysed separately (One-way ANOVA; P=0.01).
  - Initial fish were included in One-way ANOVA
- For both Soy experiments (Two factor ANOVA; temperature &diet).
  - Initial fish not included in Two-way ANOVA
- 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.

Appendix 9.6f: Dietary investigations and determining their impact on fish performance (Trent D’Antignana)

CLEANSEAS

Dietary investigations and determining their impact on fish performance and survival

Dr. Trent D'Antignana

CLEANSEAS

Background

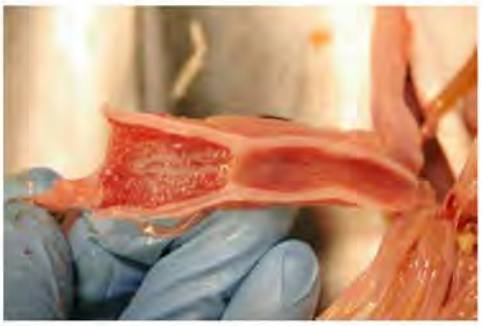
- Nutritional factors including,
  - Protein sources and inclusion
  - Lipid sources and inclusion
  - Omega 3:Omega 6 ratios
  - Amino acid contents
  - Mineral contents and interactions

  are known to influence:
  - Growth
  - Survival
  - FCR
  - Immuno – competence
  - Intestinal health

- YTK dietary formulations have changed significantly over the years

Background

- CST has little knowledge of the nutritional composition of its diets and what impact the dietary changes have had on fish performance and survival.
- The objective of this study was to identify whether dietary factors could be contributing to the disease picture. This entailed two activities:
  - Comprehensive dietary analysis
  - Commissioning of a commercial diet trial



ASX:CSS

Activity 1:

- 8 diets from both Ridley's and Skretting were analysed by a NATA accredited lab for:
  - Proximate's
  - Fatty acid profile
  - Amino acid profile including taurine
  - Minerals
- The results were reviewed by Dr. David Stone (SARDI) and CST staff.

ASX:CSS

Activity 1: Results

Proximate analysis

	Type	Units	Diet A	Diet B	Diet C	Diet D	Diet E	Diet F	Diet G	Diet H
Proximates	Ash	%m/m	9.5	11.8	13.1	12.1	8.3	8.1	10.4	10.1
	Protein		54.3	52.6	49.4	50.3	54.5	53.2	51.7	57.6
	Moisture		7.0	9.1	8.6	8.8	5.0	6.1	7.1	9.1
	Fat		18.7	18.0	19.7	18.1	19.2	22.1	24.0	17.0
	Carbohydrate		10.5	8.5	9.2	10.7	13.0	10.5	6.8	6.2
	Energy	kJ/100g	1790	1710	1730	1710	1860	1900	1880	1710

Activity 1: Results

Mineral analysis

	Type	Units	Diet A	Diet B	Diet C	Diet D	Diet E	Diet F	Diet G	Diet H
Minerals	Aluminium	mg/kg	62	43	110	86	46	20	49	86
	Antimony		<0.01	<0.01	0.48	0.11	<0.01	<0.01	0.030	<0.01
	Arsenic		1.2	2.0	1.4	1.2	1.1	1.3	1.3	2.7
	Barium		3.5	2.8	13	8.4	2.7	3.8	4.1	2.0
	Beryllium		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	Bismuth		<0.01	<0.01	0.043	<0.01	<0.01	<0.01	<0.01	<0.01
	Boron		5.0	3.6	2.5	1.1	2.8	3.1	0.6	1.9
	Cadmium		0.38	0.68	0.49	0.56	0.23	0.52	0.50	0.28
	Caesium		0.023	0.019	0.022	0.015	0.025	0.024	0.019	0.028
	Calcium		27000	35000	43000	38000	17000	17000	27000	23000
	Choline	mg/100g	178	164	133	164	181	192	165	186
	Chromium	mg/kg	0.96	0.69	0.85	1.3	0.55	0.43	0.57	1.9
	Cobalt		1.3	0.14	0.19	0.17	1.2	1.2	0.13	0.22
	Copper		7.9	9.4	12	11	12	12	13	11
	Iodide (Potassium Iodide)	ug/100g	290	300	340	390	200	160	470	760
	Iron	mg/kg	240	660	530	570	490	420	660	320
	Lead		0.11	0.22	0.43	0.32	0.068	0.068	0.26	0.16
	Lithium		0.38	0.19	0.19	0.15	0.21	0.18	0.12	0.33
	Magnesium		1800	1600	1500	1400	1500	1800	1300	1700
	Manganese		59	150	51	41	30	38	37	35
	Mercury		0.088	0.074	0.044	0.046	0.052	0.016	0.032	0.040
	Molybdenum		0.58	0.57	0.38	0.43	1.0	0.62	0.41	0.42
	Nickel		1.2	0.64	0.46	0.92	1.3	1.5	0.52	1.3
	Phosphorus		18000	20000	23000	20000	17000	14000	17000	18000
	Potassium		5200	6100	4900	5000	4900	6900	5800	9700
	Rubidium		2.9	2.0	2.2	2.3	3.3	2.9	5.0	1.7
	Selenium		1.7	1.9	1.6	1.9	1.5	1.3	1.6	1.5
	Silver		<0.05	<0.05	<0.05	<0.05	0.065	0.055	<0.05	<0.05
	Sodium		4900	6300	6900	5700	6700	7200	6900	7700
	Strontium		84	84	85	83	66	46	49	46
	Thallium		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.011	<0.01
	Tin		0.047	<0.03	0.040	<0.03	<0.03	<0.03	<0.03	<0.03
	Uranium		0.026	0.025	0.023	0.019	0.032	0.019	0.073	0.79
	Vanadium		1.6	0.24	0.26	0.26	0.80	0.25	0.26	2.3
	Zinc		140	86	110	110	120	120	170	170

Activity 1: Results

Amino acid analysis

	Type	Units	Diet A	Diet B	Diet C	Diet D	Diet E	Diet F	Diet G	Diet H
Amino Acids	Alanine	mg/100g	3426	3194	3432	3188	3231	2985	3575	3186
	Arginine		3720	3596	3639	3885	4156	3399	3921	3614
	Aspartic acid		4832	4251	4826	4690	5844	5230	4585	5561
	Glutamic acid		7460	7038	5038	5136	6491	6621	5389	8436
	Glycine		3198	3666	4225	4102	3187	3185	4106	3617
	Histidine		2371	1632	1284	1201	2148	1884	1731	1547
	Isoleucine		2241	1957	1943	2076	2177	1901	1894	2535
	L cystine		1049	1056	1339	1423	1285	1019	1177	892
	Leucine		3880	3429	4018	3851	4493	4118	3821	4161
	Lysine		3940	3165	2832	2850	4050	4065	3915	4719
	Methionine		1106	912	920	877	982	1005	934	1354
	Phenylalanine		2210	2057	2357	2240	2584	2488	2225	2434
	Proline		2224	2049	3354	3414	2258	1742	2364	2205
	Serine		2091	2187	2515	2600	2514	2291	2156	2149
	Taurine		191	223	245	158	127	234	218	331
	Threonine		2114	1944	2047	2043	2387	2143	2094	2263
	Tryptophan		649	519	510	517	665	618	575	647
	Tyrosine		2046	2025	1480	1457	1698	1495	2240	1771
	Valine		3082	2836	3694	3616	3846	3528	3283	3472

Activity 1: Results

Fatty acid analysis

	Type	Units	Diet A	Diet B	Diet C	Diet D	Diet E	Diet F	Diet G	Diet H
Fatty Acids	Butyric C4:0	mg/100g	<10	<10	<10	<10	<10	<10	<10	<10
	Caproic C6:0		<10	<10	<10	<10	<10	<10	<10	<10
	Caprylic C8:0		<10	<10	<10	<10	<10	<10	<10	<10
	Capric C10:0		<10	<10	<10	<10	<10	<10	<10	<10
	Lauric C12:0		11	18	18	18	14	22	21	17
	Tridecenoic C13:0		<10	<10	<10	<10	<10	<10	<10	<10
	Myristic C14:0		630	900	540	560	800	1020	1070	1100
	Pentadecenoic C15:0		56	87	66	69	70	83	78	89
	Palmitic C16:0		3890	5040	4110	5750	3690	4280	4680	2800
	Margaric C17:0		62	82	94	86	80	92	84	76
	Stearic C18:0		1030	720	1400	1220	960	1100	1160	560
	Arachidic C20:0		36	52	33	34	42	45	54	37
	Docosenoic C22:0		24	47	16	16	28	18	19	19
	Tetracosanoic C24:0		<10	19	<10	<10	16	<10	11	11
	Total SFA		5538	4948	6277	5747	5670	6660	7177	4709
	Decenoic C10:1		<10	<10	<10	<10	<10	<10	<10	<10
	Myristoleic C14:1		43	46	60	50	46	62	56	37
	Pentadecenoic C15:1		<10	16	13	11	<10	16	12	13
	Palmitoleic C16:1		1050	1230	1130	1170	1380	1640	1730	1160
	Heptadecenoic C17:1		<10	22	<10	10	<10	19	<10	17
	Octadecenoic C18:1n-6		12	19	16	15	<10	18	15	19
	Octadecenoic C18:1n-7		460	480	470	440	580	610	600	420
	Oleic C18:1n-9		6040	5010	6300	5260	4220	5170	5820	1560
	Eicosenoic C20:1n-9		120	310	120	110	140	150	170	410
	Eicosenoic C20:1n-11,13		16	19	17	17	12	17	22	37
	Docosenoic C22:1n-9		14	46	10	10	18	16	15	41
	Docosenoic C22:1n-11,13		<10	<10	<10	<10	<10	<10	<10	<10
	Tetracosenoic C24:1		24	66	24	24	36	38	38	82
	Total MUFA		7777	5274	8147	7096	6382	7756	8483	3798
	Linoleic C18:2n-6		1930	870	1440	1210	1280	1440	1430	640
	Gamma Linolenic C18:3n-6 (GLA)		27	41	28	29	42	51	48	37
	Stericonic C18:4n-3		94	290	110	130	220	270	230	380
	Eicosadienoic C20:2n-6		24	36	23	20	19	29	24	31
	Dihomo-gamma-linoleic C20:3n-6		18	22	16	17	20	24	29	21
	Arachidonic C20:4n-6		97	140	72	94	150	120	130	160
	Docosatetraenoic C22:4n-6		30	59	37	34	58	33	38	67
	Docosapentaenoic C22:5n-6		25	76	29	34	62	69	94	88
	Total n-6 PUFA		2153	1243	1645	1438	1621	1766	1793	1054
	Alpha Linolenic C18:3n-3		330	210	270	240	220	250	280	190
	Eicosatrienoic C20:3n-3		<10	17	<10	<10	<10	11	10	17
	Eicosatetraenoic C20:4n-3		57	200	47	58	88	110	160	280
	Eicosapentaenoic C20:5n-3 (EPA)		560	1730	640	290	1420	1630	2130	2190
	Heneicosapentaenoic acid C21:5n-3		<10	<10	<10	<10	<10	<10	<10	<10
	Docosapentaenoic C22:5n-3		120	240	98	110	170	210	260	270
	Docosahexaenoic C22:6n-3 (DHA)		200	1800	680	830	1540	1170	980	1680
	Total n-3 PUFA		1767	4180	1735	2028	3438	3370	3790	4610
	EPA+DHA		3918	3423	3080	3466	5068	5136	5583	5664
	Total LC PUFA		1382	3707	1418	1730	3130	3020	3350	4140
	n-3/n-6		0.8	3.4	1.1	1.4	2.1	1.9	2.1	4.4

Discussion

- Taurine may be deficient in *all* of the diets considering 0.5-1% taurine is thought to be required for optimal performance.
  - Taurine deficiencies have caused anaemia and green liver in yellowtail and can result in reduced growth and immuno – competence and increased disease susceptibility
- LC PUFA appear to be low in several diets (NRC 2011 suggests that levels of LC PUFA should be at least 2% for fish > 50g) – *3 diets were below this limit*
- There appears to be considerable variability in the nutrient profile within a particular type of diet.

Action

- ***Undertake activity 2: Conduct a feed trial to determine whether 2% supplemental taurine (top coated) improves fish performance.***
- ***Top coat existing diets with 1% crystalline taurine and administer to production fish.***

Activity 2:

Feed trial to determine whether 2% supplemental taurine (top coated) improves fish performance

- Experimental design
  - Facility: SBT hatchery (4 x 9m tanks containing, 4 x 1.5m Ø cages)
  - Water temp: 20°C
  - Duration: 3 months, started on the 22/6/12
  - Fish: 2012 year class fish from Port Lincoln with the lowest mortality.
  - Treatments:
    - D1 - Ridley Triumph (high FM/FO)
    - D2 - Ridley Marine CST
    - D3 - Skretting Optima YT100
    - D4 - Skretting Optima YT100 + 2% crystalline taurine
    - D5 – Ridley Marine CST + 2% crystalline taurine

Note: all diets were top coated with 2% FO, 3 reps (cages) / treatment randomised design

- *Feeding: Fed twice daily according to feed model, based on starting weight*
- *Biometrics:*
  - The individual weight and length of each fish was recorded
  - Starting weight of the cohort ≈ 257g
  - CV of the cohort ≈ 14% (graded twice to eliminate small and large fish)
  - Standard deviation in average weight between treatments ≈ 1.9g

CLEANSEAS

Activity 2:

- Sampling points: start, middle and end
- Analyses:
  - Growth
  - FCR
  - Survival
  - Histology (all organs with an emphasis on intestinal health)
  - Haematology (haematocrit, blood smears, RBC fragility)
- Progress to date
  - Baseline histology taken but not analysed
  - Baseline haematocrit value was 38.9% ± 8.2% (*n=20*), 60% of the fish were below this value.
  - Suggests the fish may in fact be anaemic, a condition associated with taurine deficiency.
  - Baseline data from "normal" YTK are required to validate this claim.

ASX:CSS

Appendix 9.6g: Minutes from the Australian Seafood CRC Yellowtail kingfish health workshop (Erin Bubner)

Australian Seafood CRC – Yellowtail kingfish health workshop

Thursday 12th July 2012, 9:00 am – 5:00pm

SARDI Aquatic Sciences, 2 Hamra Ave, West Beach, SA 5024

DRAFT MINUTES

1.0 **Welcome (Craig Foster) (9am**)

1.1 Thanks given to all attendee's for coming to the workshop.

1.2 The aim of workshop is bring attendee's together, brief them on what CST has found regarding the health issue currently impacting YTK production and discuss where to from here. CST wants to come up with an investigative pathway that has been fully considered to overcome the current health issue. Draft plan available to attendees.

1.3 The current health issue threatens the YTK industry as a whole. If we don't resolve it there is likely to be no YTK industry in Australia. This will impact largely on the customers. Performance of YTK has declined over the past 3 years. There has been a decline in growth and FCR and an increase in mortality. It has got particularly worse in the last 6 months and is now threatening financial viability of CST. In the last 6 month it has cost the company $10 million in growth and $1 million per month in mortalities. The whole disease investigation that CST has been focussing on in the last 6 month has cost $0.5 million to get where the company is today (i.e. treatment costs, pathology costs, vet service fees and getting experienced personnel together). Cost of lead up to workshop to get information on the table for consideration was $70,000.

1.4 Acknowledge Australian Seafood CRC and FRDC for sponsoring the workshop and for the collection of information up to this point.

1.5 CST is in a very urgent situation to get this problem solved to give some confidence back into the industry to rebuild it. There will be no way to move forward without an answer to this problem, as there will no confidence for future investment in the industry. Therefore, there is a need to finish today with well consulted and considered plan.

1.6 Participants introduced themselves giving brief background of their expertise and involvement with work to date.

1.7 **Present**: Erin Bubner (Flinders University), John Carragher (Logifish Consulting), Michelle Dennis (QML Pathology), Fran Stephens (Department of Fisheries in WA), Richard Smullen (Ridley Aquafeed), Mark Crane (CSIRO), Graham Mair (Australian Seafood CRC), Shane Roberts (Aquatic Animal Health Program, PIRSA Fisheries Aquaculture), Kristen Just (Ridley Aquafeed), James Munro (SARDI), Rebecca Forder (University of Adelaide), Mark Booth (NSW Fisheries and Aquaculture), David Stone (SARDI, Aquatic Sciences), Matthew Bransden (Skretting), Rhys Hauler (Skretting), Marcus Stehr (CST), Mehdi Doroudi (PIRSA), Mike Thompson (CST), Erica Starling (WA), Masashi Maita (Tokyo University and Marine Science and Technology), Marty Deveney (MISA Marine Biosecurity), Mark Shepard (Veterinarian consultant), Gavin Begg (SARDI Aquatic Sciences), Damian Crinchley (CST), Len Stephens (Australian Seafood CRC), Matt Landos (Future Fisheries Veterinary Service), Trent D'Antignana (CST), Andy Bell (University of Queensland), Craig Foster (CST), Charles Caragell (School of Animal and Veterinary Sciences, The University of Adelaide), Grant Richards (Parasite Diagnostic Services, All Farm Animal Health).

1.8 **Apologies**: Christine Huynh, Chris H and Lyndon Giles

2.0 **Health workshop program overview (Trent D'Antignana) (9.05am)**

2.1 Changes to agenda**:**

2.1.1 Mark Shepards presentation originally scheduled for 1.30pm changed to 9.10am. All presentation moved down one time slot.

2.1.2 Matt Landos's presentations on 'viral research conducted to date' and 'treatment plan to control protozoan parasites' were removed from agenda to allow more time for open discussion.

3.0 **Considerations and comparative pathology (Mark Shepard) (9.10am)**

3.1 Presentation is a general overview, looking at different aspects of enteritis over the past several years to set the frame work for today's workshop.

3.2 Background – since the mid 1990's there was a shift in complete use of FM and FO in fish diets to substitutes. At the same time started to see

changes in the intestine. Anecdotal and clinical diagnostics, but not collected much data.

3.3 Overview of what we know about enteritis, general signs of enteritis and anatomy of the intestine.

- 3.3.1 Wide spread condition. Mostly focus on villi in today's discussion (i.e. inflamed intestine).
- 3.3.2 Example - early observations in chinhook salmon in Canada, which caused a bloat like condition (gastric dilatation). Related to feeding (i.e. breakdown of food too quickly in the gut. To try and compensate for that the fish gorge on food, the food gets regurgitated and it irritates the gut. Also the shape and physical characteristic of pellet and a stressful environment can also influence this). A thickened intestine was also observed and there were many different schools of thought relating to its cause –microsporidiosis?, retroviral?, general inflammation?, A syndrome?, or plasmacyte lucemia?.
- 3.3.3 Example – Red intestine observed in carcasses observed in Atlantic salmon in the mid 1990's, which was initially thought to be a change post mortem; however, over time observations of moribund fish showed acute redness at vent. This condition could also be detected through observing the gills, as there was a pathognomonic lesion related to it (i.e. thymic reaction). Subsequently, initially thought condition was caused by toxic reaction, as each time they tried to collect bacterial sample from intestine, cultures showed monoculture only. Presented, histological images of epithelium and lamina propia of Atlantic salmon exposed to soybean meal in their diet. Non-infection reaction to feed itself, considered initial irritants in the gut. Results referred to have been published.
- 3.3.4 Example - wild sockeye salmon was also observed to have red intestine. Highlights important to consider how much has changed after death.

3.4 YTK

3.4.1 Observed thin red gut, often occurs in cooler temperatures. However, there appears to be lots of compounding factors, such as water temperature, stress, types of feed and secondary opportunistic lesions.

3.4.2 Antibiotics transiently useful.

3.4.3 Enteritis observed in YTK was similar to what is observed in the Atlantic salmon and Chinook salmon. Thin, necrotic intestine.

3.4.4 Visits to cages this week with PL. Observed 20 month old fish. Paper thin intestinal wall, thicken intestine observed, highly likely that the mucosal layer is also thickened limiting movement of food through intestine, no abdominal fat, dark green gallbladder observed suggesting that it has not been eating properly for a long time, diarrhea, no lumen. Green liver was also evident, commonly mentioned in the literature from Japan and will be discussed later in workshop.

3.4.5 Important to consider what is the association and what is the causation. We need to ask ourselves if we are asking the right questions. Provided example from the literature.

3.5 Figure on cellular metabolism presented.

3.6 List of virtues of taurine. Taurine has been shown to be essential to *Seriola* spp. (e.g. conjugates with bio-acids, emulsifies fatty acids to make them available to the fish, effects membrane stability and facilitates ion gradient). Literature indicates YTK cannot perform well when deficient in taurine.

3.7 Figure showing growth of aquaculture and use of FM presented. Observe a divergence in the two lines in the mid-1990's when people wanted Aquaculture to become more sustainable and subsequently had to stop using so much FM. Thus alternate proteins and oils started to be used.

3.8 Overview of the anti-nutritional considerations of the diet.

3.8.1 Example – studies have shown large reactions in Atlantic salmon when presented with anti-nutritional factors in the diet. It is not as well understood for YTK.

3.8.2 Example – it is known that *Seriola* spp. do not perform well at low water temperature (<17°C). Their metabolism starts to shut

down and the bio-availability of products and digestion may become diminished. Australian study has shown at lower water temperatures gut transit times decreases. This will have an effect on an osmolality level in the distal gut.

3.8.3 Example – in the recent past Japanese primarily used non-extruded feed (i.e. frozen products), which showed good survival. Now moved largely to extruded pellets. Subsequent problems observed include dark fish, dry fish, green livers and winter syndrome.

3.8.4 Posed question of what is the environment introducing to these animals? What are the influences of these interactions on the cultured fish?

3.9 Summary

3.9.1 If you have a primary or entropathogens in your system, one of four things is likely to happen: 1) toxin production, 2) overgrowth of the epithelium, 3) lesion or 4) complete disruption of function. The basics suggest that there is a primary cause and the rest is just an exacerbation, which causes a snowball effect.

3.9.2 Working hypothesis. There is some sort of primary nutritional challenge to the animals. From this a non-infectious irritation might be happening in the intestine, causing a thickening of the intestine wall, which may be leading to mal-digestion or mal-absorption so the fish are not getting as much as they want to out of the feed. Subsequently, there may be a real imbalance or functional imbalance. If that is happening, then this is the creating cascade effect. First, get a host response, which is exposed to range of stressor that will exacerbate the response, than get secondary exacerbations again and this creates a ‘perfect storm’. What can we do to break this cycle? This is the question we are posing and trying to answer today.

3.9.3 Posed question for physiologist, biochemistry nutritionist and pathologists present – within 30 seconds of killing fish that have a thickened intestine, it instantly goes completely hard. Does it

fit with underlying osmotic or ionic crisis in the fish that would stimulate rigor?

3.10 Questions and comments

3.10.1 Rhys Hauler - What were the agents for non-infectious changes in intestine? Mark Shepard - Suspicious of feed changing and therefore assumption were then made as potential cause. Initially it was observed in many large fish that were eating a lot of the feed, not just the small and poor performing fish. Meetings were then had with the feed company to try and come up with an affordable feed that doesn't compromise the health status of the fish. Didn't investigate to same level as CST, where their investigation was initially based on a potential bacterial invasion.

3.10.2 Richard Smullen – Clarified that results presented today that showed enteritis caused by inclusion of soya in the diet, was defatted soya, not soya bean concentrate. Important to make that distinction.

3.10.3 Grant Richards - In reference to thickened gut, what is the cellular change in an atrophic intestine? Matt Landos - Primary change observed in all YTK is caused by lymphocytic enteritis.

4.0 **Disease overview in the 2011 year class (Matt Landos) (10am)**

4.1 YTK culture history – both feed companies have supplied various amounts of feed in many different feed formulation at various times. Whole feed formation has never been disclosed from either feed company.

4.2 Problem outline – poor growth, elevated morality since at least early 2010. Affected year classed 2009 2010, 2011 and 2012. Fish transferred to sea at 8 g and starts to affect fish sizes 30 to 3000 g. All three sites effected (AB, W, PL). 2011 and 2012 year classes have been worst performing – slowest growth and highest mortality.

4.3 Farm locations – all shallow sites (20m or less). Each site has their own problems (e.g. other industry operating in PL in close proximity, AB exposed to wind and swell and W exposed to high salinity, low DO, industrial proximity).

4.4 Investigation methods – standard template used. Clinical signs, data collection (e.g. mortality, water quality data, epidemiology), gross pathology, microscopy, microbiology, virology (all negative results to date), clinical trials, trials with diseased fish (i.e. tank trials to try and exacerbate problem – good survival in these fish), histopathology (all reports available to attendees), feed analysis to ascertain if feed is causal agent, clinical pathology looking specifically at some selected blood parameters and collaboration with other researchers and staff.

4.5 Histological findings (2011) – summary of report.

4.5.1 Lymphocytic enteritis. Most common across all effected fish, all year class and highest prevalence. It has been commonly associated with coccidian like and myxidia like infestations in the 2011 YTK. Have not been able to identify them to species level. Association with the level of enteritis and the amount of parasites present and thought initially to be large contribution factors to the problem.

4.5.2 Fluctuating gill and skin fluke infestations. Primary pathogen in YTK and have observed some very heavy infestations and challenges with levels going up and down depending on bathing cycles/regime.

4.5.3 Periodic epitheliocystis infections of the gill have been associated with spikes in mortality, over the top of chronic baseline of elevated mortality. Elevated level of mortality, irrespective of the presence of absence of eopitheliocystis, but these then make it worse.

4.5.4 Kidney/renal myxosporidiosis, tubular proteinosis – loosely linked with protein being reabsorbed out of the lumen of the kidney into the kidney tubule cells.

4.5.5 Photobacterium septicaemia – affected in particular young fish after transfer offshore.

4.5.6 Mycobacteriosis – W only, which was resolved after moving to AB.

4.5.7 Disseminated protozoan infection possibly coccidian/myxidia, blood fluke – W only.

4.5.8 Severe lymphoid depletion.

4.5.9 Fibrosing pancreatitis, hepatic necrosis – different liver cell dying (prompted viral examination – results negative).

4.6 Mortality patterns – conducted feed trial between two companies with 2011 fish (Skretting and Ridley). Mortality data presented.

4.6.1 2.2 and 2.9kg fish, all pipe feed, with spinning fed, trial cages were located on grid system on feed barge – ruled out as being influential factor.

4.6.2 9mm diet distinct difference in mortality (March to July 2012).

4.6.3 Difference in fluke intensity between treatments was also observed.

4.6.4 Strong indication that feed influencing factor – many external factors considered.

4.6.5 Question. Mehdi Doroudi – Did you start seeing a problem in one area initially, have you seen a link to size of fish or source of fish? Matt Landos – Differences in intensity has been observed. Mortality was initially worst in W compared to AB and was one of the main reasons why these fish were moved from W. Concerns there were too many stress factors in W regarding to their environment that may have been compromising the fish. Enteritis was already present at AB.

4.7 Treatment trials

4.7.1 Oxytetracyline - Controlled photobacterium sepsis. Saw clinical response in treated fish.

4.7.2 Trimethoprim sulphonamide - Negligible effect on mortality rate or presence /absence or levels of coccidian on histologically examined fish.

4.7.3 Amprolium – To treat myxosporean. Negligible response.

4.7.4 Amprolium/salminmycin – investigation based on European results with sea bass, when thought primary pathogen in the gut was playing major role in problem. Possible response in coccidian/myxidia. Had difficultly bathing the fish at time of treatment and subsequently had high gill fluke infestations, so mortality benefits of the treatment may have been overwritten by

the problems associated with high fluke levels. However, pathology results showed that parasite load may have been lowered by the treatment.

4.7.5 Diet trial (mid Dec 2011 to early June 2012) – examined Ridely and Skretting 9mm diets. Ridley diet had higher crude protein, Ridley diet lower crude lipid level, FM confidential, FO confidential, Taurine level to be tested, but assume deficient, n3:n6 ration to be tested, but expected to be deficient.

4.7.6 Question - Mehdi Doroudi – Was there any big difference in the feed in previous years to 2010 onwards. Matt Landos - Yes, FO generally declined and FM variable over the years (ranging from 25-60% in diets). Suspect problem here is that information on macro levels of nutrients is insufficient. Within each raw material there is great divergence in quality and micro-nutrients so it is important to know what these are. We need to know what they are consuming completely, where at the moment most of this information is unavailable. If we want to understand micro-nutrition in fish we need to know these details. Then, need to take the next step to look at what the fish is taking from the feed (i.e. bioavailability). Need this information to move industry forward.

4.8 Immunosupression (caused by cage stresses, dietary deficiencies)

4.8.1 Diet trials have shown that they are in a stressful environment at the sea cages – i.e. if fish grown in tanks, it has been shown that they do not develop enteritis and don't generate much mortality when fed the same diets that they were fed in the sea cages. If we move diseased fish back into tanks, survival improves. There is an overlay of environmental stress on the fish that is altering the nutritional requirements of the fish therefore assumptions of dietary needs cannot be determined from tank trials. Comment: David Stone – Tank trial not suppose to transferred directly in cage, should always conduct pilot trial in cage situation, then to commercial scale. Some things may have been overlooked in the past.

4.8.2 Lowered mucosal immunity:

4.8.2.1 Increased red cell fragility.

4.8.2.2 Mildly anaemic fish - lowered total number of red cells circulating in the blood in what appear healthy fish and in sick animals number of red cells are lower again.

4.8.2.3 Low blood cholesterol level - half that of Japanese YTK. Taurine is required to produce endogenous cholesterol, which is needed in various physiological places in the body such as the cell membranes and mucous. Hypothesis – low blood cholesterol, altering the integrity of mucus lining of the gut, inflamed gut because we are getting more bacteria presented through the gut to immune system. Subsequently increased antigen presentation through gut mucosa.

4.8.3 Osmoregulation dysfunction in the fish – in moribund fish, pathologists do not see enough wrong with the cells to have caused them to die.

4.8.4 Increased disease intensity:

4.8.4.1 More gill/skin/gut parasitism – i.e. gill mucus is not as protective as it should be, fish will re-infect faster, which means they need to be bathed more often, which can cause mortality.

4.8.4.2 Decreased resilience to oxidative stress and chronic lymphocytic enteritis.

4.8.5 Question:

4.8.5.1 Mehdi Doroudi – Do you think quality of fingerlings or broodstock is an influencing factor? Matt Landos – In terms of hatchery origin to mortality outcome we have seen a difference in 2012 year class with the severity of the problem. The fish that travelled from PA to AB to be stocked had much longer transportation stress and those populations were worse than AB populations, but very noisy data. Sampled fish from each hatchery before transfer to sea and histology results showed no evidence

of enteritis. There are several broodstock that contribute to each year class of fish from several tanks, but we don't see a great difference between performance of larval runs and thus don't think we have a genetic factor.

4.8.5.2 Mehdi Doroudi – Have the same broodstock been used since 2010? Matt Landos - Yes. Mehdi Doroudi suggests that we should not discount this as playing a role. Also, suggests need to be more specific in terms of what we are referring to as cage stressors versus tank stressors, as there are stressors associated with each system.

4.8.5.3 Marcus Stehr– Is there a difference between size of fish at stocking and outcomes – Matt Landos – One trial stocked at 40g after vaccination (normally stock 8 -10 g). Had lower mortality in vaccinated fish compared to fish stocked at 8-10g, but eventually succumb and is now not much difference in mortality.

4.8.5.4 Graham Mair – Are there currently any progeny of F1 broodstock or are they all from wild stock? Matt Landos – Yes all from wildstock. Graham Mair - Is there a difference in salinity between locations and is there any correlation? Matt Landos - There has been differences noted between W and AB, where salinities differed.

4.9 Flow chart - Renal proteinosis (i.e. protein droplets appearing in cells lining renal tubular lumen). Flow chart available to attendees.

4.10 Flow chart - Hepatic necrosis (i.e. death of live cells). Flow chart available to attendees.

4.11 Flow chart – Enteritis. Flow chart available to attendees.

4.12 Analysis of feed:

4.12.1 Evidence of taurine deficiency (free taurine) (analysed by laboratory in NZ and confirmed by laboratory in Europe).

4.12.1.1 Confirmed anaemia, evidence of fragile blood cells, which will be discuss in more detail later today, and low blood cholesterol. Suspect immunosuppression as a

response. Green livers in starved fish (5 days) recently observed after being freshly harvested, which was only sporadically evident in the past. Observed as segmented green, not a diffuse green as observed in Japanese YTK.

4.12.2 Omega 3 fatty acids – show low, variable levels. We know we have had a low inclusion level in the diet.

4.12.2.1 Low taurine = low bile salts = low lipid digestion/absorption. Low levels are made in comparison to NRC requirements (3 out of 8 diets were below NRC requirements).

4.12.2.2 Low temperature decrease pancreatic lipase activity, high lipid diet drives increased bile salt demand. Low taurine = low cholesterol, which impacts on mucous production. Low n3 uptake is going to compound the impact on those cell membranes. Low taurine is associated with osmoregulatory impairment.

5.0 **Disease overview in the 2012 year class (Matt Landos) 11.30am**

5.1 Histological findings in 2012 year class:

5.1.1 Similar to 2011 fish; however, did not identify gut protozoa as playing a role and absence of gill fluke on most of the population. In addition observed epicarditis, which was a new lesion, which initially could not be explained but after extensive sampling it was eventually established that it was probably associated with the myxozoa.

5.2 Trial – Examine effect of environmental stress on health and survival of YTK, in particular the effect of high winds and swells in combination with varying diets. This was deemed an important factor to examine as the company found that on days when it was extremely windy, mortalities peaked from approximately 10 per day to up to 500 per day. It is believed the observed spike in mortality was related to stress, where the fish may have already been weak, possibly due to insufficient diet, and the high winds and swell compounded that issue causing further stress to the fish and subsequent mortality.

5.2.1 It was decided to stock two cages in PL in sheltered site to facilitate feed supply and minimise exposure to rough seas. 20,000 fish in total were involved in trial.

5.2.2 Results showed that enteritis still developed and mortality continued in both cages. Evaluated up to 6 different diet formulations from each feed company, which changed overtime according to what they believed at the time, were the factors that were deficient. Fish in each cage were medicated at one time point to treat bacterial gill disease (late May). A decrease in mortality was observed in one of the cage after a mortality spike in late April, early May. FM and FO were requested to increase in the diets from both companies and improvements were seen in survival following this, towards the end of the trial.

5.2.3 Key differences in the diets – protein Ridley > Skretting, lipid Ridley < Skretting, FM and FO confidential, other protein/oils confidential.

5.2.4 Early Ridley and Skretting were deficient and new diets were also considered deficient in some components based on analysis conducted by CST.

5.3 Suggestions to try and resolve health issue:

5.3.1 Complete formulation disclosure from feed companies to veterinarians, physiologist, and pathologist etc to understand what I happening here.

5.3.2 Raw material QA on each feed run.

5.3.3 Post milling QA to determine manufacturing losses – i.e. how much of the raw material ends up in the diet.

5.3.4 Monitoring fish and uptake of nutrients in fish (i.e. bloods, tissue levels of taurine, PCV, MCV, cholesterol, bilirubin).

5.3.5 Longer feed trials (i.e. 5 week is inadequate, as fish appear to sail along for 30 days, then after 60 days they and start to diverge). Major reasons why longer trials have not been performed in the past (i.e. cost, tank size etc).

5.3.6 Investigate if fish have deficiencies of other amino acids.

5.3.7 Conduct gut biota culture study.

5.4 Questions posed by Matt Landos for discussion:

5.4.1 What level of FM origin taurine is sufficient in diets for fish of different sizes in different water temperatures? How much taurine is in our FM supplied, what is the origin, has our raw material taurine level changed? What level of supplementation should be added at different times of year/season and for different sized fish?

5.4.2 What level of supplementation of crystalline taurine is therefore required?

5.4.3 Is the level for taurine supplementation required altered by other dietary components?

5.4.4 What is the optimal DP level in diet for YTK different sizes in different water temperatures?

5.4.5 What are the optimal DR levels in diet for fish of different sizes?

5.4.6 Changed to diets requested to increase FM and FO inclusion for PL 2012 fish. Why have the analysis results not reflected an increase in n-3 fatty acids and taurine increase compared to previous formulations?

5.4.7 What is the best / most rapid method for identification of gut protozoa, which have been labelled coccidian and myxidia, based on histological appearance?

5.4.8 Is it feasible to undertake infection trials with these organisms on fish which are on a range of diets to explore the role of diets in assisting infections?

5.4.9 Are treatment trials for organisation able to be organised / warranted and commercially viable?

5.4.10 Are there any alternative explanations?

5.5 Discussion will be held after last scheduled presentation.

6.0 A Japanese perspective on yellowtail health and our existing disease profile (Masashi Maita) (11.45am)

6.1 Overview of Japanese perspective on YTK health.

6.1.1 Particular focus on the problem of taurine deficiencies.

6.1.2 May be some relationship between problem in Japan and Australia.

6.2 Recent mass mortality of farmed YTK in Japan – two diseases were determined to be the cause.

6.2.1 Issue may be related to diet composition.

6.3 Presented results from experiment from Murata et al.

6.3.1 Experimental diets - NTS (i.e. no taurine supplement), LTS (i.e. low taurine supplement), HTS (i.e. high taurine supplement) and FM (fish meal). Inclusion levels of taurine were 0.73, 2.5, 21.4 and 4.08 g/kg respectively.

6.3.2 41 week trial, with 240 g fish.

6.3.3 Haematocrit levels varied between treatment groups, although in some cases not significant.

6.3.4 FM showed no incident of green liver.

6.3.5 Increased in incident from HTS, LTS and NTS, respectively.

6.3.6 Bile pigments (ug/ml) contents in liver of YTK increased in NTS groups, unlike other groups.

6.3.7 Results of study showed that taurine supplementation can reduce incident of green liver.

6.3.8 The chain of events leading to the appearance of green liver in YTK non-FM diets may be because bilirubin cannot be excreted adequately in the bile. The lack of taurine leads to greater hemolysis. These events result in bilirubin accumulations in liver causing green liver and / or inadequate taurine biosynthesis by the fish.

6.3.9 Haematological parameters continued to change up to 60 days.

6.3.10 Artificial infection with *P.piscicida* led to higher mortality when fed the non-FM diet compared to control group. Therefore showed that non FM diet caused decrease in disease resistance. Mortality was significantly higher between groups.

6.3.11 Summary of change between groups – non FM diet decrease Ht values, TC/PL, urea nitrogen, disease resistance at 30 and 60 days, expect urea nitrogen increased at 60 days. Shows these

values are good indication of taurine deficiencies in fish. Urea nitrogen good indication to diagnose deficiencies.

6.3.12 Lipid content in liver of YTK – 60 days of feeding. Cholesterol lower in control and phospholipids higher in control. Both important components of bile membrane. May be related to membrane combination and function.

6.4 Presented flow chart of taurine, bile salt, cholesterol and phospholipids and relationship between components.

6.5 Presented data from second experiment.

6.5.1 Experimental diets - control diet, non-FM, non-FM plus cholesterol, non-FM plus taurine and non-FM plus taurine and cholesterol.

6.5.2 Effect on Ht levels in YTK showed differences between diets.

6.5.3 Did not show taurine supplementation have positive effect after 4 weeks, but after 8 weeks showed normal Ht levels.

6.5.4 Non FM group and NF plus cholesterol continued to show lower values. Cholesterol content in liver of YTK showed high levels in group supplemented with cholesterol after 4 weeks and increasing again in 8 weeks in comparison to all other groups.

6.5.5 Mortality of YTK following artificial infection with *L. garvideae* showed NF plus cholesterol has very low levels at 4 and 8 weeks compared to all other groups.

6.5.6 Taurine deficiency cause decrease in disease resistance. Also, deficiency of lipid in liver cause decrease in stress resistance.

6.5.7 In the first day of challenge (not caused by infection - N and C showed few deaths. None in other groups.

6.5.8 Increased number of *benedenia seriolae* parasite significantly higher in non-FM diet, compared to FM diet. Suggests resistance against parasite in FM diet compared to NFM diet.

6.6 Summary of two experiments.

6.6.1 Presented a table on the composition of experimental diets and posed question ‘what is the critical level in FM diet in YTK diet?’

6.6.2 Results suggested - experiment one showed significantly higher number of mortality when only 0% FM was used. Experiment

two showed significantly higher number of mortality when <30% FM was used. Not that used Ht has indicator of taurine deficiencies.

6.6.3 Can conclude that the critical inclusion of FM in diet of YTK is 30-40%, where <30% may decrease disease resistance. Is was also stated that Taurine deficiency will not kill the fish itself, but cause infection by secondary pathogen and thus result in higher mortality.

6.7 Ability of taurine biosynthesis in YTK liver presented. Showed that taurine is essential.

6.8 Fundamental health problem in PL.

6.8.1 Possibly insufficient taurine, due to feeding lower FM diet.

6.8.2 Cause low disease resistance, with low stress resistance and accumulation of fat being contributing factors as well.

6.8.3 Examined this week Ht data from PL fish (2 year old fish). Normal levels of cholesterol are 250-300mg/100ml. All PL fish examined showed low levels in plasma. Cages 11-11, 12-11, 12-11, GL, 11-12 and 12-12 were examined. Ht level also lower in PL fish. Normal levels are 38-45%. Osmotic fragility also examined. Normal levels are 0.5 to 0.47%. Higher in PL fish (i.e. subsequently cells were easy to destroy). Urea nitrogen. Normal levels in adults 12 -17mg/ml and 20-25 in juveniles. Low levels observed in PL fish, except for moribund fish.

6.8.4 Necessary to consider taurine deficiency as cause of health problem observed at CST.

6.9 Questions

6.9.1 Mark Shepard – Can you comment on role of taurine and cholesterol on the quality or thickness or amount of mucus? – Masashi Maita - Don’t know the relationship between those factors.

6.9.2 Len Stephens – Are kingfish feeds regularly supplemented with taurine for Japanese industry? Masashi Maita – Yes, all feed mill companies supplement taurine in both non FM diet and FM diets.

6.9.3 Craig Foster – How much do they supplement feeds? Masashi Maita – Confidential information.

6.9.4 Erica Starling – Request clarification if the low FM diet provided by Skretting is still 30% provided by Skrettings? - Masashi Maita Yes.

6.9.5 Richard Smullen – Clarified details regarding Ridley diets trialled by CST. Data was presented by Matt Landos.

6.9.6 James Munro – Did you have a chance to see the analysis of healthy fish? Matt Landos – Yes, all fish were hook caught. 5 moribund fish sampled only.

6.9.7 Erica Starling – Have you tested flesh for taurine levels to see how they would use it and if there is a difference in utilisation between the diets tested? Masashi Maita – No.

6.9.8 Michelle Dennis – In fish that have been fed a taurine deficient diet, have you looked at serum albumin and glucose levels in addition to the other parameters that you tested? Masashi Maita - No did not look at it, but he considers plasma and serum levels to be similar. Michelle Dennis suggested serum levels may be more accurate over plasma levels.

6.9.9 Richard Smullen – Highlighted that there was very low levels of vegetable protein present in Ridley diets trialled by CST. There has been a lot of discussion of whether land animal protein and / or vegetable protein sources are causing the problem in YTK. Taurine is still present in sources of vegetable and animal protein; however, levels are lower than marine animal proteins sources (order of levels of taurine – marine > animal > vegetable). Can you explain difference between total and free taurine? Masashi Maita - presented only data of free taurine.

6.9.10 Len Stephens – highlighted need for a definition taurine. Richard Smullen – described definition to group.

6.9.11 Richard Smullen – Is green liver a classic case of taurine deficiency? Masashi Maita – other possible causes may be due to parasite.

6.9.12 Rhys Hauler – Have you ever seen enteritis develop as we are seeing here in Australia? Masashi Maita – No, not normally associated with taurine deficiency.

7.0 **YTK pathology view from Western Australia (Fran Stephens) (1.25pm)**

7.1 Health issues – only observed in fish in sea cages. No commercial sea cages in WA at the moment, only small scale trials.

7.1.1 Jurien Bay 2008 – fed commercial diet (stocked at 5g, massive myopathy event not long after transfer – diagnosed as relating to vitamin E and selenium, coated pellets with supplements)

7.1.2 Geraldton 2010/11 – Fed commercial diet, then transferred to FM/FO only to combat problem with lesions.

7.1.3 Geraldton 2011/12 – FM/FO diet only.

7.1.4 Summary of environmental conditions at both sites: water temp 18-28°C, windy rough seas and 6-13 m depth.

7.2 Lesions have been observed on fish in both SA and WA - only observed in sea cages, not hatcheries. Presented range of conditions observed:

7.2.1 Cystic and fibrotic changes - exocrine pancreas. Lesion progresses over time – get a lot of scar tissue and fibrosous.

7.2.2 Cholangitis – infection in the bile duct.

7.2.3 Fatty livers observed, but generally in fish that are doing well.

7.2.4 Protein in kidney tubules observed – possibly get it in normal healthy fish, but more prevalent in fish that are not doing well.

7.2.5 Perls positive melanomacrophages in spleen.

7.2.6 Yellow/black pigment in liver, spleen, kidney.

7.2.7 Kudoa-like myxozoan plasmodia on heart – believe to be related to enteritis - similar parasite reported in Japan, but does not appear to be a big problem.

7.3 Comment on blood sample analysis.

7.3.1 Problems with clotting and gelatinisation, some fish with low PCV and Hb, low total plasma protein and albumin. Fish with poor conditions index have high GLDH. There is a lot more work that needs to be done to work out what is going on.

7.4 Questions

7.4.1 Len Stephens - Do you see enteritis? Fran Stephens – No, I have not seen parasitic enteritis.

7.4.2 Trent D'Antignana – Comment directed to feed companies. Given how extensively vitamin E deficiencies have been studied how they end up with a diet that was considered deficient in Vitamin E – Fran Stephens - the diets were perhaps not deficient as such, but the requirement was higher than normal in the fish due to the fairly extreme environmental condition that the fish are kept in.

7.4.3 Erica Starling – Clarification on trials conducted in WA, which tried to demonstrate sea cages effect. Fish kept onshore were exposed to same water source as those at sea as it was flow through system with no filtering mechanism. Should not underestimate the sea-cage effect and wants to really re-iterate that point.

7.4.4 Richard Smullen – Do you anything about the ceroid – is it something the animal is producing? Fran Stephens – no I am not sure. Thought for a long time that it was a fixation artefact until pathologists suggested it was not an artefact. Michelle Dennis has observed this in a number of species of fish, but is not aware of its significance.

7.4.5 Rebecca – Have you tried to isolate the ceroid? Fran Stephens – No.

8.0 **Impacts of FM and FO replacement on gut health (David Stone) (1.50pm)**

8.1 Assessment of soybean enteritis like condition in juvenile YTK held under different feed and temperature regimes.

8.1.1 Presented experimental results – 4 pilot trials (solvent extracted soybean meal (18 and 22°C), soy protein concentrate (18, 22°C), dietary oils – 20% oil added (22°C) and dietary oils 20% oil added (18C°)). 5 week trials. Results available in Aquaculture Journal.

8.2 Conclusions from experiments.

8.2.1 Key criteria to classify enteritis in Atlantic Salmon were measured and substantial changes in all parameters consistent with soybean enteritis was not observed in YTK. Extensive changes to Atlantic salmon intestinal structures occur at these inclusion levels.

8.2.2 No major inflammatory responses were observed in response to the different diets.

8.2.3 Goblet cells proliferation were high across all experiments and treatments. Fish growth and survival did not correspond to the changes in intestinal structure. Closer examination showed mucous layer erosion, altered mucous composition and increased goblet cell numbers in digestive tract.

8.2.4 Growth, feed efficiency and digestive trypsin were significantly reduced by inclusion of canola oil.

8.2.5 Green liver was seen in YTK in response to canola oil and less extent soybean oil. Alternative lipid study showed green liver in non-FM diets. Had same taurine levels, only difference was type of oil (highest incidence of green liver with canola oil compared to all other groups).

8.2.6 Important to note that cholesterol level in diet reduced when including canola oil in diet.

8.3 Recommendations

8.3.1 Cause of GCP and high ebls of SNV warrant further investigation.

8.3.2 Need to get baseline references in these experiments, as controls in the presented experiments were not suitable, possibly due to pre-existing condition.

8.3.3 Confirmation of normal tissue morphology.

8.3.4 Provide basis for developing scoring system specific for YTK, rather than assuming equivalent to salmon.

8.3.5 Differences in pre-feeding history and culture conditions may have influenced GC and SNV levels in fish prior to studies.

8.3.6 More research is needed.

8.4 Questions and comments

8.4.1 Len Stephens – Questioned the relevance of some of the results presented.

8.4.2 Michelle Dennis – What was your classification of green liver? David Stone – was based only on visual classification. Michelle Dennis – Did you record the kind of pattern of distribution of the green colour? David Stone – It was close to the junction where we cut the liver away and it just spreads out from there. Michelle Dennis – was it in close proximity to the gall bladder – David Stone - Yes. Michelle Dennis – In that case it could have been a post mortem artefact? David Stone – Fish were sampled within 30 seconds after killing, so unlikely to be a post mortem artefact. Michelle Dennis – Made the point that reference to green liver disease term is not really productive in understanding the disease process.

8.4.3 John Carragher – Most of your changes were seen in the anterior of the gut? David Stone – No. Did not see similar redness of hind gut currently observed in YTK at CST.

9.0 **Dietary investigations and determining their impact on fish performance and survival (Trent D'Antignana) (2.10pm)**

9.1 Summary of work that the company has been working on currently.

9.2 Presented background information on various nutritional factors and what factors they are known to influence. Company has had limited knowledge of nutritional composition of its diets and impact of dietary changes on fish performance and survival.

9.3 The objective of the recent work carried out by CST is to determine if these factors are contributing to the current health issues.

9.3.1 Activity 1 – 8 diets from Ridley and Skretting were analysed for proximate composition (3 to 4 mm diets).

9.3.1.1 Found differences in fat but similar protein levels.

9.3.1.2 No difference in regards to minerals that stood out from company perspective. However, free taurine levels did stand out.

9.3.1.3 Following this, conducted a literature review on taurine inclusion levels (20 to 30 papers). Literature suggests requirements are 2.5-10g/kg of free taurine. The inclusion levels in the diet appear low.

9.3.1.4 Still finalising outcomes from this analysis.

9.3.1.5 Fatty acid analysis – some DHA levels were low compared to recommendations to NRC.

9.3.1.6 Summary: 1) Taurine could be deficient in all diets considering 0.5-1% taurine is thought to be required for optimal performance. 2) LC PUFA appears to be low in several diets. Should be at 2% for fish >50g. 3) There appears to be considerable variability in the nutrient profile within a particular diet type. Subsequent action was to undertake activity 2.

9.3.2 Activity 2 (in progress) – Feed trial to determine if supplemental taurine (top coated) improved fish performance. Top coating existing diets with crystalline taurine and administer to production fish.

9.3.2.1 2012 fish examined. 6 month old. 250 -3kg. Fish from all sites examined. Water temperature 20°C . Fish held in tanks in SBT hatchery. 3 month duration (started 22/6/12). 5 different diets/treatments being examined. Top coating with 2% FO, 3 replicates. Feed twice daily according to feed model, based on staring weight. 1000 fish involved, 257 g at start. CV 14%. SD 1.9g. Sample at start, middle and end.

9.3.2.2 Analyse growth, FCR, survival, histology, haematology. Progress to date – baseline histology collected but not analysed. Baseline haemocrit analysed.

9.3.2.3 Question – Richard Smullen - Did you consider making your own diets? – Craig Foster – CST is in discussions with David Stone about doing this, but limited with time so we rolled straight into this trial. The main

question is to see if by adding taurine to existing diets improves outcomes (i.e. are they deficient in taurine).

9.3.2.4 Comment - Craig Foster - this is presentation of one thing that the company is doing to try and overcome this problem.

9.4 Question and comments

9.4.1 Rhys Hauler – As taurine is soluble, what method have you considered for top-coating? Trent D'Antignana – use gelatine, but as feed intake should be relatively quick there should not be problem. Craig Foster - Also, company is using 2% to cover any potential loss.

9.4.2 Mark Booth - Where is activity two being performed – Trent D'Antignana - In tanks at SBT hatchery.

9.4.3 Graham Mair - Can you look at wild YTK to get baseline? Trent D'Antignana – Yes, tried but did not have success collecting wild specimen.

9.4.4 David Stone - How much gill flukes contribute to reduction in Ht? – Trent D'Antignana - Yes, significant impact. 2011 had trouble controlling flukes, which caused problems. 2012 have had less fluke problems. Anaemia is different between age classes, so may related to this.

10.0 General discussion (Craig Foster) (2.35pm)

10.1 The current health issue needs to be resolved for the YTK industry to get off the ground and it has to be resolved quickly. The purpose of workshop is not to be able to identify exactly what the cause is, but identify what the likely causes are and then determine how CST can proof that they are the cause or not the cause and systematically work through them. Matt Landos presented earlier in workshop what he believes are the likely scenarios based on the work that he has done with the company, but at this stage cannot proof whether they are right or wrong at this stage. He has identified what he thinks needs to be investigated further.

10.2 CST has conducted some viral research, but is unsure if enough has been done to discount this as a potential cause. Requested comment from

Mark Crane, who stated that if you are going to look for viruses you need to look at tissue with lesions, not just random samples. Paraffin blocks prepared for histology analysis can also be processed for EM. 4,5,4 sequencing is also ok for analysing tissues that have lesions. Pyro-sequencer is available at SARDI for this type of analysis, which is relatively inexpensive. Sequences all DNA, personnel interprets it and align it with known sequences of marine viruses from YTK and other marine fish species. Other ways to try and eliminate if there is it a viral related matter. If it is caused by a virus you will see it via EM on tissue lesions in sufficient quantities. Cannot be determined by analysing random non-diseased tissue, need to analyse tissue with lesions. However, it is not clear what tissues should be targeted?

10.3 Michelle Dennis suggested anaemia is a new lead worth following up.

10.4 Len Stephens suggested enteritis could be secondary type problem to stress, nutritional deficiency etc.

10.5 Mark Booth raised question of whether health issue can be linked back to broodstock (i.e. vertical transmission). CST has tried to track to see if the disease has been associated with any particular batch of fish and results suggest it is not. However, as a pre-cautionary matter CST has removed all existing YTK broodstock.

10.6 If a virus is found to be the cause CST would have to get new stock and start again and if that is the case the company would like to find it sooner rather than later before more money is spent. The current pathology and mortality pattern is not consistent with a virus and the likelihood of it being a virus is very low, thus why the company has turned attention to nutrition. The company is comfortable that it is not a viral issue. The company knows that there is multiple pathology and that is why it is taking so long to try and resolve. Large number of experts that the company gets advice from suggests that there has to be some nutritional basis to it. Focus has moved to deficiency in the diet, as not helpful to look at nutrition generally. For example target possible deficiencies in taurine or essential fatty acids.

10.7 Richard Smullen commented on the remarks made by CST regarding the lack of nutritional composition provided to company. Originally,

discussions were had between the feed manufacturers and the company regarding the composition of feeds that they needed for their fish. The feed company did not just present them with a feed and that was it. Historically there have been big mortality events in the past that have been put down to handling, over-feeding or under-feeding. It is ok to claim that certain year classes have done well in the past, but that is not always the case. It could put it down to a nutritional problem in a multi-factorial case, where there has been an underlying problem and therefore they are not eating enough food. Trying to remove variants is a good approach to try and work out what the problem is, but peaks in mortality have occurred fairly recently yet diets have not changed that much. Craig Foster disagreed and believes diets have changed dramatically, with poor performance not just being recent but over the last 3 years and particular worse again in last 6 months.

10.8 Craig Foster posed question that if it is not nutritionally caused issue, then how can the company prove that? Richard Smullen agrees that the company is going about its investigation in the right way, but his concern is because the company has got small fish dying on diets that have not caused mortality in the past, and there does not appear to be a large difference in the diets fed to the two groups of fish, then what is causing this problem? First big mortality event occurred in PL in January 2010 following a big storm where there was a general feeling at the time that there was a lot of uneaten food etc. brought up off the sea floor that then changed the biological environment of the fish. Since then it appears the problems seen have got worse. Marcus Stehr questioned how that related to the different locations that the YTK are farmed in.

10.9 Erica Sterling made few points. Loss of growth is important in parallel to mortality (referred to as ill-thrift, i.e. fish performing poorly). It has been great to compare against problem in Japan but key thing that should be noted is that the Japanese get their fish from wild. It is a good comparison in a general sense, but need to be mindful that nature has culled fish in wild, unlike in Australia where YTK are produced in hatcheries. The ultimate point is that this industry will not go forward unless you get to the bottom of this problem, whether is it multi-factorial or not. CST needs to

start on a pathway to start discounting some of these things, one by one. Transparency from the feed companies is needed to be able to move forward, as CST needs to be able to adjust their feed in response to environmental stressors (e.g. season, weather, storm events). Richard Smullen said that Ridley will be working with companies on this issue. Erica Sterling made comparison to food companies, where we know exactly what is in the product because everything is on the label. It should not be this difficult to find out what is in the food that we are feeding to the fish. Rhys Hauler said Skretting will be collaborative with CST to try and work though feed factors in relation to this problem; however, through this would like to see the use of diet codes, rather than distinction made between Skretting and Ridley when presenting data.

10.10 Craig Foster posed question, if we want to eliminate taurine as a contributing factor, what should the company do? Rhys Hauler suggested collaborating with the Protein Research Centre in Norway, who are steering some fairly fundamental science around bile salt nutrition and physiology currently with Salmon. They recognise if the intestine is compromised, there is measurable decreases in circulating bile salts and also taurine. The Centre could assist with providing physiological measures to substantiate if it is taurine deficiencies causing problem or not. Contact person is Astrid Krogahl.

10.11 Len Stephens asked if there is someone or a research group in the world that looks at taurine deficiencies specifically in humans or land based animals in order help get a quick answer to this problem. No attendees were aware of anyone.

10.12 Craig Foster posed question if CST runs a trial with 2012 fish and show a response to taurine supplementation, what does that mean and when would I do next? Len Stephens suggested doing a dose trial. Determine what the most sensitive indicators are. Rhys Hauler suggested taurine alone may be too simplified, but agrees that you do need to break down the various factors. Richard Smullen suggested to look at what point they start to show the clinical signs, even when they are being fed diet that is considered sufficient in taurine. Rhys Hauler said that time and time again they are seeing differences between tanks and the sea cage

environments. Suggest getting back to the environment that we are seeing this problem in, which in this case the sea-cages. Suggest doing a dose trial is both a tank and sea-cage environment at the same time. The work that Skretting has done in house on taurine supplementation is not freely available. Basically, the company decreased FM to dilute taurine levels until the fish become compromised. Taurine was then provided as a supplement and the results showed that the fish did not respond to it, reiterating why looking at taurine on its own may be a little bit too simplified. Richard Smullen said he had similar feedback from Japanese company, where they just routinely add it when FM content is low as assurance that the fish are receiving required nutrients. Rhys Hauler added on request from Matt Landos that 25% FM was the threshold that *Seriola lalandi* can tolerate under experimental conditions. Rhys will confirm duration of the trial.

10.13 Craig Foster posed question of what else the company can look at other than taurine? Should company explore choline, or fatty acids?

10.14 Len Stephens asked whether the company could gain anything by measuring taurine levels in a large number of fish to determine if they are on a taurine deficiency diet. Could it be measure in the plasma or other different tissues? Trent D'Antignana said it could be measured in the liver. Richard Smullen highlighted that it was interesting in one trial they only saw an effect after 76 days. Len Stephens asked if CST has measured taurine in flesh of CST fish and if not suggested it should be done. Craig Foster agreed. Erica Sterling has looked at this in fish fed FM diet and marine diet. Too early for them to be conclusive about this. Graham Mair highlighted that the company needs to be careful not to limit on factors, as fish in different classes may already be compromised.

10.15 Mark Stehr questioned whether there should be a focus to look at what CST was doing in 2006 compared to what they are doing today. Craig Foster stated that CST to Skretting and Ridley and asked them if they could provide them with the feed they used back then to feed out now to their fish (i.e. high FM diets). Both companies agreed to this. However, as CST has had so many problems the company is essentially chocked with feed, where they have $1 million worth of feed sitting around at the

moment. Question is do CST sit on that feed and let it get older and possibly run the risk of it becoming mouldy, supplement it with taurine, switch to new feed, use old feed in experimental work? Matt Landos highlighted that when comparing the diets fed in 2006/07 to present diets, the company has gone back to similar nutrient formulation (i.e. inclusion rates of ingredients). However, asked whether it is possible to determine exactly what those profiles were back then and compare to what they are now? CST needs to know what the nutrient supply is and made the point that knowing just the percentage of FM is not enough information. Concerned that CST is trying to interpret at too big a level and they need to be determining is at nutrient level. Richard Smullen said that it is possible, but is it really worth doing as it involved a lot of work and is complicated. Rhys Hauler asked if there is anything specific that CST should be looking at. Matt Landos said taurine is very much still considered an important component, but agrees that it is possibly not the only factor, as the fish don't look like they just have a taurine deficiency. Rhys Hauler and Richard Smullen said taurine was not specifically determined in past diets, as it wasn't monitor but may be able to back calculate from raw material used. It is known that fatty acids profiles have changed as 100% fish oil was used in the past and is not now. Matt Landos suggested that even if we can start putting things into categories of what has and has not definitely changed then CST can start moving things off the list. Richard Smullen suggested it would be worth looking at choline chloride levels in the diet, but then it was not added to the diets in the past. Craig Foster pointed out that choline chloride levels decrease with a decrease in FM inclusion in the diets. Is it possible then for feed manufacturers to specify what the minimum choline chloride concentration is in the diet? Richard Smullen explained that if they decreased FM from 40 to 20% in the diet, they would then supplement with equivalent fraction of choline chloride that it decreased by (i.e. 1500 mg).

10.16 Erica Sterling proposed putting together a timeline of proposed actions.

10.17 Marcus Stehr asked if sanitisers are used in their feed. Rhys Hauler said Skretting did a trial in 2009, but currently do not use them. Richard

Smullen said Ridley use sanitisers in specific diets (i.e. barramundi diets for farms in northern Australia) but not in YTK feed.

10.18 Rhys Hauler highlighted that in Japan they do have a vaccination program in place against streptococcus. The industry is supported by vaccination program and without it they were getting 30% plus mortality.

10.19 Graham Mair said that they have been doing a lot of research on larval rearing, but given the timeline available to try and overcome this problem suggest larval rearing is not an option here. However, shifting to F1 broodstock is a move in the right direction. Matt Landos added that CST did a big grade and extensive culling this year to remove weak fish.

10.20 Len Stephens highlighted the point that during the workshop there has been a lot of time spent and discussion on taurine. Posed question to company if they are happy that it is not other nutrients causing the problem? Craig Foster advised that CST has evidence suggesting vitamin E deficiency is not a problem.

11.0 General discussion – review and development of a coherent and comprehensive R&D strategy to elucidate the cause for poor fish performance and high mortality (Craig Foster) (4pm)

11.1 Request for suggestions from attendees on what they consider the most valuable actions for CST in the next 6 months.

11.1.1 Erica Sterling suggested putting the fish on a deluxe diet. Use fish as a control group, regardless of costs, to understand what parameters that gives the company and use that as a baseline. Do it as a commercial based trial. Craig Foster said that the CST is currently using a Ridley deluxe diet with fish held onshore. Problem is that when the company trials a deluxe diet at sea, they have problems. Erica Sterling said that this is why the trial needs to be done in sea cages. Rhys Hauler asked if CST can do it in their offshore R & D facility at PL. Damian Crinchley said that the R & D facility will be ready in approximately two weeks. Minor problem is CST does not currently have small fish nets, only large fish nets. The type of diet we would like at, Craig Foster considers a deluxe diet to be all FM and FO, taurine level

included at required level, high vitamin C and E, choline and cholesterol. Trent D'Antignana has a concern that the fish are already compromised. Therefore, Craig Foster suggested CST would need to use November 2012 fish, which means smaller nets for cages will need to be sourced to house the fish.

11.1.2 Marcus Stehr suggested CST sourcing pellets from Japan and look at composition of those diets. Richard Smullen said they supply Japanese companies and already know the feed composition; however, information is not freely available.

11.1.3 Trent D'Antignana suggested performing QA on diets coming into nursery.

11.1.4 John Carragher suggested doing some trials looking at amino acids and fatty acid levels in the blood after the various diets have been consumed. This can show if those amino acids and fatty acids are in excess in the diet or not. Richard Smullen said that taurine can be looked at in the same way.

11.1.5 Len Stephens is still concerned with enteritis issue and how CST plans to deal with it. Craig Foster said one thing the company can look at is the pyro-sequencing to see what material is in the gut, as suggested earlier in the workshop. Michelle Dennis said in some cases they have bacterial colonises have been present but it appears to be secondary. There does not appear to be a primary bacterial agent causing infection. Shane Roberts asked if CST has found an increase in bathing frequency for flukes over time and if so do you think more regular bathing is disturbing the gut? Matt Landos commented that it is unlikely as 2012 year class fish have not been bathed, as they don't have flukes, yet they still have enteritis.

11.1.6 Marcus Stehr questioned if can we get someone to do analysis of how YTK are farmed in Japan (i.e. size of sea cages, big water flow etc.) and make comparison to Australian practise to see if there is something that is been done fundamentally different that we can then use to improve our production

techniques. Craig Foster said that CST knows roughly what Japanese practises are. Erica Sterling said that there is clear differences to Japan (i.e. we have shallow water etc.), but we have had success in the past so not necessary to follow their lead. For example, they are very heavy handed with antibiotics, which we cannot do here.

11.1.7 Erica Sterling posed question to group that if these fish are immuno-supressed, it not the same mechanism attacking the intestine? Matt Landos commented that it is more complicated than that. Micelle Dennis said a better term is immuno-disregulation.

11.1.8 Mike Thompson asked if the feed companies agreed to look at archival data. Richard Smullen and Rhys Hauler both agreed that they will do what they can; however, they know that they do not have taurine levels recorded in past batches of feed. In that case, Matt Landos asked if the origin area of the FM could be provided as it may be useful. Richard Smullen said that it may be possible to supply that information.

11.1.9 Marcus Stehr posed the question whether CST can discount broodstock have been a contributing factor. Craig Foster said CST has looked down that path already and from that investigation does not believe it is a contributing factor. YTK broodstock are F1's and no wild fish are being used this year.

11.1.10 David Stone found that YTK they received at SARDI from PA hatchery lacked the flight response, which was considered unusual. The fish transferred nicely into tanks, tanks were clean and water temperature was 20°C. This was different to fish they have received in the past. Craig Foster posed question of what we do anything about that. David Stone said that this may relate back to feeding a good diet early in developed (i.e. immediately post-weaning). Perhaps have a look to see if there was a difference in diet fed to 2012 fish in the hatchery.

11.1.11 Len Stephens suggested having a diagnostic regime in place.

11.1.12 Len Stephens suggested performing a small tank based diagnostic trial on sick fish. Craig Foster said that it may be possible to run tank based trial in AB hatchery, as it is not being used in this year's production run. John Carragher asked if they uptake taurine from the water and if so it might also be useful to look at in this type of trial.

11.1.13 Len Stephens suggested speciating the bugs in the gut and anaerobes. Marty Deveney said that there is a quantitative measure of what is active. It is a very complicated area to examine and try and get definitive outcomes from, as the environment and diet can change and there may be differences observed between individuals.

11.1.14 Masashi Maita said his research showed that supplementation of taurine can improve physiology conditions, such as anaemia, but cannot prevent infection. Therefore, CST should consider the use of immuno-stimulants in the diet. Craig Foster said that CST has used diets in past (e.g. Pro-tech), but have not seen positive response to date.

11.1.15 Mark Shepard suggest having a tissue/blood bank to be able to go back and look at osmotic conditions for example, to have a reference point if the fish improve. Is there any plan? Craig Foster said that this can be done but it is a matter of who would do it and who would store the samples. David Stone suggested the vet school at Adelaide University might be good place to store it.

11.1.16 James Munro highlighted the need for a clear case control study to rule out some factors (e.g. fish might die from poor health where they cannot handle the conditions, rather than from a specific lesion or enteritis). Some structure in research approach is warranted.

11.1.17 Richard Smullen asked if hose piping effect has been seen in pigs. John Carragher said this can been observed in pigs, which is regarded as a coccidian issue called hose pipe syndrome. John Carragher has tried to encourage conversion

between people at vet school at Adelaide University and CST, as they are very keen to be involved. Broad based vet school with people that have expertise with a number of mammalian species. Matt Landos said attempts were made to identify coccidian.

11.2 Matt Landos to provide summary document to attendees on important points raised during workshop.

12.0 Closure of workshop (Craig Foster) (4.45pm)

12.1 Thanks given to all attendees. Special thanks given to Mark Shepard and Masashi Maita for travelling long distances to attend the workshop.

12.2 Acknowledged Australian CRC for funding work leading up to the workshop and the workshop itself.

12.3 Encouraged attendees to pass on thoughts to the company that may arise from this workshop.

12.4 The company must now consider where to from here based on available timeframe and cost.