Growing a profitable, innovative, collaborative Australian yellowtail kingfish aquaculture industry: bringing 'white' fish to the market.

WA Component

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Executive Summary

This set of experiments compared the growth performance, survival and health of yellowtail kingfish (YTK) sourced from two different hatcheries (Strain 1 and Strain 2) fed on various diets over a commercially relevant time period of 56 weeks (ca. 13 months). Fish health was assessed routinely throughout the trial using histology, haematology, blood biochemistry and by measuring various immune parameters via both flow cytometry and more classical techniques.

The 56 week trial was split into four blocks investigating different dietary treatments in each block. One diet (Diet B) was fed throughout all four blocks and during an interim period between Block 2 and 3.

Block 1 was conducted over a three month period and compared three commercially available diets from an average starting fish weight of 67 grams. Diets A & B are diets formulated specifically for marine fish, whilst Diet C is a barramundi diet. Block 2 followed immediately after Block 1 with an average fish starting weight of 938 g. This trial ran for four months and compared the performance and health status of fish fed Diet B with and without the addition of two commercially available immunostimulants. A fourth treatment was included in this block in which those fish fed Diet C in Block 1 were switched to Diet B in order to gauge their recovery from this diet. Block 3 compared the growth performance of fish fed 9 mm pellets of the aforementioned Diets A and Diet B as well as a 9 mm diet of another commercially available diet, Diet D. This trial commenced with an average fish weight of 2.6 kg and was conducted over a three month period. Block 4 was a four week continuation of Block 3 comparing Diet B and Diet D, with a fish starting weight of 3.5 kg.

Over the 56 week trial, those fish fed continuously on Diet B grew from an average size of 73 grams to 3636 grams, with an overall FCR over this size range of 1.70. In order to compare our data with the national FCR targets of <1.5 for fish up to 1.5 kg and <2.2 for fish between 1.5 - 3.5 kg, we have broken down the FCR into fish of these two size classes. Fish fed Diet B up to 1.5 kg achieved an FCR of 1.2, better than the national target, however the FCR in fish from 1.5 to 3.6 kg was slightly worse than the national target at 2.3. At the start of Block 1, Strain 2 fish were significantly larger (72 grams) than those from Strain 1 (64 grams), and this statistical difference remained throughout the 56 week trial with the final weight of fish from Strain 2 (3908 grams) remaining significantly larger than those from Strain 1 (3575 grams).

In Block 1, absolute weight gain was significantly affected by both fish strain and diet. Fish from Strain 2 had significantly greater absolute weight gain (884 grams) than those from Strain 1 (770 grams). Fish fed Diets A and B had equal weight gain over the three month period (937 and 884 g, respectively, pooled across strains), significantly greater than those fed Diet C (659 g). Those fish fed Diet A had a significantly better FCR (1.05 ± 0.01) than those fed Diet B (1.11 ± 0.01) and Diet C (1.27 ± 0.02), which were also significantly different to each other. In Block 2, there was no effect of immunostimulant inclusion on growth, food intake or FCR over the four month trial period. At the end of this four month block, fish in the ex-Diet C treatment and fish from Strain 1 remained significantly smaller than all other fish, however the rate of growth of fish in both groups were significantly faster than the other diets and those fish from Strain 2, demonstrating that they were closing the gap in terms of their initial weight difference. In Block 3, fish fed Diet D had significantly greater weight gain than those fed Diet A and Diet B. Whilst Diet D had the lowest FCR of the three diets in Block 3 (1.90 ± 0.03) this value was not significantly different to that obtained in Diet A (2.27 ± 0.16) and Diet B (1.96 ± 0.02).The significantly higher weight gain of fish fed Diet D was due to their significantly higher food intake; most of which occurred in the first two months of the three month block. In Block 4, fish ate equal amounts of Diet B and Diet D and as a result, growth and food conversion ratio was equal in fish fed these two diets.

Most diets were within the specifications stated by the manufacturers. Due to the many differences in nutritional composition outlined in this report between diet types, it is not possible to determine exactly which differences (or combination of differences) in the diets led to differences in the fish's performance.

The types of protein sources used in all diets and their digestibility values were unknown, however all diets appeared adequate in terms of their essential amino acids content and their ratios of essential to non-essential amino acids. There was some evidence, however, that the lower methionine content of Diet C may have contributed to the cataracts seen in fish that were continued to be fed on this diet over a longer term period.

Each pellet size of Diet C contained a higher percentage of total lipid than the corresponding pellet size of Diet A and Diet B, which were similar to each other. The 9 mm pellets of Diet D contained a similar level of lipid to the other 9 mm diets. There were significant differences in the percentages of fatty acids and key fatty acid ratios investigated between all four diets, which are likely to have been contributing factors to the differing performances between diets. These analyses demonstrated that different lipid sources were used in each diet and suggested that fish

oil substitution probably occurred in all diets except Diet D. As the manufacturers of Diet A claimed to have only used marine oil in the 3, 4 and 6 mm pellets of this diet, the high level of linoleic acid in these diets may have been the result of inclusion of trimming oil from farmed salmon, which has been demonstrated to be high in this fatty acid. In Block 1, Diet A and B had similar nutritional profiles and performed similarly in terms of the growth, but the former gave significantly better FCR. Based on evidence from studies with Japanese yellowtail, the higher percentage of long-chain omega-3 fatty acids and higher DHA:EPA ratio of Diet A may have been a contributing factor to this superior food conversion efficiency.

The 4 and 6 mm pellets of Diet B contained much lower levels of cholesterol than the 3 mm pellets of this Diet and of all sizes of Diets A and C. These differences are likely to be the result of differing levels of cholesterol in the raw materials, as cholesterol supplementation is not usually practiced in commercial aquaculture diets. Despite these differences in the diets, plasma biochemical analysis revealed that it was fish fed Diet C that were hypocholesterolemic (not those fed Diet B which was lower in cholesterol). The hypocholesteremia seen in fish fed Diet C is likely due to the lack of dietary taurine, which plays a key role in cholesterol metabolism. Histologically, fish fed Diet C exhibited fattier livers with a greater extent of vacuolation than those fed Diets A and B. This may have been attributable to the slightly higher lipid content of this diet but is also likely to have been caused by the dietary taurine deficiency and hypocholesterolemia exhibited in these fish; both of which impact on lipid metabolism. No other histological observations could be attributed to diet or fish source with the exception of cataracts which developed in fish were maintained in Diet C for a further 20 weeks past the end of Block 1. Diet D contained a similar low level of taurine to Diet C, which caused problems in the fish fed this latter diet. Despite the low level of taurine in Diet D, fish fed this diet performed very well and did not exhibit hypocholesteremia. We therefore suggest that Diet C probably contained soy products and that Diet D did not, as soy actively strips taurine from the body, necessitating much higher inclusion levels in such diets.

Dietary selenium varied considerably between diets with Diet A > Diet B > Diet C = Diet D. No major benefits of the higher level of selenium inclusion were seen in fish fed Diet A, however the target value of >5.5 $mg.kg^{-1}$ of organic selenium to maximum growth was not reached in this diet. Vitamin C content could not be accurately measured in the diets. Vitamin E content was similar in Diets A, B and D and much higher than in Diet C.

During Block 1, Strain 2 fish exhibited an average natural mortality of 6.0 ± 1.6%, twice that of Strain 1 fish (2.6 ± 0.7%). The average mortality rate by diet was 5.5 ± 1.8% for Diet A, 3.2 ±

1.8% for Diet B and 4.1 ± 2.2% for Diet C. Due to the high variation between replicates, no significant effect of diet or strain was found for mortality. Based on evidence from the literature, the hypocholesterolemic fish fed Diet C, should have been significantly more prone to bacterial infection than those fed Diet A and Diet B. The bacterium *Photobacterium damselae* subsp. *damselae* was isolated from the gut of all fish examined, both healthy and moribund, yet the presence of this bacterium did not lead to greater mortality in Diet C fish. This suggests that this bacteria was not the primary cause of mortality and indeed symptoms typical of Photobacteriosis were lacking in the moribund fish examined. A set of preliminary bacterial challenge trials on fish fed Diets A and B resulted in no mortality, regardless of the biotype of *Photobacterium damselae* subsp. *damselae* used; the route of administration (intubation, intravenous or intraperitoneal) or the dose rate applied (up to 10^7 CFU.fish^{-1}). That these fish had previously been exposed to *P. damselae* subsp. *damselae* in the tanks would likely have led to them acquiring natural antibodies against it and therefore some resistance, however the complete lack of mortality supports literature evidence that this bacteria is a secondary pathogen and therefore not necessarily the cause of mortality in the tank trials. Despite the difference in mortality between Strain 2 and Strain 1 fish not being statistically significant, it is noteworthy that the more rapidly growing Strain 2 fish exhibited twice the mortality of those from Strain 1. This may indicate a relationship between very high growth rate and susceptibility to mortality, however this requires further investigation.

In the first month of Block 2, another natural bacterial infection resulted in mortality. On closer inspection of the microbiological data, *Vibrio harveyi* also occurred in most of the moribund fish, as well *Photobacterium damselae subsp. damselae*. This suggests an interaction between these two bacteria may possibly occur to increase virulence and this hypothesis will be tested in FRDC project 2016-117. Those fish being fed Diet B supplemented with Aquaguard experienced significantly less mortality (3.3%) during Block 2 than those in all other treatments, which ranged from 8.8% (Diet B + Actigen) to 13.8% (Diet B). Many of the measured nutritional parameters in the 6 mm Diet B diet fed during the first month of this block were out of specification, including a low level of several key fatty acids. This may have been a contributing factor to this mortality, however as there was no other diet tested in this block in which to make a direct comparison of survival, we cannot be sure of this. Whilst survival was significantly improved in fish fed the diet supplemented with Aquaguard during this early natural mortality event, the various measurements of immune response in the fish at the conclusion of the trial could not determine any significant benefits of the two immunostimulants. Indeed we found consistent evidence that those fish fed Diet B supplemented with Aquaguard actually had a more compromised immune system than

those in all other treatments. This suggests that the extended administration of Aquaguard had a negative impact on the fish after four months of feeding. Whilst there is varying evidence on the optimum feeding schedule of immunostimulants, our data support the hypothesis that continual feeding is detrimental. The natural recovery of the fish from infection occurred in conjunction with a reduction in the occurrence of *Vibrio harveyi* and an increase in the incidence of *Vibrio scopthlami*, suggesting a potential probiotic function in the latter, which is supported by the literature. This will be investigated further in FRDC 2016-117.

This project demonstrated significant differences in the performance of YTK fed different diets across a commercially relevant time frame. It has generated growth and FCR data that are superior to those reported previously and that will be used to generate more industry-relevant growth models for optimising feeding and food conversion ratios in warm-water environments. The project has been highly successfully in generating large volumes of baseline data on health and has generated new techniques and skill sets within Western Australia that will be of key importance to the developing YTK industry in this state. The project has identified several areas for future work that have potential in improving YTK health and subsequently the profitability and sustainability of the industry.

Keywords

Yellowtail kingfish, *Seriola lalandi*, aquaculture, commercial diets, nutrition, bacterial disease, Photobacterium, Vibrio, immunostimulant, histology, immunology, haematology

1. Introduction

This project integrated with the Rural Research & Development for Profit Programme, project number RnD4Profit-14-01-027 1. This overarching project was designed to enable industry to grow its position by developing more cost effective, sustainable feeds and feeding strategies to enhance growth and health of yellowtail kingfish (YTK)(*Seriola lalandi*

This WA component of the project had the specific objective of benchmarking the performance of YTK grown in waters representative of the warm waters of the mid west of Western Australia and to determine the effect of different commercial diets on the health status of yellowtail kingfish.

In order to achieve these objectives and to provide industry relevant data, this study compared the growth, FCR and health of YTK sourced from two different hatcheries fed a number of different diets during a commercially relevant farming period of 56 weeks. The trial was split into four blocks investigating various diets in each block. Throughout the trial we were also able to compare the performance of YTK sourced from two different hatcheries, by microchipping individual fish and co-locating them within the same tanks during each feeding experiment. In addition to comparing different commercially available diets, one of the blocks compared the performance and health of YTK which were fed on one commercially available diet to which different immunostimulants had been added in an effort to improve their health.

2. Objectives

1. Benchmark the performance of YTK grown in waters representative of the warm waters of the mid-west of Western Australia.
2. Determine the effect of different commercial diets on the health status of YTK.
3. Compare performance of YTK derived from two strains of broodstock

3. Methods

The trial was split into four blocks investigating various diets (Table 1). One diet (Diet B) was fed throughout all four blocks and during an interim period between Block 2 and 3, when the experimental tanks were being utilised for commercial nursery production.

Table 1: Details of experimental treatments investigated in each block of the 56 week trial.

Block	n	Diets	Pellets Sizes (mm)	Average Starting Size (g)	Duration (weeks)
1	4	A B C	3, 4 & 6	67	12
2	3	B B + Aquaguard B + Actigen B_exC	6 & 9	938	16
Interim		B	9	1985	12
3	3	A B D	9	2663	12
4	2	B D	9	3494	4

3.1. Diets

The general specifications of the diets tested in the four blocks, as provided by the diet manufacturers, are shown in Table 2. Nutritional composition analysis was performed on a subsample of each diet by various laboratories (Table 3).

The immunostimulants tested in Block 2 were Actigen^{TM}, a yeast-based mannan oligosaccharide (MOS) (Alltech, Kentucky USA) and Aquaguard, a 1,3 beta glucan product supplied by Aquatic Diagnostic Services International Pty Ltd. Both products were applied to the diet at the manufacturers' recommend rate of 0.1% and both were top coated to the diet using 50 mL.kg^{-1} of a 20% w/v solution of gelatin according to Partridge et al. (2014).

Table 2: Proximate composition of the different diets tested in the study according to their manufacturers.

Block	Diet	Pellet Size (mm)	Manufacturers Proximate Composition							
			Crude protein (%, min)	Lipid (%, min)	Fibre (%, max)	Moisture (% max)	Carb. (%)	Ash (% max)	Phosphorus (%)	Digestible Energy (MJ/kg)
1	Diet A	3	48	15	1.5	11	11.5	13	1.5	17.0
1	Diet A	4	45	20	1.5	11	9.5	13	1.4	18.0
1	Diet A	6	45	20	1.5	11	9.5	13	1.4	18.0
1	Diet B	3	50	18	4	8	nr	nr	1.4	19.8
1	Diet B	4	50	18	4	8	nr	nr	1.4	19.8
1	Diet B	6	48	20	4	8	nr	nr	1.4	20.1
1	Diet C	3	50	17	nr	7	17	9	1.3	19.0
1	Diet C	4	50	17	nr	7	17	9	1.3	19.0
1	Diet C	6	45	20	nr	7	19	9	1.3	19.3
2	Diet B	6	48	20	4	8	nr	nr	1.4	20.1
2, 3 & 4	Diet B	9	44	24	4	8	nr	nr	1.4	20.7
3	Diet A	9	42	24	3	10	nr	13	nr	18.5
3 & 4	Diet D	9	42	24	2	nr	nr	15	1.00	nr

*nr = not reported

Table 3: Analytes measured on trial diets and the laboratories which conducted them.

Analyte	Laboratory
Moisture (%)	Agrifood Technology (AGT)
Crude protein (%)	Murdoch University (MFRL)
Crude lipid (%)	Agrifood Technology (AGT)
Fibre	Agrifood Technology (AGT)
Ash	Agrifood Technology (AGT)
Fatty acid profile	Department of Agriculture & Food, WA (DAFWA)
Amino Acids (including taurine)	Department of Agriculture & Food, WA (DAFWA)
Cholesterol	Agrifood Technology (AGT)
Maco and micro minerals	Murdoch University (MFRL)
Vitamin C	National Measurements Institute (NMI) & Agrifood Technology (AGT)
Vitamin E	National Measurements Institute (NMI) & Department of Agriculture & Food, WA (DAFWA)

3.2. Fish

Fertilised eggs of the two strains of YTK were received by ACAAR from two different hatcheries and cultured separately in the ACAAR hatchery. The Strain 1 batch comprised of fish from two spawns which were seven days of age apart and which were pooled on 25th September 2015 when the batches were 30 and 37 days post hatch (dph), respectively. Strain 2 comprised of fish from a single spawn which were four days younger than the average age of the pooled Strain 1 batch. The two strains were kept separate until they were microchipped between December 1st and 3rd 2015. All trials were conducted in 10 m^3 tanks with each treatment replicated as described in Table 1. Each tank operated on flow through with a water flow rate of 100 $L.min^{-1}$. Central aeration and vertical inlet manifolds maintained dissolved oxygen at safe levels and assisted in self-cleaning of the tank floors. Dissolved oxygen and temperature were measured once daily in each tank. Fish were fed daily to satiety and the amount of food consumed was recorded.

3.2.1. Block 1

Block 1 was conducted over a three month period (4th December 2015 to 29th February 2016) and compared three commercially available diets fed to fish with an average starting weight of 67 grams. Diets A & B are diets formulated specifically for marine fish, whilst Diet C is a barramundi diet.

After microchipping, 70 fish from each of the two strains were weighed into each of 12 x 10 m^3 tanks for the commencement of the trial and feeding on the treatment diets commenced on the 4th December when the Strain 1 and Strain 2 fish were 103 and 99 days of age, respectively. During the first month of the trial fish were fed three times per day on 3 mm

pellets, reducing to twice daily in the second and third month when the fish were fed on 4 mm and 6 mm pellets, respectively.

To maintain safe and commercially relevant stocking densities, fish were removed from each tank on the 15th January 2016 to reduce the numbers to 120 fish per tank. Sufficient fish of each strain were removed to ensure an equal number of each strain remained in each tank.

At the conclusion of Block 1, a subsample of fish from each treatment were maintained on these treatment diets in different tanks for further work on bacterial challenge trials and fillet nutritional profile as described below.

3.2.2. Block 2

Block 2 followed immediately from Block 1 and ran for four months (1st March to 20th June 2016). This trial compared Diet B with and without the addition of two immunostimulants, fed to fish with an average starting weight of 938 g. A fourth treatment was included in this block in which the fish fed Diet C in the previous block were switched to Diet B in order to gauge their recovery from the barramundi diet.

At the conclusion of the Block 1 trial, all remaining fish from Diet A and Diet B were pooled then 80 fish (40 each from Strain 1 and Strain 2) were restocked from this pool into each of nine of the 12 x 10 m^3 experimental tanks. An equal number of fish from the Diet A and Diet B treatments from Block 1 were stocked into all tanks allocated to Diet B treatments (with and without immunostimulants). Those fish fed Diet C in Block 1 were redistributed into the remaining three x 10 m^3 tanks and fed Diet B during Block 2 to determine their recovery from Diet C. This treatment was denoted as ‘ex_Diet C’. Tanks in this treatment were also stocked with 80 fish, with equal representation from Strain 1 and Strain 2.

During the first month of the trial, fish were fed twice daily on 6 mm pellets and for the remainder of the trial fish were fed once daily on 9 mm pellets.

To maintain safe and commercially relevant densities, fish were removed from each tank on the 11th May 2016 to reduce the numbers to 50 fish per tank. Sufficient fish of each strain were removed to ensure an equal number of each strain remained in each tank.

3.2.3. Block 3

For a period of 87 days following the conclusion of Block 2, the experimental tanks were unavailable due to commercial nursery production. During this period, all fish (with the exception of the ex_Diet C fish which were culled at the end of Block 2) were pooled into a 30 m^3 tank and fed once daily to satiety on Diet B.

Block 3 compared the growth performance of YTK fed 9 mm pellets of the aforementioned Diets A and Diet B as well as a 9 mm diet of another commercially available diet, Diet D. The trial ran for three months from 15th September to 12th December 2016. At the start of the trial, 40 fish (20 each from Strain 1 and 2) were reweighed into nine x 10 m^3 tanks. The average starting weight of fish was 2663 g. Fish were fed once daily to satiety.

3.2.4. Block 4

Block 4 was a four week continuation of Block 3 comparing Diet B and Diet D, fed to fish with an average starting weight of 3494 g.

On the 15th December, 18 fish (nine from each strain) were redistributed into duplicate 10 m^3 tanks for the four week trial which continued to compare Diet B and Diet D in large fish.

3.3. Fish Sampling

All fish in all tanks were weighed at the end of each month during each of the four blocks of trials. At this time, one fish from each tank was randomly selected and sampled for health assessment. Histology was conducted on major organs (gills, liver, kidney, fore-gut mid-gut, hind-gut, spleen, skeletal muscle, heart and brain). The weights of liver and visceral fat were also measured on these fish and presented as a percentage of the fish's body weight to give a hepatosomatic index (HSI) and visceral fat somatic index (VSI), respectively.

At the end of Block 2, in addition to the routine histology, samples of fore-gut, mid-gut, hind-gut were also taken from the same fish and stained with a combination of Alcian Blue and Periodic acid–Schiff. The quantification of mucous cells and measurements of villi and their abundance was conducted at the end of this trial on these three gut sections as part of an honours project by Murdoch University Animal Science student Shayla Steffanetti. These

measurements were performed using ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Multiple images of each gut section were taken using a microscope equipped with a camera and the image containing the most intact gut villi was chosen. Every intact villus was then measured from its base to the tip, and every mucous cell per villi was counted using ImageJ.

Gonad weights from the sampled fish were recorded from the end of the second month of Block 2 when gonads were first observed. Macroscopically distinguishable sexes were observed at the end of the first month of Block 3. By the end of Block 3, some maturation was noted during fish sampling and when 195 fish were culled at this time, their sex was noted and correlated with their weight.

Blood was taken from the caudal vein of each fish sampled (i.e. the same fish described above) and immediately transferred to lithium heparin foetal tubes and stored in an ice slurry for up to 60 minutes before processing. Processing included taking a sample of each whole blood for measurement of packed cell volume (PCV) by spinning capillary tubes for three minutes at 13,000 g. Foetal tubes containing the remainder of the whole blood were spun under the same conditions and blood plasma and red pellet were frozen separately prior to transfer to VetPath (Belmont, WA) where the parameters detailed in Table 4 were measured on plasma, with the exception of haemoglobin and glutathione peroxidase (GPx), which were measured on the red cell pellet. Haemoglobin in whole blood was also calculated by multiplying the red cell pellet value by (100- PCV).

Table 4: Blood plasma and haematological parameters.

Acronym	Unit	Definition
PCV	%	Packed cell volume
CK	U/L	creatine kinase
ALT	U/L	alanine aminotransferase
GGT[1]	U/L	gamma-glutamyl transpeptidase
GLDH	U/L	glutamate dehydrogenase
BT	umol/L	total bilirubin
UREA	mmol/L	urea
CREAT	umol/L	creatinine
BOHB[1]	mmol/L	beta-hydroxybutyrate
CHOL	mmol/L	cholesterol
TRIG[2]	mmol/L	triglycerdies
LIPASE[2]	mmol/L	lipase
Na	mmol/L	sodium
K	mmol/L	potassium
Cl	mmol/L	chloride
PROT	g/L	protein
ALB	g/L	albumin
GLOB	g/L	globulin
Ca	mmol/L	calcium
P	mmol/L	phosphorus
Mg	mmol/L	magnesium
GPx	U/g of Hb	glutathione peroxidase per gram of haemoglobin
$Hb_{(red)}$	g/L	haemoglobin per litre of red cell pellet
$Hb_{(w)}$	g/L	haemoglobin per litre of whole blood

[1] Measured in Block 1 only

[2] Measured in Blocks 3 and 4 only

In addition to the fish sampled at the end of each month, samples of moribund fish were also taken as they arose and were analysed histologically and for the parameters in Table 4. In addition, samples of healthy (n=8) and moribund (n=4) fish sampled during a mortality event in Block 2 (29th March 2016) were taken for gel electrophoresis for determination of immunoglobulin types.

During Block 2, VetPath also developed and optimised methods for the set of haematological parameters for YTK shown in Table 5 and from the end of Block 2, haematology parameters were also measured routinely on the same fish sampled at the end of the each month. As the haematology analyser measured $Hb_{(w)}$ directly, this parameter was no longer measured in the biochemical suite described above.

Table 5: Haematological parameters optimised for YTK by VetPath.

Parameter	Unit	Definition
RBC	$x10^{12}$/L	Red blood cell concentration
HGB	g/L	Haemoglobin concentration in whole blood
MCHC	g/L	Mean corpuscular hemoglobin concentration (concentration of HGB per volume of RBC)
PCV	L/L	Packed cell volume
MCV	fL	Mean corpuscular volume (average volume of an RBC)
MCH	pg	Mean corpuscular hemoglobin (concentration of HGB per RBC)
Platelets	$x10^{9}$/L	Platlet (thromocyte) cell concentration
WBC	$x10^{9}$/L	White blood cell concentration
Neutrophils	% of WBC	% of the white bloods cells which are neutrophils
Lymphocytes	% of WBC	% of the white bloods cells which are lymphocytes
Monocytes	% of WBC	% of the white bloods cells which are monocytes

At the conclusion of Blocks 2, 3 and 4 samples of serum (collected from unheparinised, clotted blood samples) were frozen at -80˚C for analysis of lysozyme activity (either via an EnzCheck, Lysozyme Assay Kit E-22013 or using a turbidimetric method adapted from (Kumari and Sahoo, 2006; Sahoo et al., 2004) and complement pathway (ACH50) using a method modified from (Costabile, 2010; Ferriani et al., 1999; Yano et al., 1988a, Matsuyama, 1988 #3218; Yano et al., 1988b). Samples of heparinised blood were also taken for flow cytometry at the end of Block 2 (three fish per tank to give n = 9 for each treatment) to investigate immune parameters described below under ‘Bacterial Challenge Tests’. For these analyses, whole blood was lysed (BD Pharm Lyse) to remove red blood cells and the leucocytes were resuspended in culture media.

At the conclusion of Blocks 1, 2 and 3, fillet samples were also taken for biochemical analysis of total lipid, fatty acids, cholesterol, vitamin E, selenium and amino acid profile (including taurine). For those fish from Block 1 the fillet samples were taken from fish that had been maintained on their treatment diets past the end of the trial. All parameters with the

exception of cholesterol were measured on fillets taken from fish at the end of the live bacterial challenge trials described below which occurred in April 2016, whilst cholesterol was measured in fillets from fish sampled following the challenge trial with dead *Streptococcus iniae* described below and which occurred in July 2016. In addition to fillet biochemical composition, fillet quality was measured in fillets from these Block 1 fish which were sampled on 19th July 2016. Fish were killed by overdose with AQUI-S and bled in an ice slurry. Fillet pH was recorded immediately after death and the fish were then sent to a local seafood wholesaler for filleting and vacuum packing. The fish were filleted one day after culling. The parameters used to determine fillet shelf-life and quality include the quality index assessment (QI), sensory assessment, appearance and taste, and microbiological quality evaluation by examining the total anaerobic bacteria or total plate count (TPC) on day 1, 4, 8 and 12. The sensory evaluation was conducted by a panel on Wednesday the 25th July 2016. The fillet pH was also measured at days 5 and 12. Each measurement was taken from a previously unopened fillet pack to avoid contamination.

Samples of gut from these fish were also sent to the SARDI microbiome project.

Food conversion ratios (FCR) were calculated at the end of each month and for the entire trial duration as the food consumed per tank divided by the biomass gain per tank. All fish removed from each tank (samples, culls and mortalities) were weighed and accounted for in FCR calculations.

3.4. Bacterial Challenge Tests

In an effort to quantify the effects of diet and strain on the immune response of YTK, two preliminary bacterial challenge trials were conducted during Block 1 using live *Photobacterium damselae* subsp *damselae* on fish fed the two marine fish diets (Diets A and B) in order to determine a 10 day dose that would affect 50% of the fish (ED_{50}) for conducting more refined bacterial challenge studies at the end of this block and in all subsequent experiments.

For the preliminary studies a subsample of 36 fish (18 from each of the Diet A and Diet B treatments and with an equal number of Strain 1 and Strain 2 fish within diet) were removed from the experimental tanks on the 1st February 2016. These fish were equally split across two x 10 m^3 tanks (18 fish per tank, with nine per diet type comprising approximately equal numbers of fish from Strain 1 and Strain 2) in a quarantine room separate to the research facility. The average size of the fish at this time was 624 grams. For the first preliminary study, fish were administered either a high or low virulence strain of *Photobacterium damselae* subsp *damselae* at one of five concentrations prepared by the Department of Agriculture and Food Western Australia's microbiology laboratory. Both of these strains had been isolated from fish in the seacage. Strain 'virulence' was assigned by the extent of haemolysis exhibited by these bacteria on agar plates containing 3% horse red blood cells (blood agar) purchased from PathWest Media Laboratories, Perth. The dose rates ranged from 10^3 to 10^7 CFU.$fish^{-1}$ in 10^1 increments. The bacterial challenge was applied on 12th February 2016 as an intraperitoneal injection (IP). Following injection, fish were monitored for signs of bacterial disease for a period of ten days. They were fed once daily during this period on a reduced ration comprising equal amounts of Diet A and Diet B. The second preliminary study utilised the same fish as the first preliminary study. This second study investigated three different strains of *Photobacterium damselae* subspecies *damselae* administered either via intubation or via intravenous injection (IV). The three strains included the same two investigated in the first trial in addition to a third strain which had previously caused an acute mortality event of pink snapper at ACAAR. The fish were monitored as described for the first preliminary trial.

As a result of these preliminary trials, no live bacterial challenge tests were conducted at the completion of Block 1. On the 4th April 2016, the fish from the challenge trial were culled

and a subsample of six fish taken for measurement of PCV and bacterial isolation from kidney, gut and liver.

Using a further subset of fish which had been maintained on Diets A, B and C past the conclusion of Block 1, a new challenge trial using heat-killed *Streptococcus iniae* was conducted on 12th July 2016. Each fish was injected with 7 x 10^8 CFU and samples of blood were taken at time 0 (i.e. prior to injection) and then 4 and 24 hours post injection for measurement of complement and lysozyme activities.

At the conclusion of Block 2, an alternative bacterial challenge test was trialled in which isolated leucocytes were challenged with either live, dead or no *Streptococcus iniae* and the response of the leucocytes measured via flow cytometry following the lysing of the whole blood samples as described above. These cultures were then exposed to either no bacteria (control) or live or dead *Streptococcus iniae*, isolated from a natural fish kill in Broome. Based on previous experiments, the ratio of blood cells to bacteria was set at 1:1. Cell mortality was measured in all samples as well as the intracellular oxidative activity (DCFH-DA) and lysosome presence and activity.

3.5. Statistical Analyses

Growth, survival and the blood biochemical parameters from Block 1 were analysed via two way analysis of variance, with diet type and fish strain as the two factors, followed by Tukey's HSD on least square means. All means presented from the outcomes of two-way ANOVAs are least square means. Insufficient replicate blood samples were obtained in Blocks 2, 3 and 4 to include strain as a factor in the statistical analysis of blood biochemistry and parameters were therefore pooled between strains.

As fish from each strain were pooled in the same tank it was not possible to determine the effect of strain on food consumption or food conversion ratio and therefore these factors were assessed for diet only with one-way analysis of variance, followed by Tukey's HSD test. All statements of significance refer to the 0.05 level and all errors presented are standard errors.

In Block 2, mortality rates were compared among treatments using a generalized linear model, assuming a binomial distribution with a logit link function. Tank nested within treatment and strain of fish were included as effects in the model. As treatment had a significant effect on the risk of mortality, differences in survival percentages between each

pair of treatments were tested by Chi-square, using a Bonferroni correction for multiple comparisons. In addition, survival times were compared among treatments by the Kaplan-Meier method, with a Chi-square approximation to the log-rank test.

4. Results & Discussion

4.1. Water Quality

Water temperature throughout the trial ranged from 18.0°C to 23.3°C and averaged 20.4°C (Figure 1).

During Block 1, water temperature increased from 20.9°C to 23.0˚C and averaged 21.8˚C. During Block 2, water temperature declined from 23.3°C to 18.4°C with an average of 21.0°C. In Block 3 water temperature ranged from 18.0°C to 19.7°C and averaged 18.7°C and in Block 4 temperature ranged from 19.8°C to 21.2°C with an average of 20.5°C.

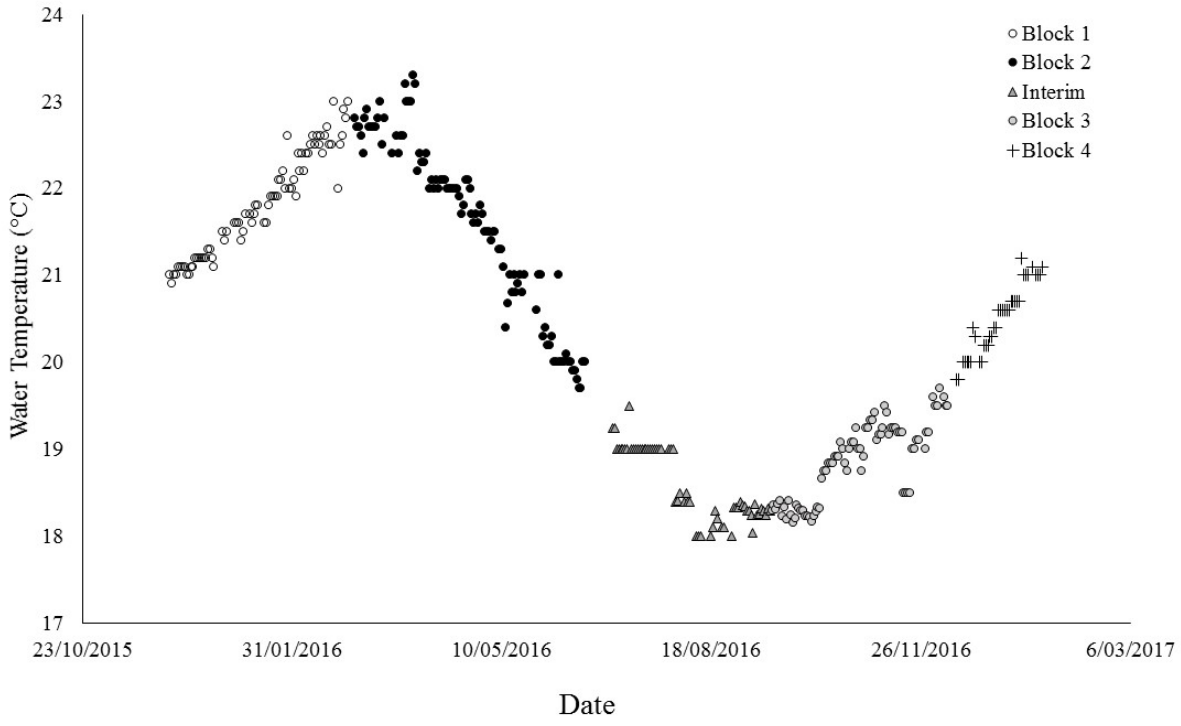

Figure 1: Water temperature in trial tanks over the full 56 week trial period.

Adequate dissolved oxygen was maintained at all times. In Block 1, dissolved oxygen saturation averaged 87% across all tanks. The average daily minimum value across all tanks over the three month period was 75% and the lowest absolute minimum value was 68%. Block 2 dissolved oxygen saturation averaged 86% across all tanks. The average daily

minimum value across all tanks was 81% and the lowest absolute minimum value was 65%. In Block 3 dissolved oxygen saturation averaged 88% across all tanks, with an average daily minimum of 82% and an absolute minimum of 80%. In Block 4 the dissolved oxygen saturation averaged 92% with an average daily minimum of 91% and an absolute minimum of 87%.

4.2. Growth

Over the 56 week trial, those fish fed only on Diet B grew from an average size of 73 grams to 3636 grams (Figure 2).

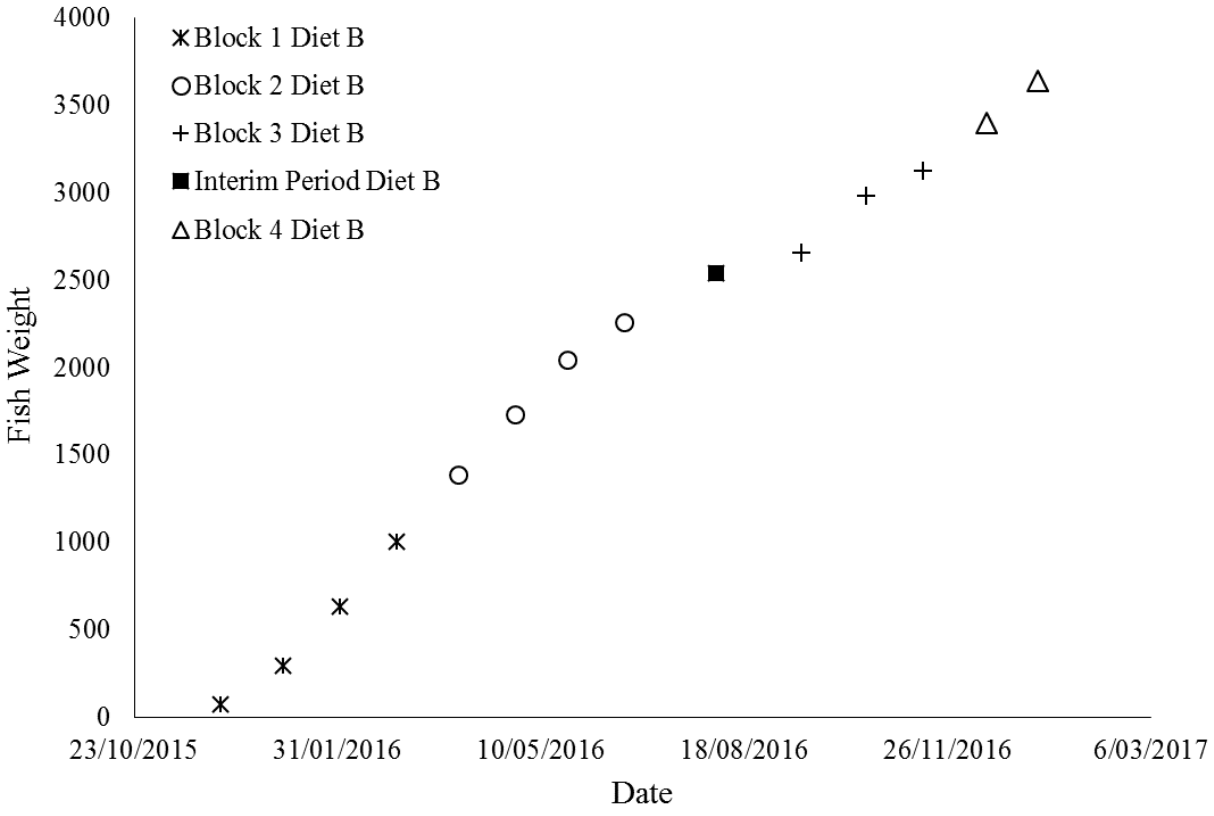

Figure 2: Growth of YTK fed Diet B over the full 56 week trial period.

4.2.1. Block 1

At the commencement of the trial, Strain 2 fish were significantly larger (71.7 grams) than those from Strain 1 (63.9 grams) ($P < 0.0001$), despite those from Strain 2 being four days younger (Figure 3).

Despite fish being randomly allocated to each dietary treatment, the average starting weights of both the Strain 1 and 2 fish allocated to the Diet B treatment were significantly greater than those allocated to Diets A and Diet C (Figure 3). We believe this difference was due to the fact that the initial period of microchipping all of the fish spanned three days and Diet B fish were tagged last. Therefore during the first two days of the tagging period these fish were still receiving a full ration, whilst those which had been tagged earlier did not eat a full ration in the first few days after being tagged.

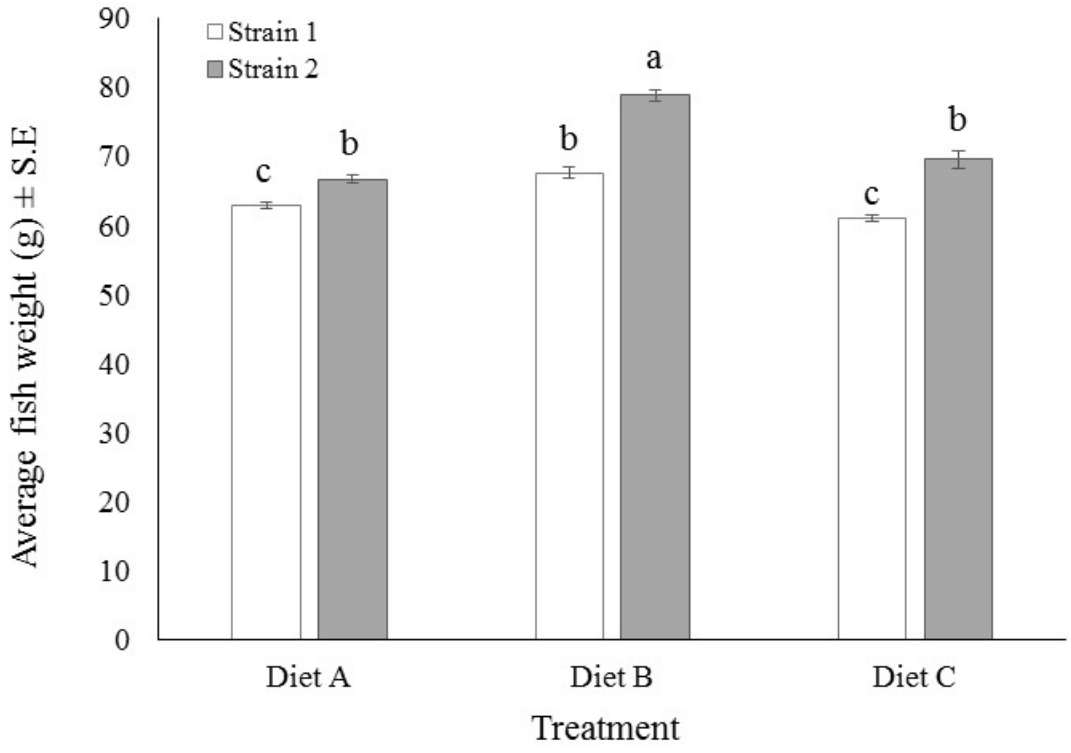

Figure 3: Average staring weight (g) of YTK in each treatment of Block 1.

At the end of each month, the relationship between fish weight and both diet and strain was consistent (Figure 4). The effect of diet and strain on average fish weight were always significant ($P < 0.001$), but not the interaction of these two terms ($P > 0.1$). Those fish fed

Diet C were always significantly smaller than those fed Diets A and B, which never differed from each other. Those fish from Strain 2 were always significantly larger than those from Strain 1.

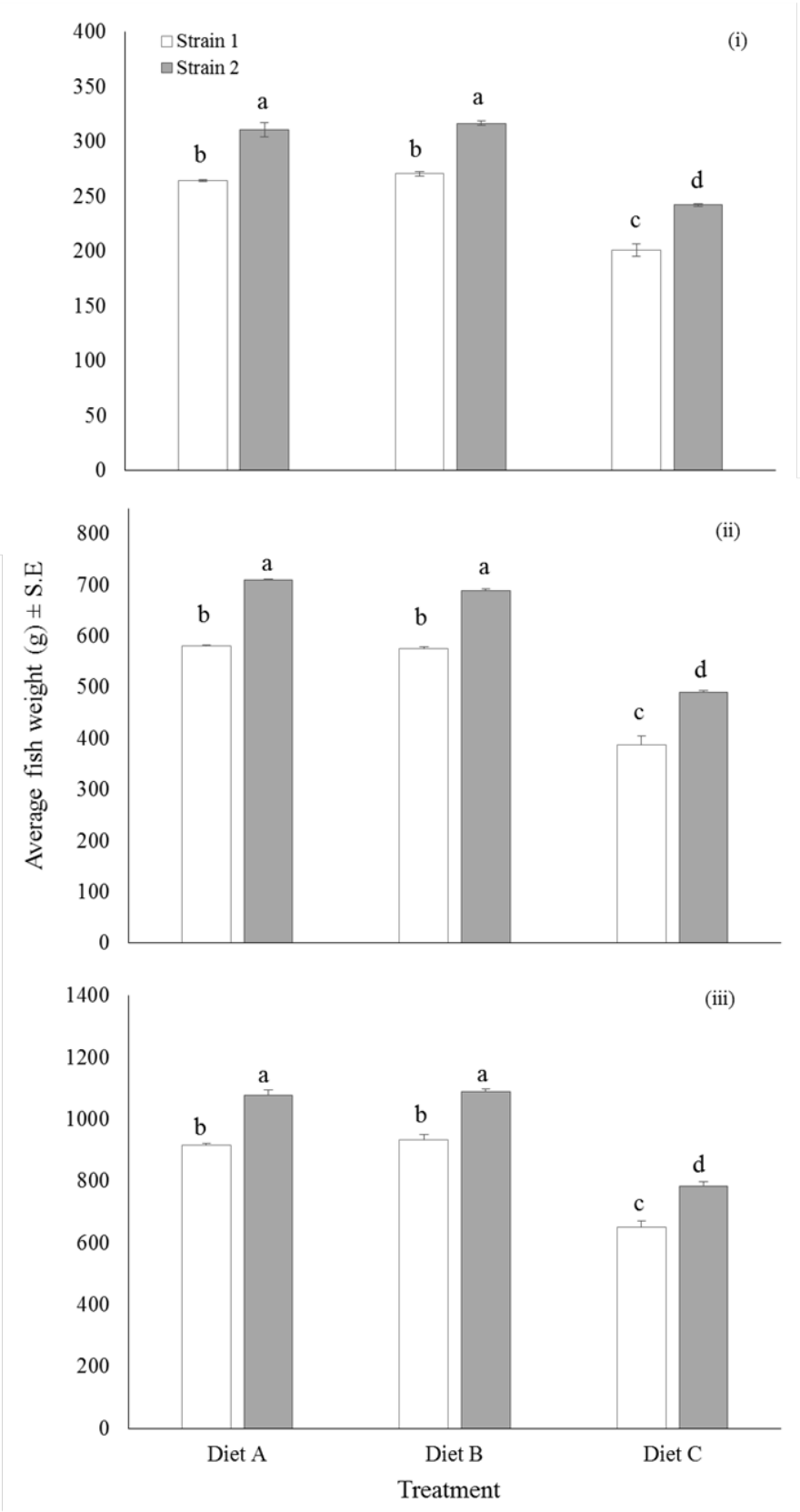

Figure 4: Average weight (g) of YTK in each treatment of Block 1 at the end of month 1 (i), month 2 (ii) and month 3 (iii).

Data collected over the three month trial (Figure 5) shows that the best performing fish were from Strain 2 that were fed either Diet A or B, whilst the poorest performing fish were those from Strain 1 fed on Diet C.

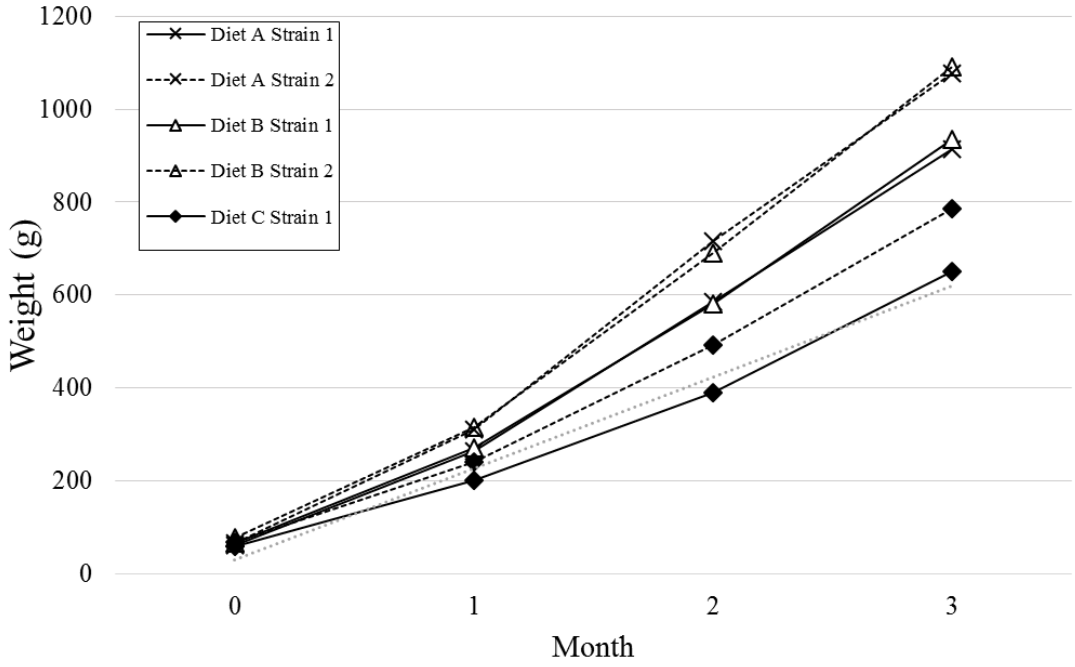

Figure 5: Growth of YTK from different strains and fed different diets during Block 1.

Specific growth rates (SGR) over the three month period are shown in Figure 6. There were significant effects of both strain ($P < 0.0001$) and diet type ($P < 0.0001$) as well as their interaction ($P = 0.002$) on SGR. The reason for the relatively poor SGR of the Strain 2 fish fed Diet B is due to their significantly larger size at the start of the trial (Figure 3). For example, whilst the difference in starting size between Strain 2 fish fed Diets A and B was only 11 grams, this resulted in a significant difference in SGR between these two treatments at the conclusion of the trial, despite them having a similar final weight (1088 vs 1079 grams, respectively). This demonstrates the sensitivity of the SGR function to slight differences in starting weight and given that all fish in all subsequent blocks begin with significant differences in weight, no further SGR analysis was undertaken.

Given the differences in starting weight, absolute weight gain is a more commercially relevant comparative measure of the fish's performance and these data are presented in Figure 7. Absolute weight gain followed the same response as the average weight by month i.e. it

was affected by both diet (P <0.001) and strain (P = 0.006) but not the interaction (P = 0.33). Strain 2 fish had a significantly greater absolute weight gain (883 g) than Strain 1 fish (770 g) and Diet C fish exhibited significantly lower absolute weight gain (659 g) than those fed Diet A (937 g) and Diet B (883 g) that did not differ from each other.

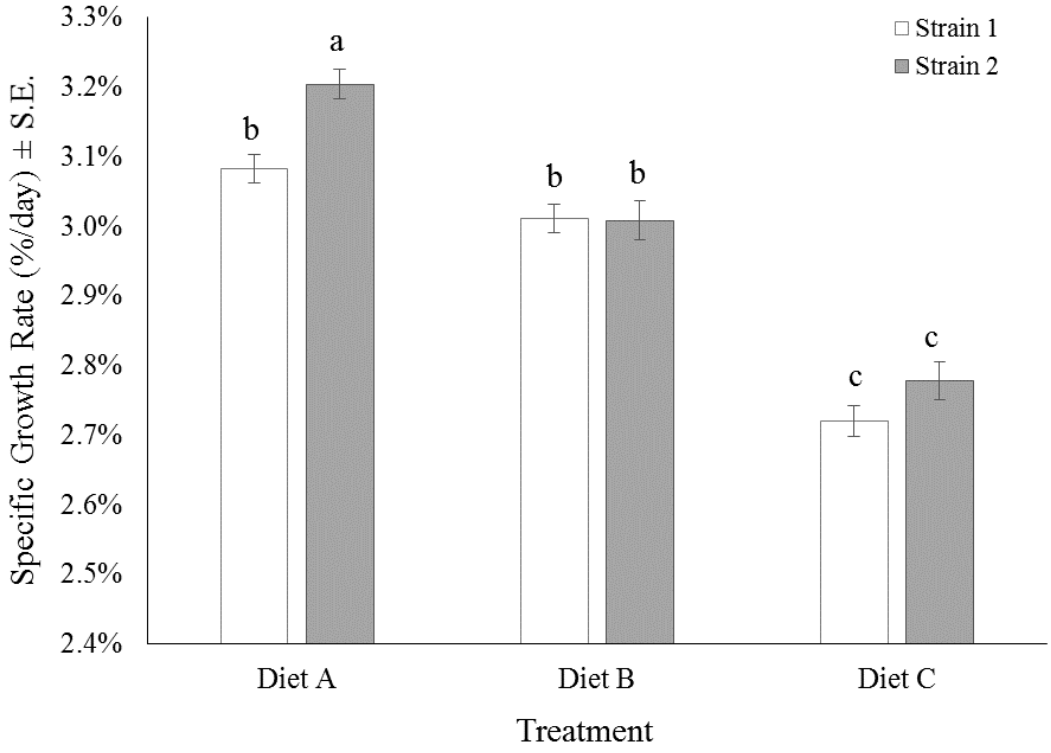

Figure 6: Averaeg specific growth rates (SGR; %.day^{-1}) of YTK from different strains and fed different diets during Block 1.

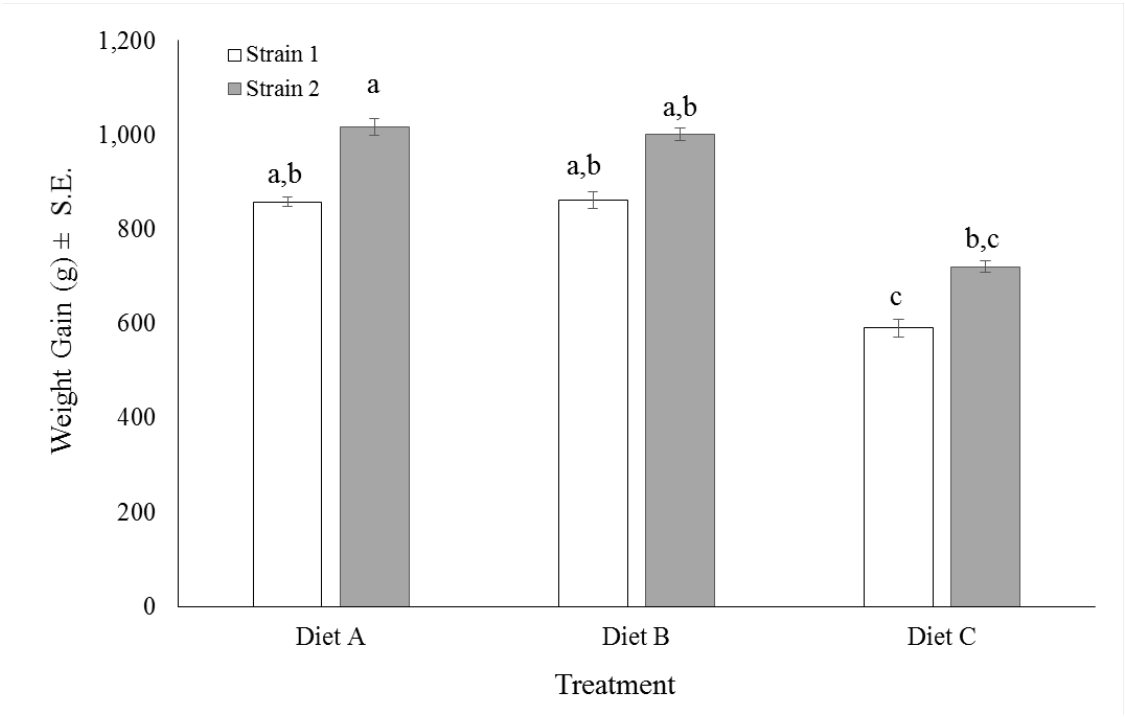

Figure 7: Average absolute weight gain (g) of YTK from different strains and fed different diets during Block 1.

Booth et al. (2010) presented the following growth equation for YTK in the size range of 50 to 2000 grams grown at temperatures between 20 and 25˚C.

Equation 1: Growth (g fish^{-1} d^{-1}) = 0.268 ± 0.33 x BW $^{0.52\pm0.2}$

Where BW = body weight (g).

More recently, Booth et al (2011) presented the following updated growth function which includes water temperature (T) as an input parameter.

Equation 2: Growth (g fish $^{-1}$ d^{-1}) = (1.353758 + 0.1437899 x T + 0.0029999 x T^2) x BW $^{0.4804985}$

Using our average starting weight of 67 grams and using the upper and lower confidence intervals presented in Equation 1, we have added ‘high’ and ‘low’ growth rates modelled to the data presented in Figure 5 below as well as the growth rates predicted by Equation 2 at our average water temperature of 21.8˚C (Figure 8). These data demonstrate that we achieved superior growth to that predicted by both models. The growth rate predicted by the upper

range of the first model was similar to that achieved by our poorest performing treatment, i.e. Strain 1 fish fed Diet C. Given that the work by Booth et al (2011) was conducted using commercial diets at a similar time when the Australian industry was using taurine deficient diets, this may be a contributing factor to the lower growth rates predicted by these models.

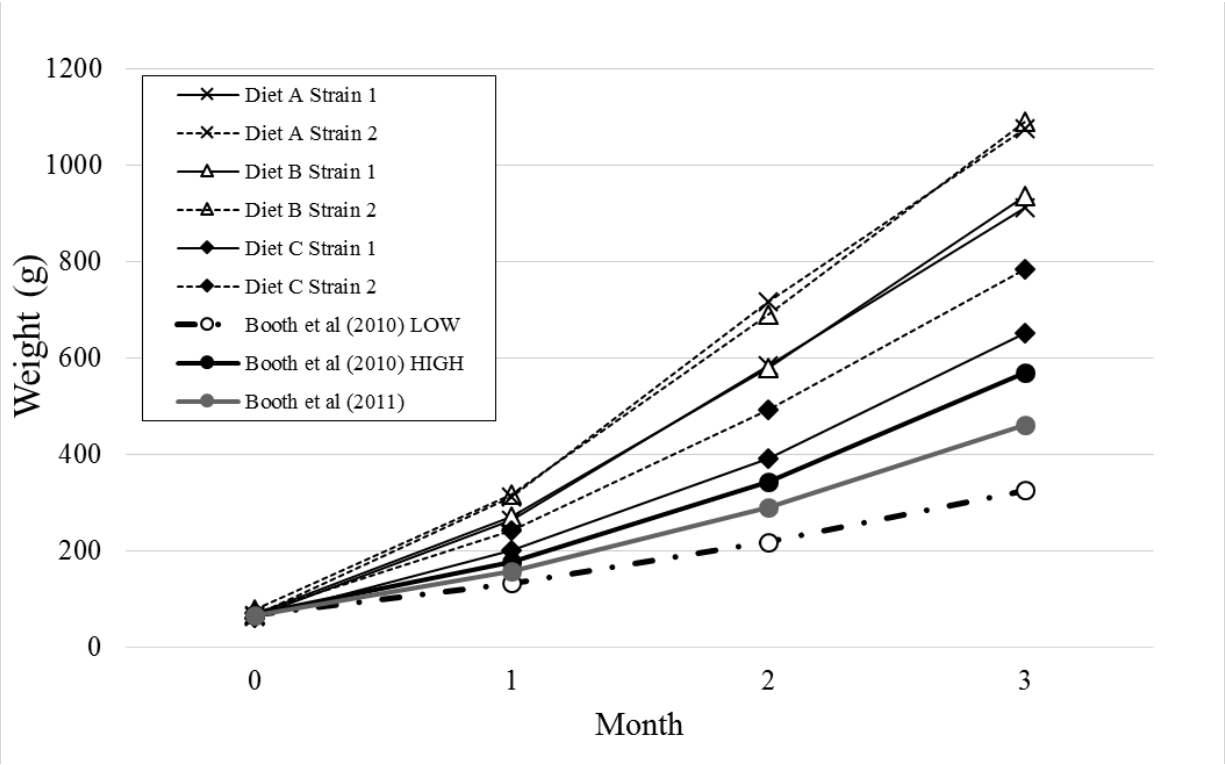

Figure 8: Growth of YTK from different strains and fed different diets during Block 1 including modelled growth from Booth et al. (2010) and (2011).

4.2.2. Block 2

At the commencement of Block 2, those fish in the exDiet C treatment were significantly smaller those in the other three treatments as a result of their history (Figure 3 and Figure 9). The average starting size of fish in this treatment were 646 ± 8 grams for the Strain 1 fish and 796 ± 6 grams for the Strain 2 fish. There was no significant difference between the average starting weights of fish assigned to the other three treatments. Within these three treatments Strain 2 fish were again significantly larger (1089 grams) than those from Strain 1 (931 grams) ($P < 0.0001$).

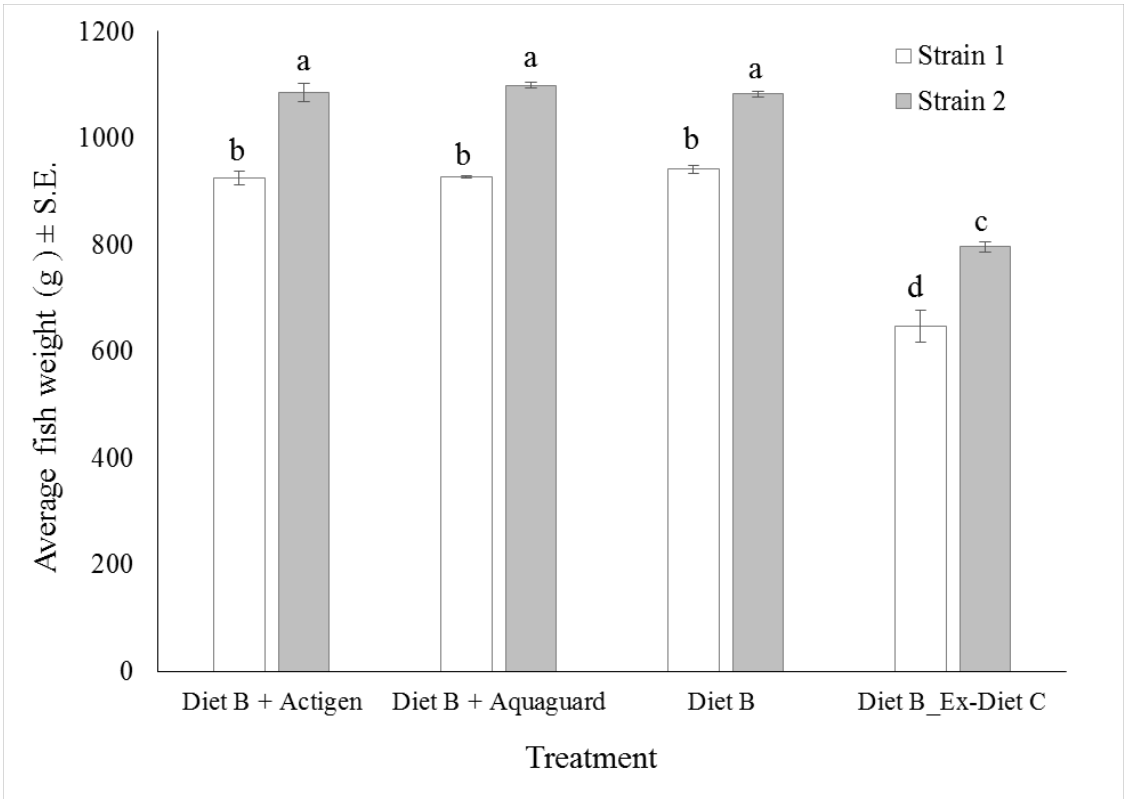

Figure 9: Average starting weight (g) of YTK in each treatment on Block 2.

Average weight of fish in each treatment at the end of each month of Block 2 is shown in Figure 10. At the end of the first month, the relationships between strain and diet on fish size remained the same as at the start of the trial. That is, fish in the exDiet C treatment remained significantly smaller than those from the other three treatments and Strain 2 fish remained significantly larger than those from Strain 1. By the end of the trial those fish in the exDiet C had closed the weight gap considerably. Two factor ANOVA revealed significant effects of both diet ($P = 0.007$) and strain ($P = 0.0001$) but no interaction ($P = 0.17$) on the final weight. Whilst those fish in the exDiet C treatment were still the smallest (2083 g), they were not significantly different in weight to those fed Diet B alone (2255 g) or those fed Diet B with Actigen (2261 g) but were significantly smaller than those fed Diet B supplemented with Aquaguard (2350 grams). There were no significant differences in final weight between those fish fed on Diet B or those fed on Diet B supplemented with either Aquaguard or Actigen. Whilst beta-glucans and oligosaccharides have been reported to have growth promoting effects, there is a wide body of literature that also demonstrates no benefits of these products to fish growth. Bagni et al (2005), for example, reported no improvement in growth when a commercial beta glucan was fed in cycles (15 days on then 45 days off) to sea bass for 8 months. Welker et al (2007) fed four commercial preparations of yeast or yeast subcomponents to channel catfish for 4 weeks, with none of the products resulting in growth improvement. Similarly, Shelby et al (2009) reported no growth benefits to Nile tilapia when fed similar commercially available yeast and yeast products for two to four weeks. Sealey (2008) achieved no growth benefit of feeding a commercial beta glucan, or various barley strains with varying levels of beta glucan to rainbow trout. Similarly Whittington et al (2005) found no benefit to the growth of tilapia after feeding beta-glucans for 10 weeks. When Li and Gaitlin (2004) fed diets supplemented with a commercial preparation of partially autolysed brewer's yeast or a different preparation containing partially autolysed brewer's yeast, dairy components and dried fermentation products to hybrid striped bass, they did not obtain any significant improvements in growth. Furthermore, there were no growth benefits of supplementing Atlantic salmon diets with 10 g/kg of mannan oligosaccharide, fructooligosaccharide or galatooligosaccharide for 16 weeks (Grisdale-Helland et al., 2008). Cook et al (2003) achieved faster growth of pink snapper during winter, but not summer, when fed on a diet supplemented with commercial beta glucan. These data suggest that beta glucans may have a more positive effect on growth at sub-optimal temperatures and this may

account for the lack of growth promotion in the current trial, where temperatures were not sub-optimal for YTK.

On the whole, fish from Strain 1 remained significantly smaller (2120 grams) than those from Strain 2 (2355 g) at the end of the trial, however there were cases where there were no significant differences in weight between Strain 1 and Strain 2 fish at the end of the trial (i.e. Diet B + Aquaguard and exDiet C,

Overall weight gains for each treatment are shown in Figure 11. Absolute weight gain was not affected by diet ($P = 0.25$), strain ($P = 0.12$) or the interaction of these terms ($P = 0.17$).

Whilst the weight gain of fish in Strain 1 was equal to that of Strain 2, it is clear from the data above that neither the Strain 1 fish, nor those from the exDiet C treatment caught up to the fish in the other three treatments over this four month period. However it does appear that the exDiet C fish exhibited some compensatory growth during this block.

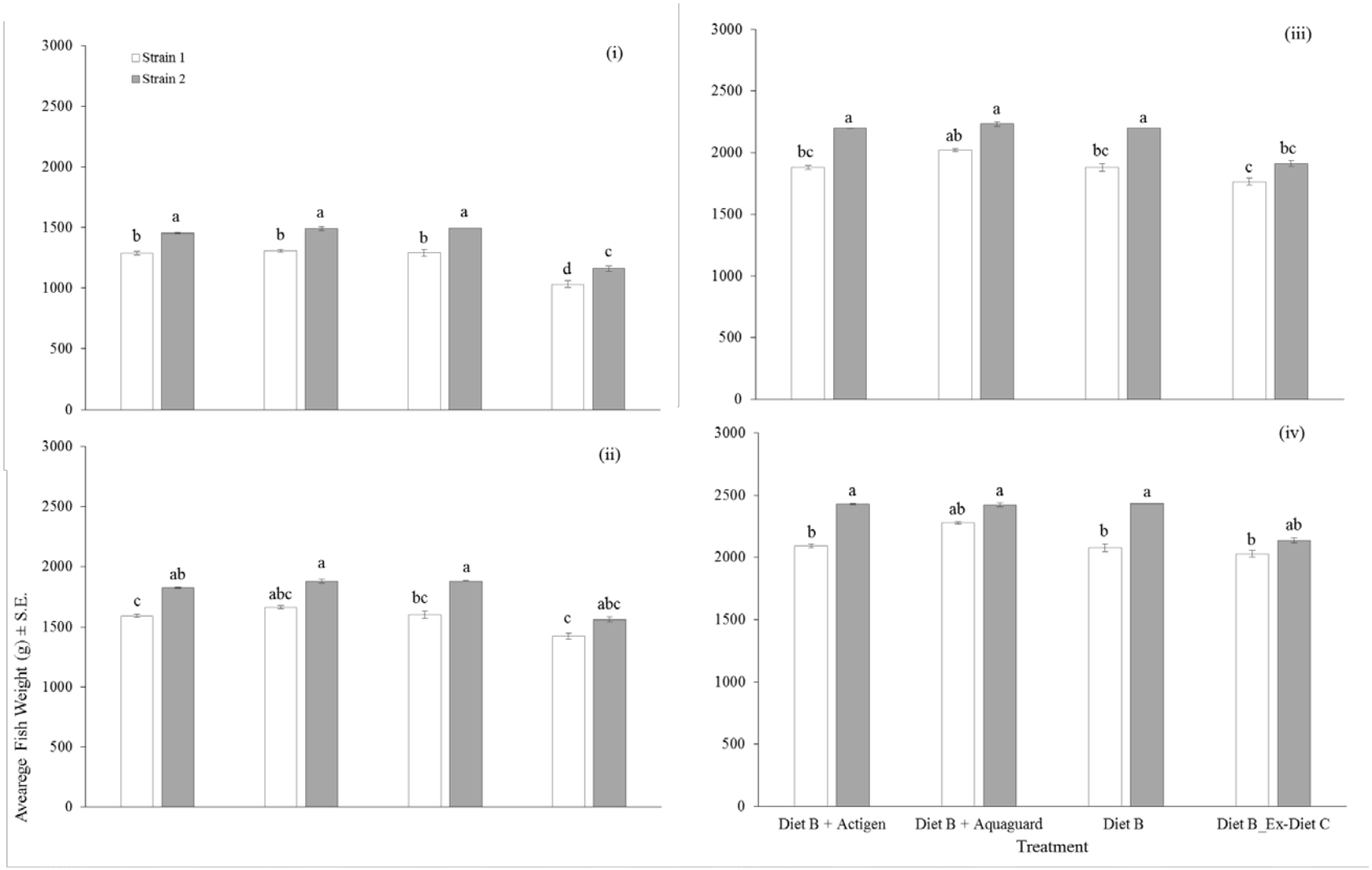

Figure 10: Average weight (g) of YTK in each treatment of Block 2 at the end of month 1 (i), month 2 (ii), month 3 (iii) and month 4 (iv).

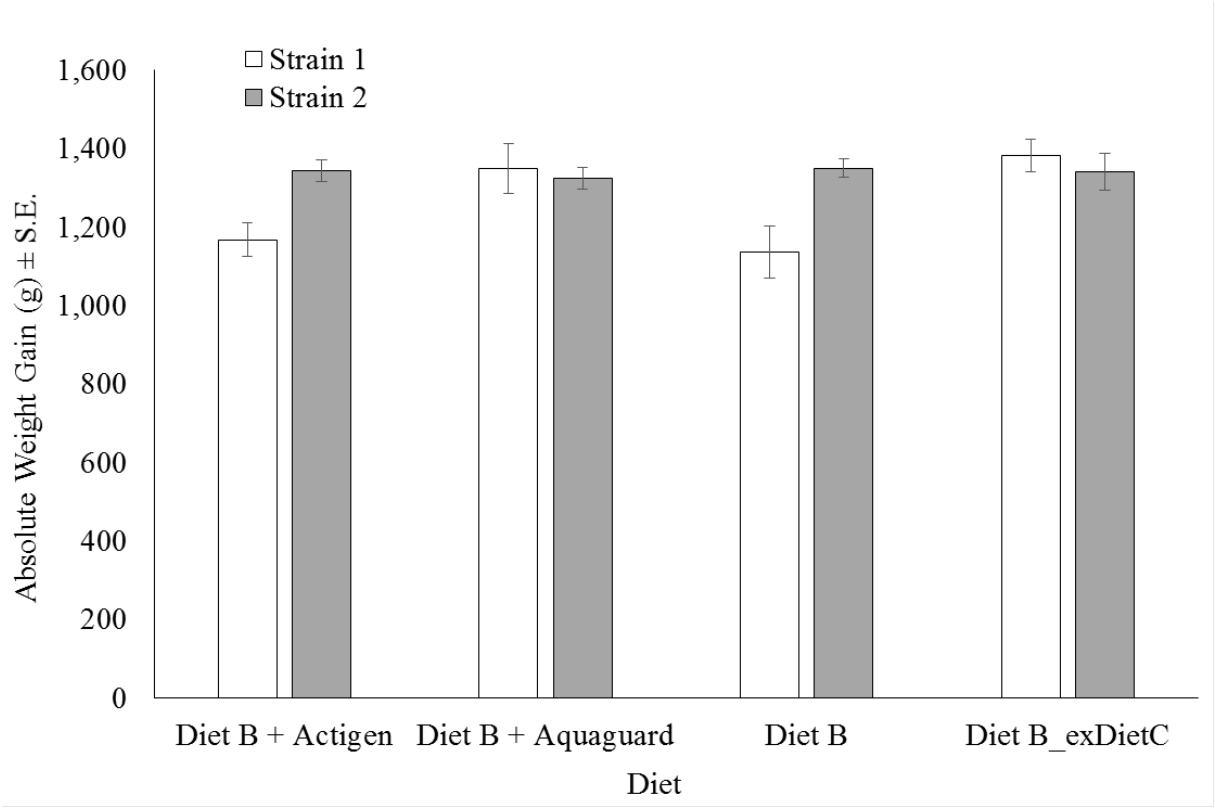

Figure 11: Average absolute weight gain (g) of YTK from different strains and fed different diets during Block 2.

The effect of diet and strain on fish weight over time is shown in Figure 12

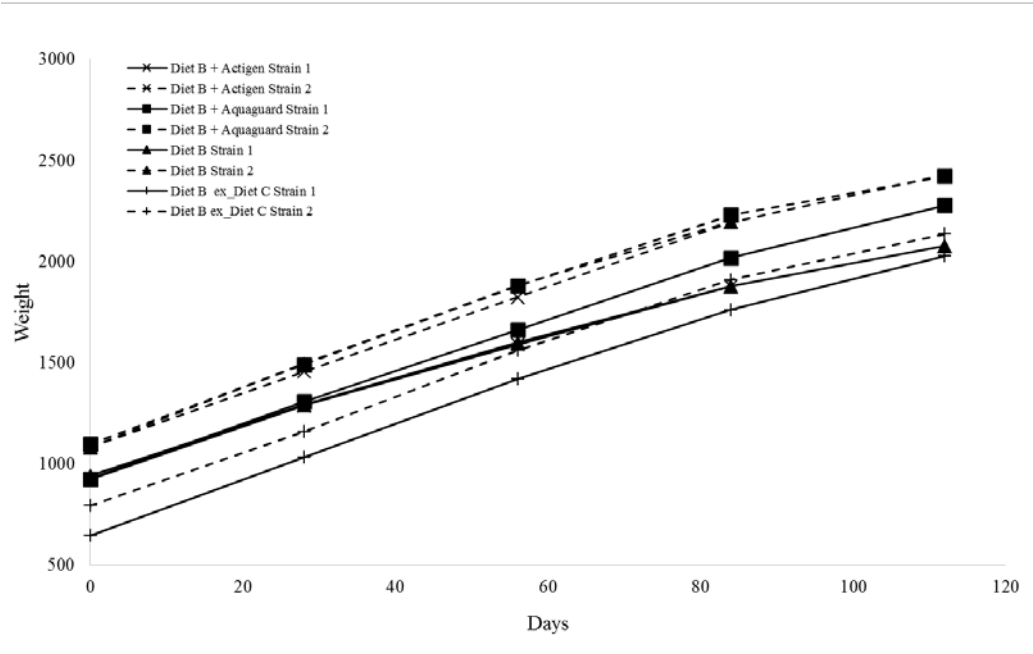

Figure 12: Growth of YTK from different strains and fed different diets during Block 2.

Using our average starting weight of 1009 grams for fish in three treatments (i.e. excluding those from exDiet C due to their significantly smaller starting size), we have added 'high' and 'low' predicted growth from Equation 1 to Figure 13. We have also added the growth predicted by Equation 2 at our average water temperature of 21.0˚C to this figure. These data demonstrate that we achieved a very similar growth rate to that predicted by the temperature compensated Equation 2 and within the range predicted by the high and low rates from Equation 1. This is in contrast to the Block 1 data presented above where we achieved far superior growth to that predicted by both models. This suggests that these models are more accurate for larger fish than the smaller fish in Block 1.

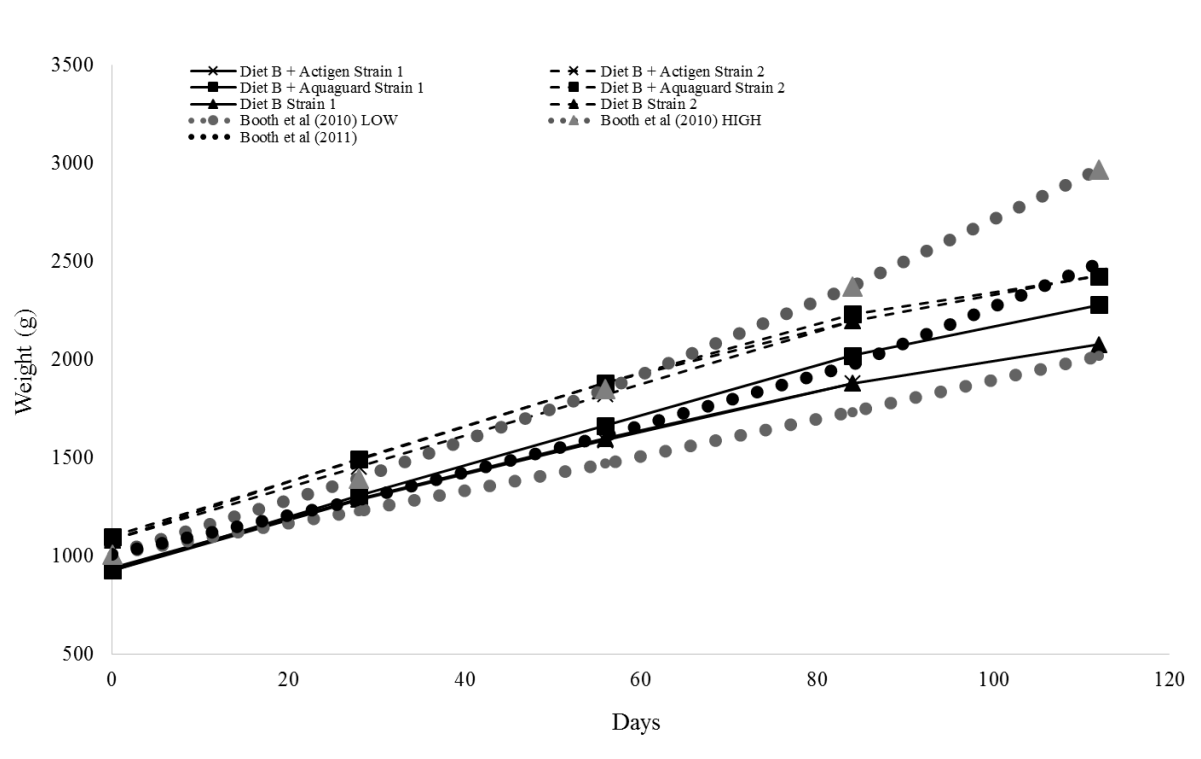

Figure 13: Growth of YTK from different strains and fed different diets during Block 2 including modelled growth rates from Booth et al. (2010) and (2011).

4.2.3. Block 3

At the commencement of Block 3, fish from Strain 2 (2827 ± 20 grams) remained significantly larger than those from Strain 1 (2490 ± 30 grams)($P<0.0001$), but there was no

difference in the starting size of fish between the different dietary treatments (P = 0.10)(Figure 14).

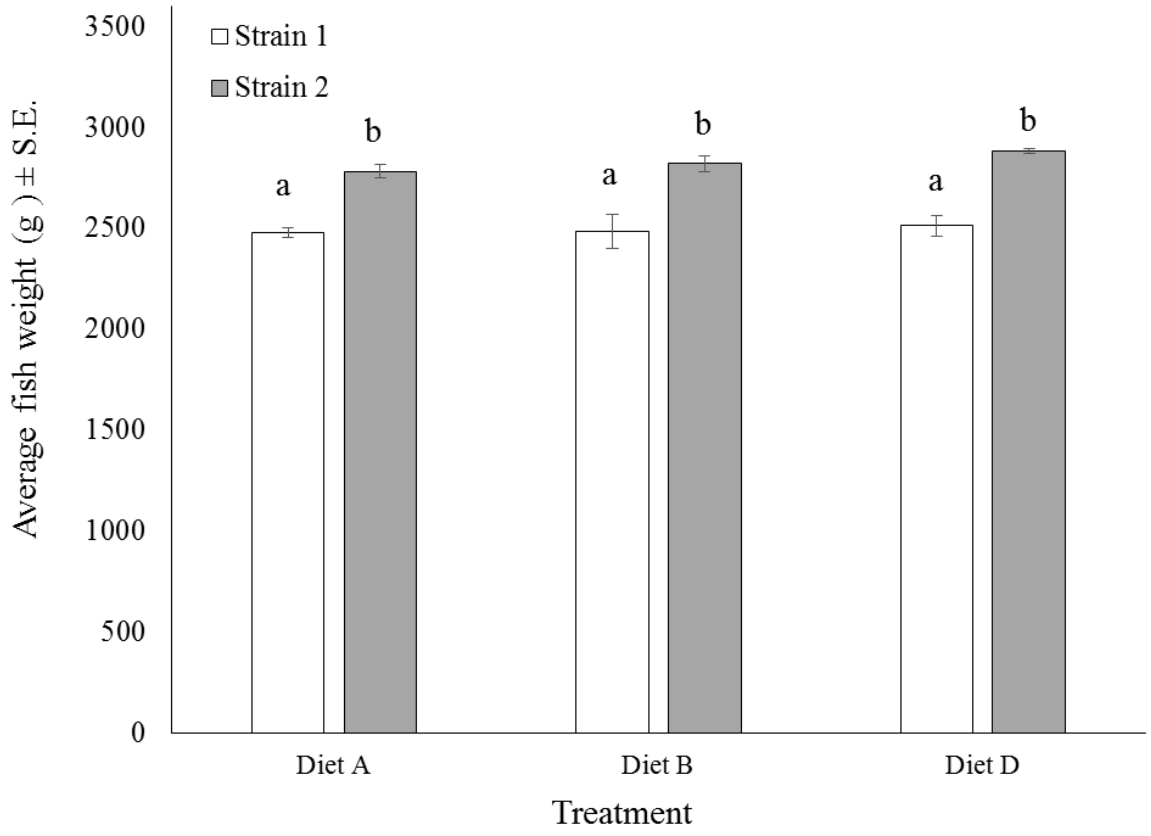

Figure 14: Average starting weight (g) of YTK in each treatment of Block 3.

At the end of each month, fish from Strain 2 remained significantly larger than those from Strain 1 (Figure 15). By the end of the first month, there was a significant effect of diet on fish weight (P = 0.0003), with those fish from Diet D being significantly larger (3057 grams) than those from Diet A (2861 grams), but not from Diet B (2975 grams). This relationship remained the same at the end of the second month, however by the end of the trial those fish from Diet B (3351 grams) had become significantly larger than those fed Diet A (3184), whilst fish fed Diet D remained significantly larger than both other treatment groups (3525 grams).

Absolute weight gain differed between diets ($P < 0.0001$), but not by strain ($P = 0.19$) or the interaction of these terms ($P = 0.83$). Those fish fed Diet D had significantly higher weight gain (829 grams) than those in both Diet A (555 grams) and Diet B (699 grams), which also differed from each other (Figure 16).

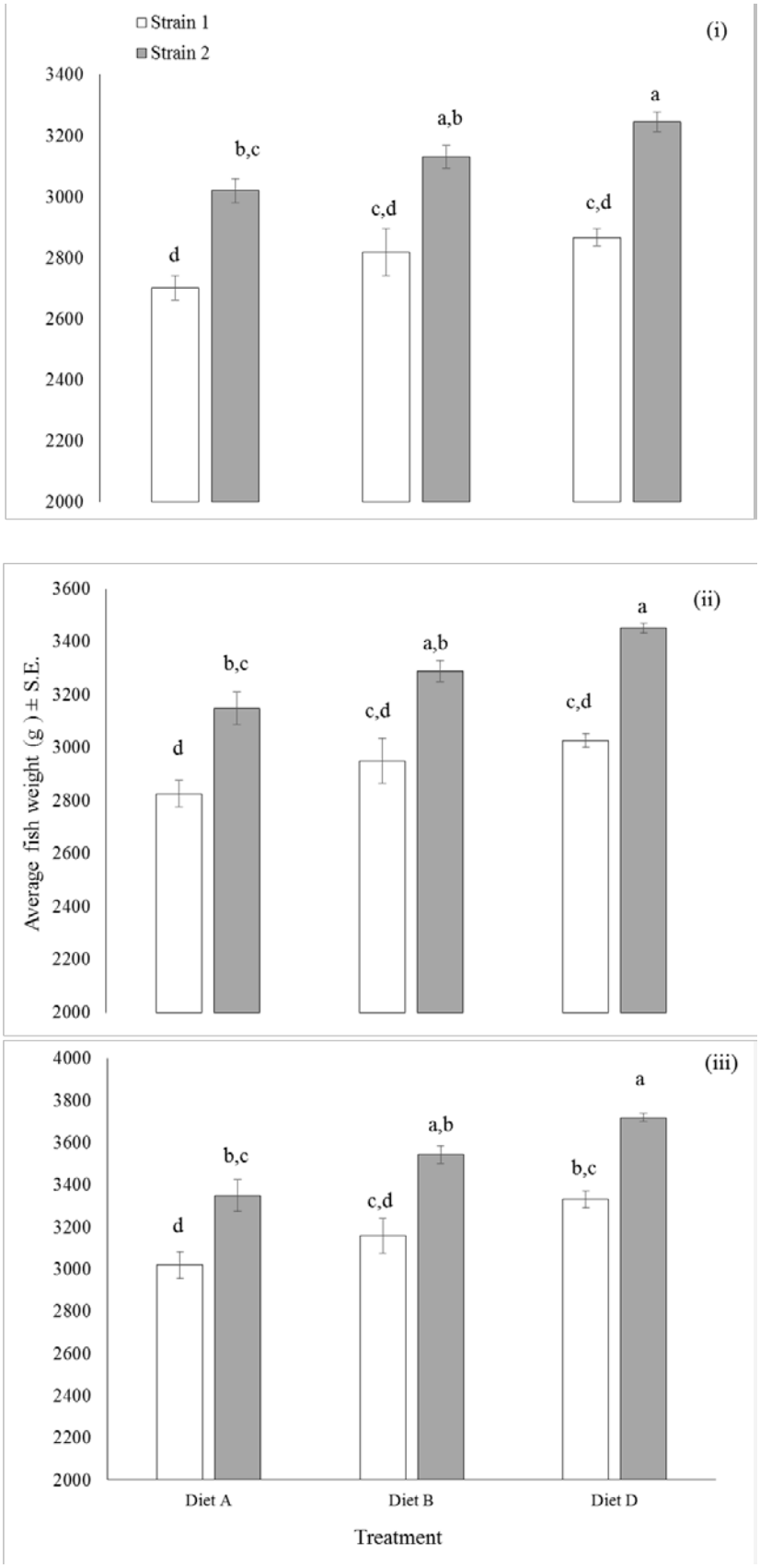

Figure 15: Avearge weight (g) of YTK in each treatment at the end of month 1 (i), month 2 (ii), month 3 (iii) of Block 3.

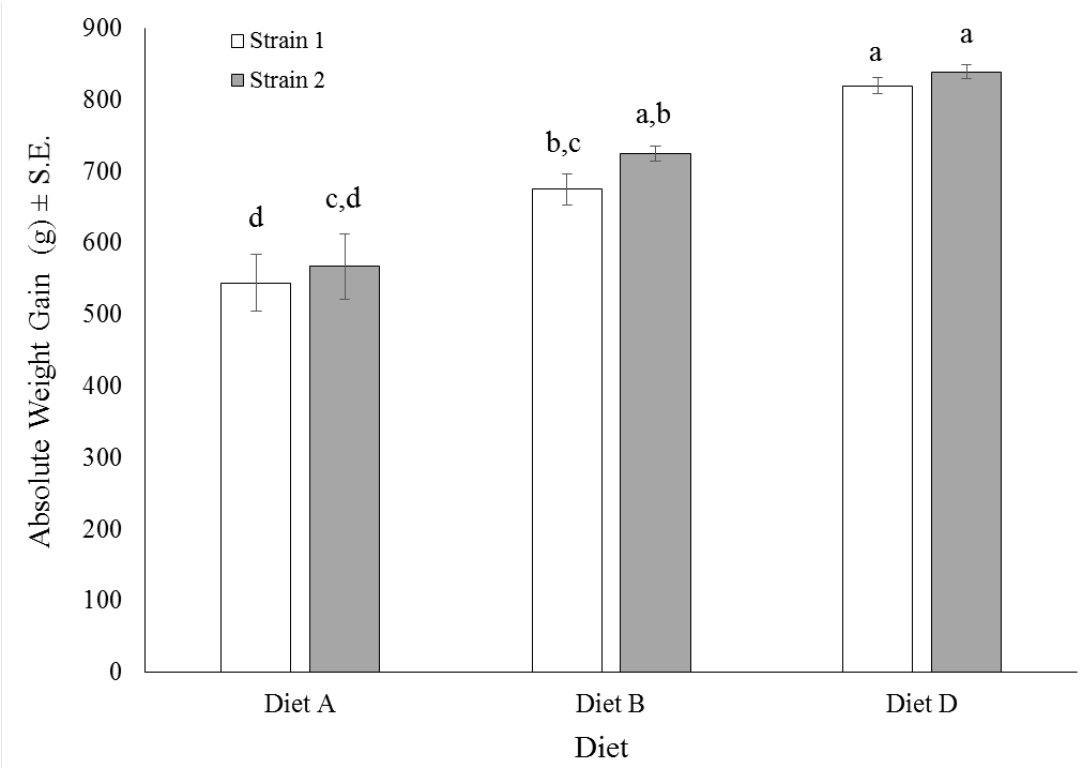

Figure 16: Average absolute weight gain (g) of YTK from different strains and fed different diets during Block 3.

Using our average starting weight of 2659 grams, we have added ‘high’ and ‘low’ predicted growth from Equation 1 to the data collected during Block 3 (Figure 17) to Figure 18 as well as the growth predicted by Equation 2 at our average water temperature of 18.7˚C. These data demonstrate that we achieved a very similar growth rate to that predicted by the second equation and to that predicted by the low rate from the first equation. This suggests that the second model is accurate at this water temperature and despite these models being validated only at fish sizes up to 2 kg, the second model remains reasonably accurate for fish up to 3.2 kg and the first is accurate at the lower levels of confidence limits within it.

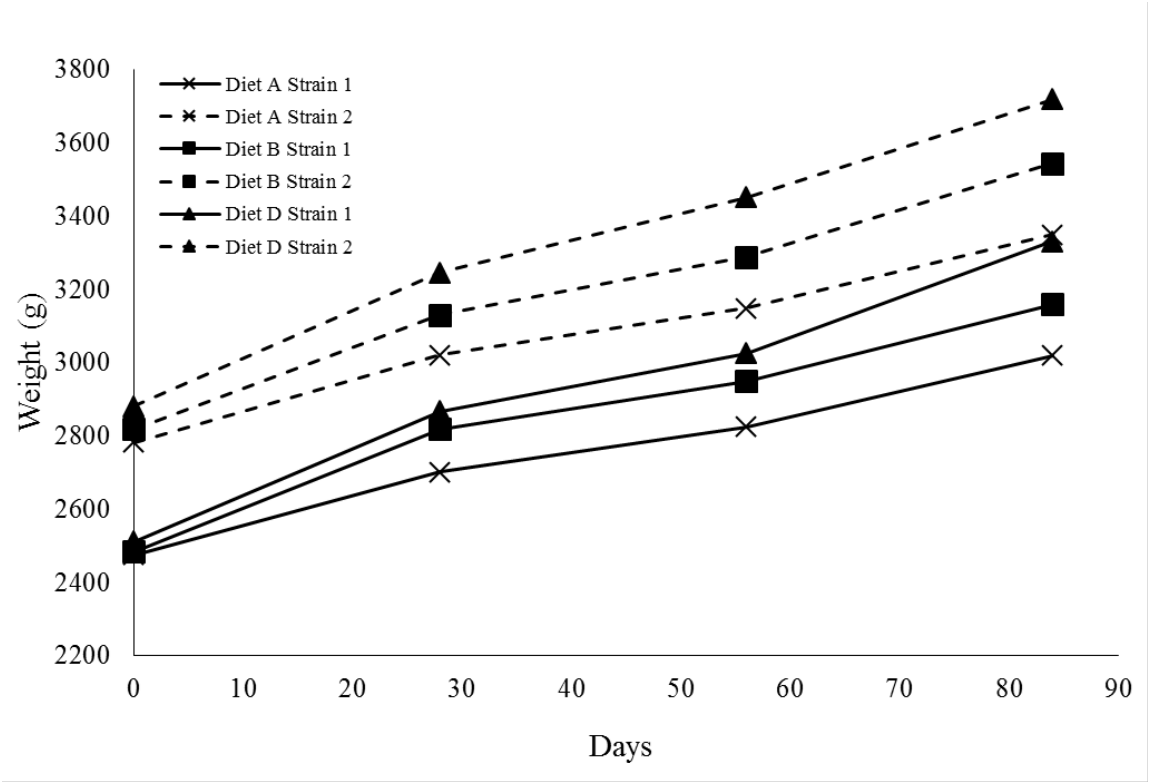

Figure 17: The effect of YTK strain and diet on weight over time during Block 3.

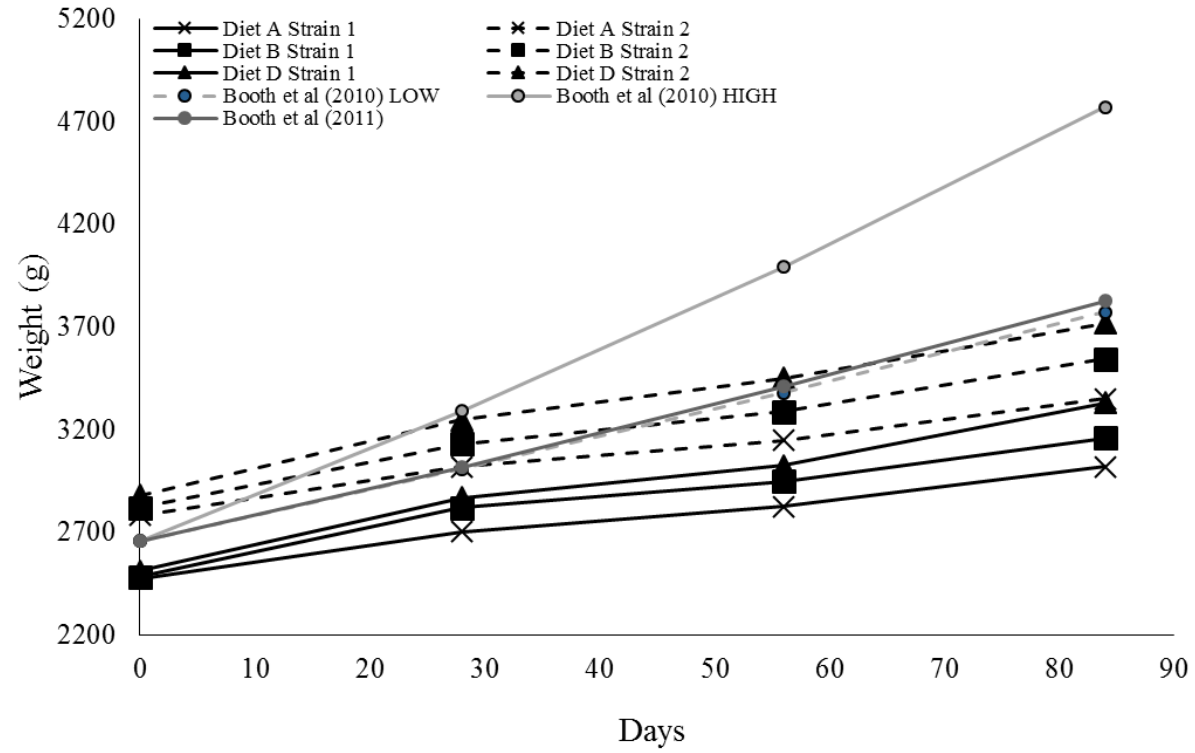

Figure 18: Growth of YTK from different strains and fed different diets during Block 3 including predicted growth modelled from Booth et al. (2010) and (2011)

4.2.4. Block 4

Fish were redistributed into Block 4 such that the fish sizes were as similar as possible to those at the end of Block 3. The average starting weight of fish was therefore effected by diet (P = 0.0001) and strain (P = 0.001) and with no interaction effect (P = 0.96) (Figure 19). The average starting weight of fish in Diet D treatment was 3590 g and for Diet B fish was 3398 g. The average starting weight of Strain 1 fish was 3315 grams and of Strain 2 fish was 3673 grams.

At the end of the trial, these relationships and their significance remained the same. The average size of fish fed Diet D (3847 grams) was significantly greater than those fed on Diet B (3635 grams) and Strain 2 fish remained significantly larger (3908 grams) than those from Strain 1 (3574 grams) (Figure 20). In terms of absolute weight gain, there was no effect of diet (P = 0.47), strain (P = 0.37) or the interaction of these terms (P = 0.21)(Figure 21). Fish fed Diet D gained 256 grams, whilst those fed Diet B gained 237 grams. Strain 1 fish on average gained 259 grams, whilst those from Strain 2 gained 234 grams. These data suggest that the beneficial effects of Diet D seen in Block 3 were no longer as pronounced as during Block 3 and the reasons for this are discussed below under the Food Conversion Ratio and Food Consumption section.

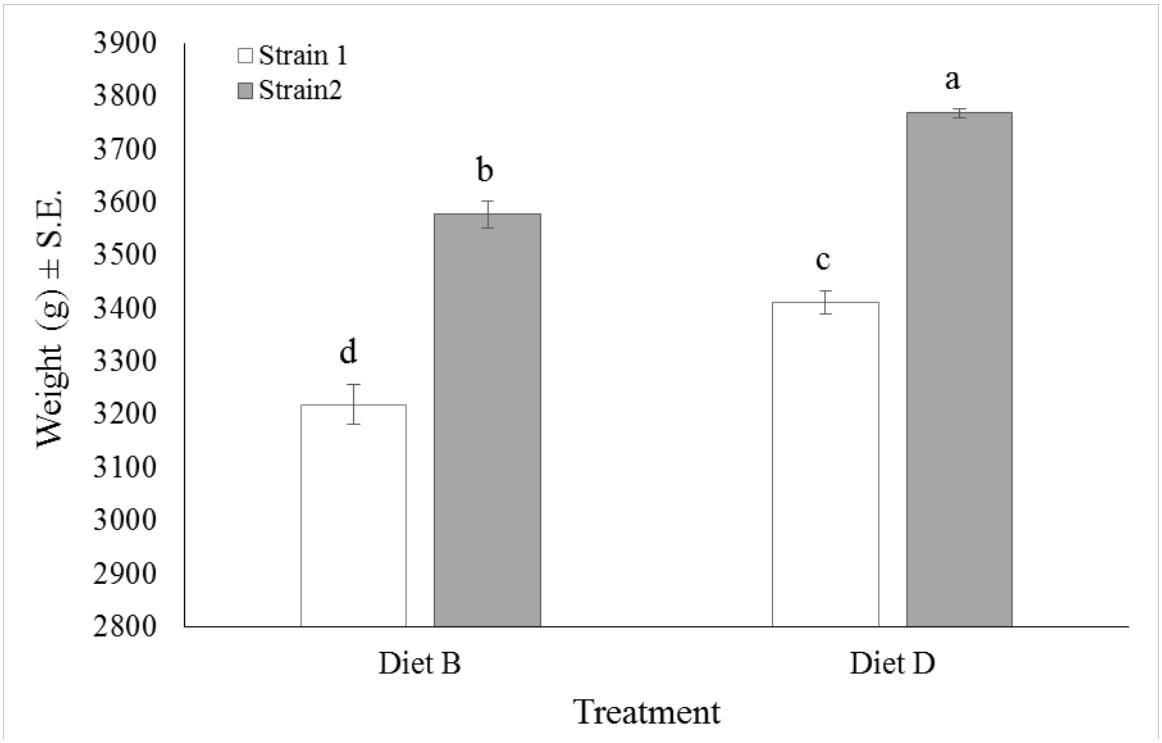

Figure 19: Average starting weight (g) of YTK in each treatment of Block 4.

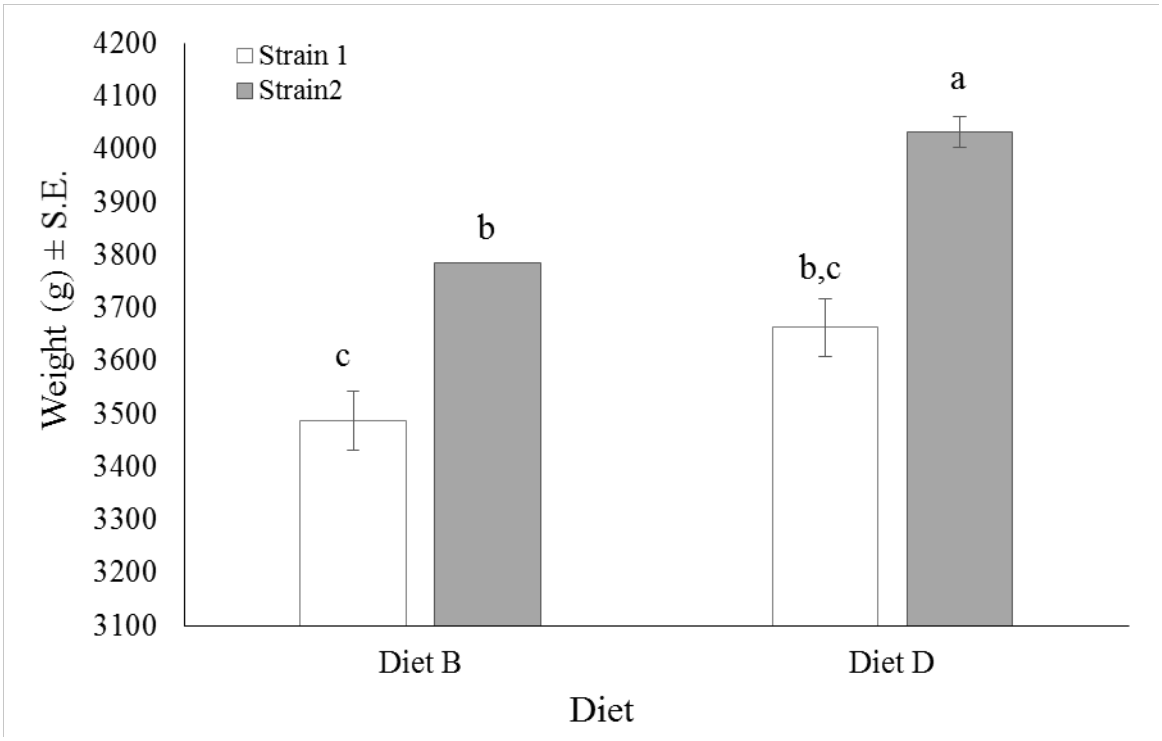

Figure 20: Average final weight (g) of YTK in each treatment of Block 4.

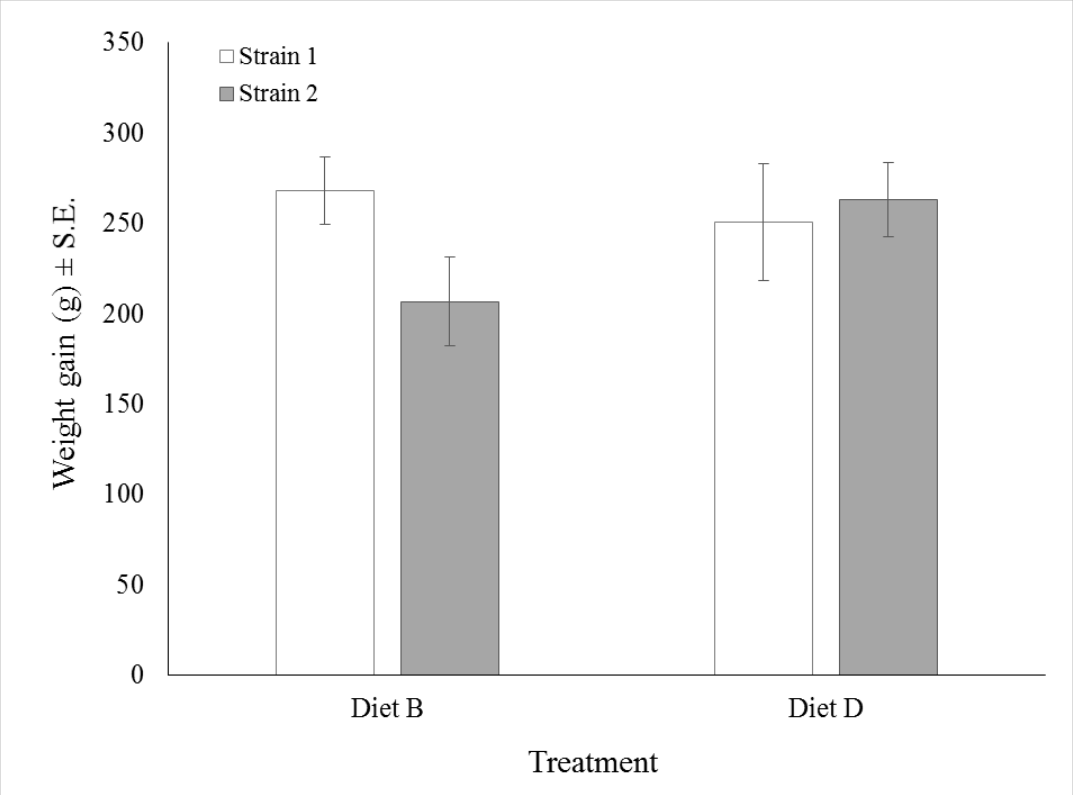

Figure 21: Average absolute weight gain (g) of YTK from different strains and fed different diets during Block 4.

Using the same approach described previously, we have added ‘high’ and ‘low’ predicted growth from Equation 1 (Booth et al 2010) to the data presented in Figure 22 below as well as the growth rates predicted by Equation 2 (Booth et al 2011) at our average water temperature of 20.5˚C. The similarly of the slope of our growth lines to the ‘low’ model demonstrates that the model is relatively accurate with the lower range of parameters, despite these models being validated only at fish sizes up to 2 kg. The steeper slope of the ‘high’ model parameters and the Booth et al (2011) temperature compensated model shows that these models did not reflect the growth rates of these fish.

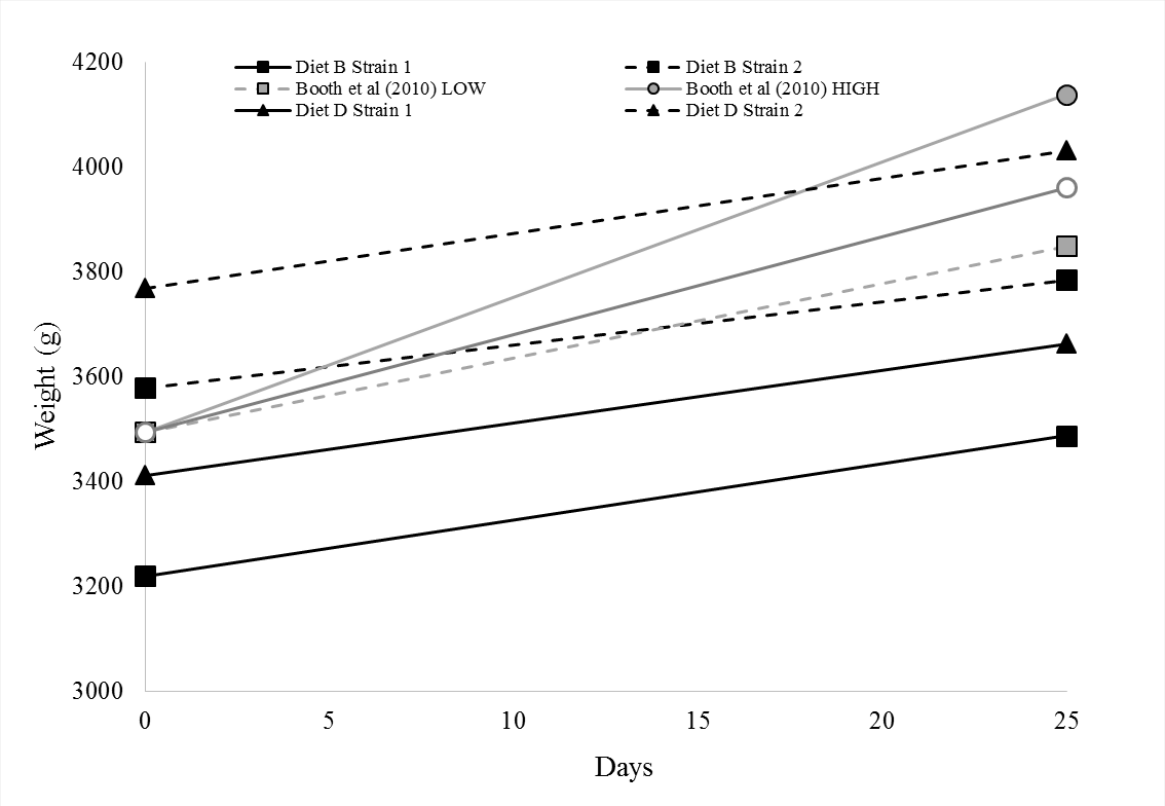

Figure 22: Growth of YTK from different strains and fed different diets during Block 4 including predicted growth rates from Booth et al. (2010) and (2011)

4.3. Food Conversion Ratio and Food Consumption

Over the 56 week trial, those fish fed continuously on Diet B had an overall FCR over this period of 1.70. In order to compare our data with the national FCR targets of <1.5 for fish up to 1.5 kg and <2.2 for fish between 1.5 - 3.5 kg, we have broken down the FCR into fish of these two size classes. Fish fed Diet B up to 1.5 kg achieved an FCR of 1.2, better than the national target. However, the FCR in fish from 1.5 to 3.6 kg was slightly worse than the national target at 2.3. Month-to-month FCR and the average daily food intake on a percent body weight basis (%BW.day^{-1}) for fish fed Diet B throughout the trial are shown in Figure 23, along with the growth of these fish, which was also presented above in Figure 2

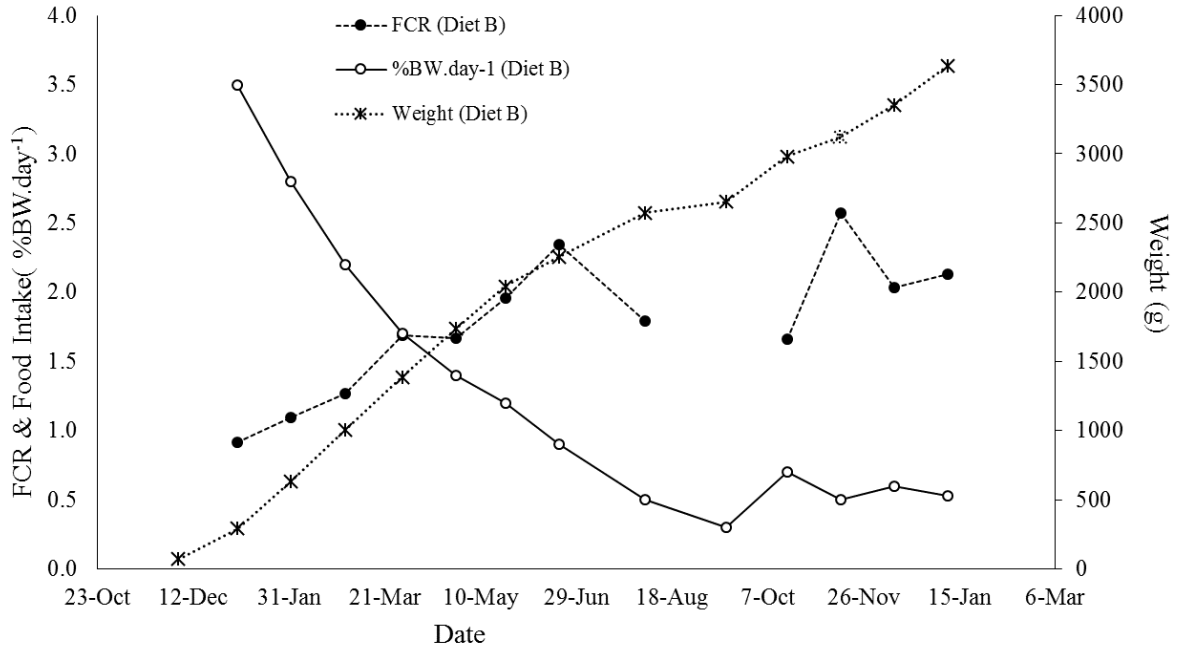

Figure 23: Food conversion ratio, food intake (%BW.day^{-1}) and growth over the full 56 week trial for fish fed Diet B.

FCR data were not obtained during the interim period between Block 2 and 3. These data may firstly suggest that the fish were underfed during this interim period as the growth curve flattens a little at this point. Furthermore, the slight increase in feed rate (%BW.day^{-1}) in the first month of Block 3 may suggest that the fish were overfed in this month, however the FCR during this month was good (~1.5) but jumped to 2.5 in the following month. This may be the result of a period of compensatory growth following the interim period when the fish were underfed.

The raw food intake data (on a %BW basis) for fish fed Diet B throughout the trial are shown in Figure 24, along with a power curve fitted to this data. A power curve was fitted on the basis that the same curve type was used by Booth et al (2010) and this curve is also plotted. These data show that the power curve is probably not the most appropriate to our data set, as the majority of points throughout most of the trial period are above the line of best fit. They also demonstrate that our fish ate more than that predicted by the Booth et al (2010) model when they were small (< 1 kg) but less than the model when they were larger. This was also reflected in the growth of the fish in the current trial compared with the models (see Figure 8,

Figure 13, Figure 18 and Figure 22) and suggests that the inaccuracy of the model for small fish is the result of its underestimation of food intake by these fish.

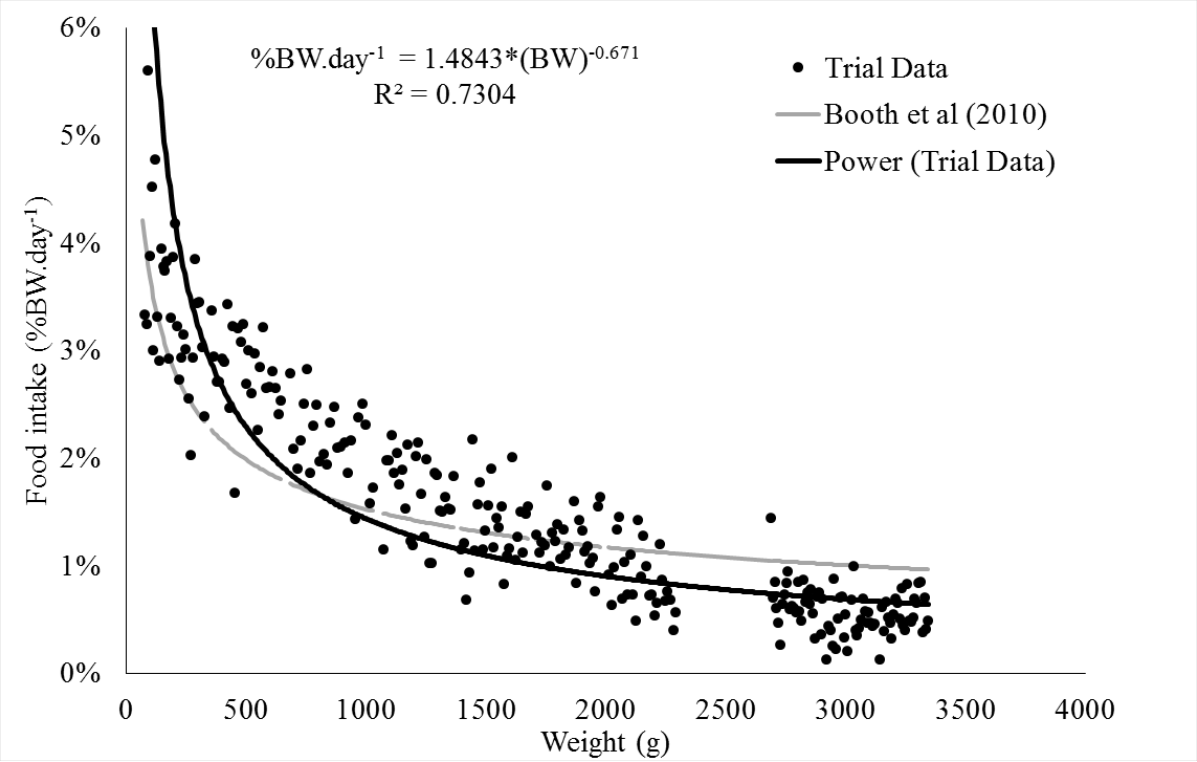

Figure 24: Relationship between fish weight (g) and food intake (%BW.day^{-1}) for YTK fed Diet B for 56 weeks and modelled data from Booth et al (2010).

4.3.1. Block 1

The total amount of food eaten by fish in each treatment over the three month Block 1 period shows that fish fed Diet C ate significantly less food (101 ± 1 kg) than those fed Diets A (119 ± 3) and B (127 ± 2 kg), which did not differ from each other (Figure 25).

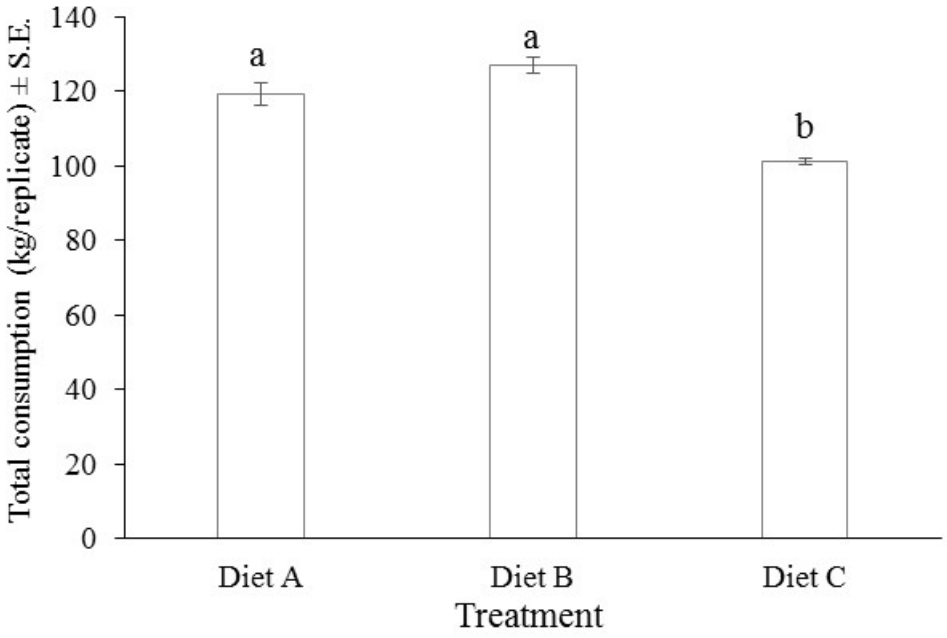

Figure 25: Average total consumption (kg) of each diet by YTK during Block 1.

The amount of food eaten on a %BW basis as a function of fish size for fish in Block 1 is shown in Figure 26. Also shown in this figure are the high and low range values for relative food intake derived from the following equation from Booth et al. (2010).

Equation 3: Relative feed intake (% BW.d^{-1})= 21.14 ± 1.02 x $BW^{-0.3805 \pm 0.012}$

As expected, based on their superior growth, this figure demonstrates that the food intake by YTK in the current trial was always higher than predicted by the model.

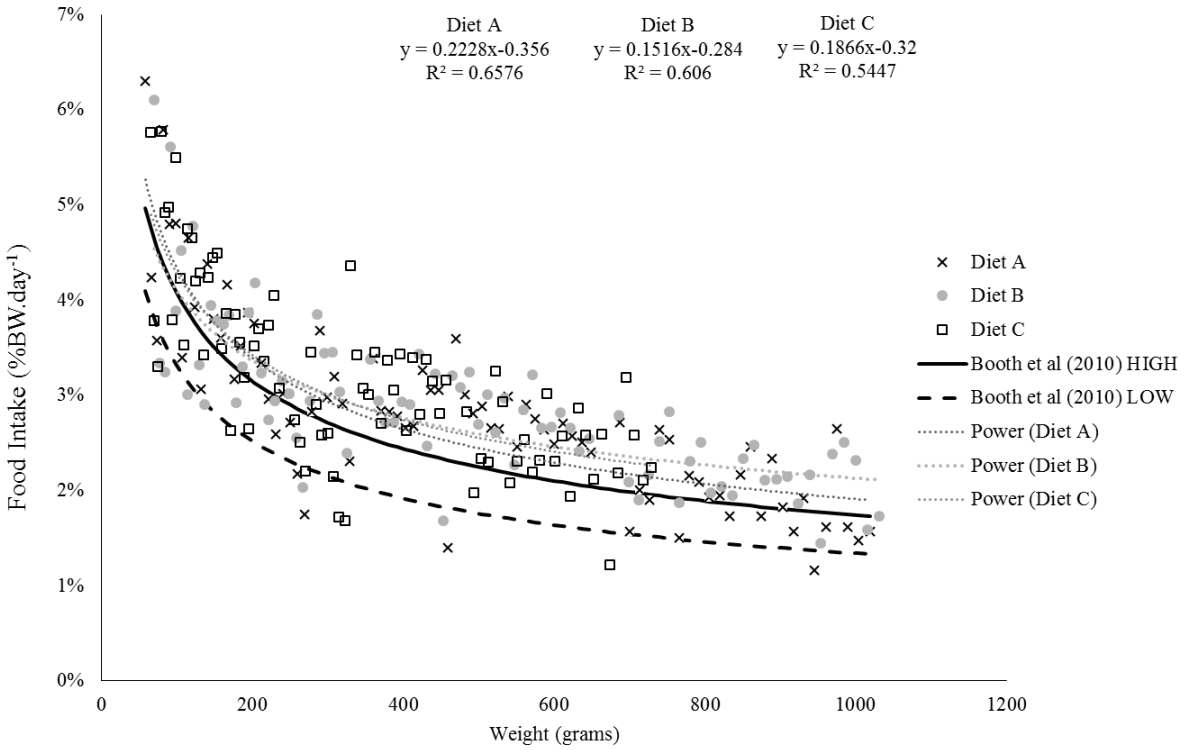

Figure 26: Relationships between fish weight (g) and relative food intake (%BW.day^{-1}) for YTK fed different diets during Block 1 and modelled data from Booth et al. (2010).

Food conversion ratio (FCR) over the full three month period was significantly affected by diet ($P < 0.0001$)(Figure 27). Those fish fed Diet C had a significantly worse (higher) FCR (1.27 ± 0.02) than those fed Diet A or B. Fish fed Diet A had the best (lowest) FCR (1.05 ± 0.01), significantly better that those fish fed Diet B (1.11 ± 0.01).

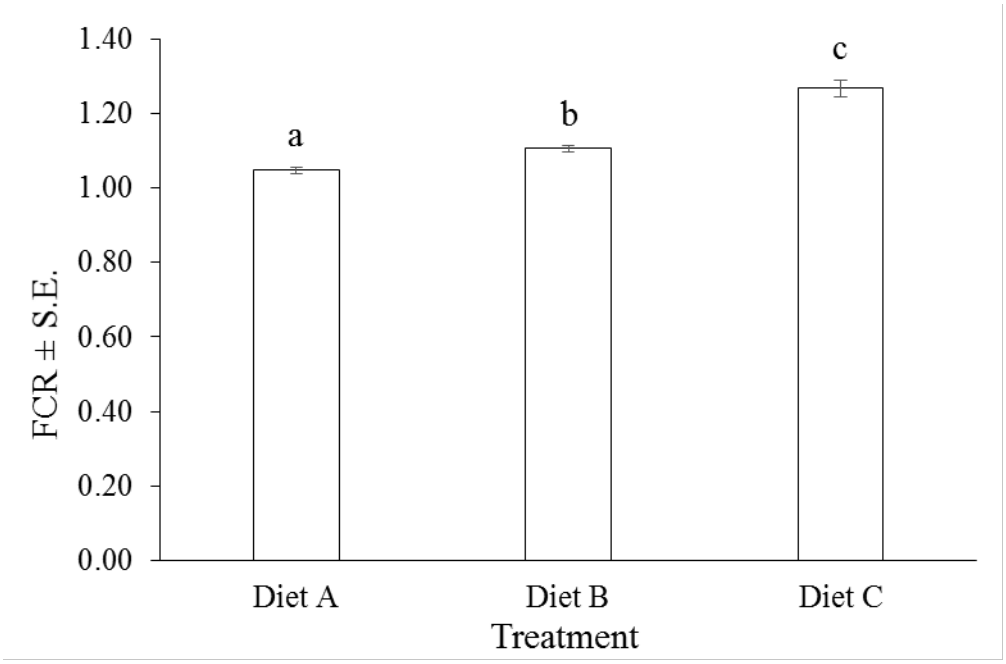

Figure 27: Average food conversion ratios (FCR) of YTK fed different diets during Block 1.

The change in FCR over time for each diet is shown in Figure 28. This figure demonstrates a linear increase in FCR over time on Diets A and B, but a levelling of FCR between months 2 and 3 in fish fed Diet C. This figure also shows that whilst there was never any significant difference in FCR between Diets A and B from month to month, the consistently better FCR on Diet A resulted in the significantly better overall FCR achieved using this diet (Figure 27).

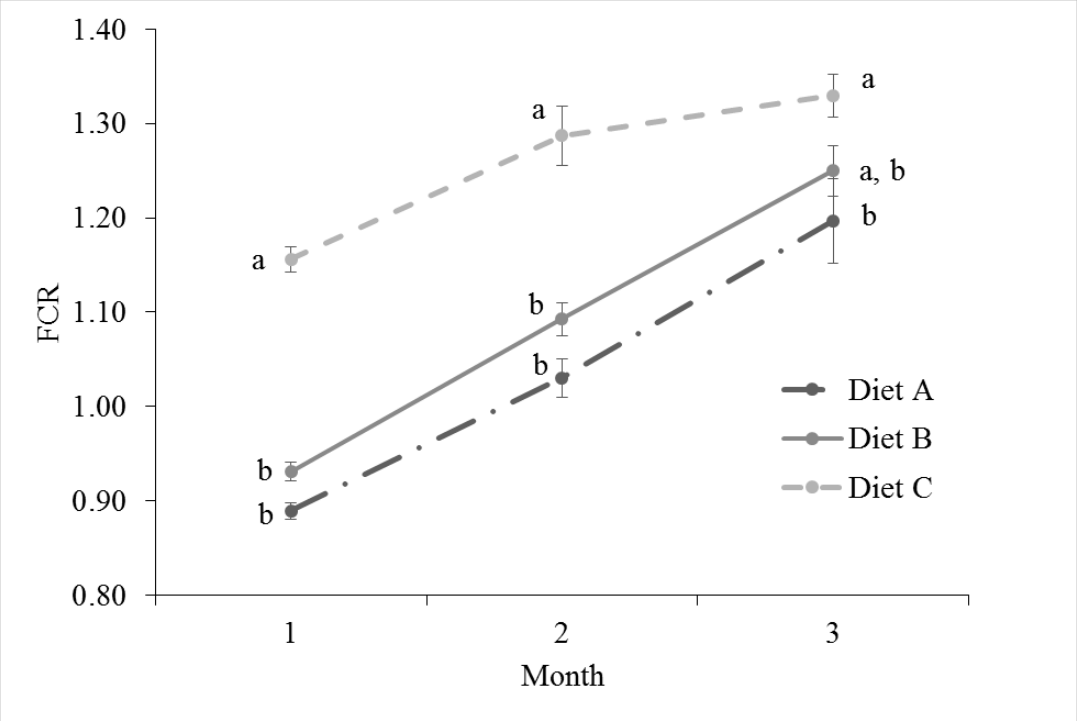

Figure 28: Change in FCR with time for YTK fed different diets during Block 1.

Booth et al (2010) achieved FCR values ranging from 1.39 to 1.68 over a 35 day period in fish that were approximately equivalent in weight to those grown between month 1 and month 2 in the current trial. FCR obtained during this period in the current trial ranged from 1.05 (Diet A) to 1.31 (Diet C). These trials demonstrate that achieving an FCR <1.5 can be achieved with YTK of this weight.

4.3.2. Block 2

There was no significant effect of diet on the total consumption of food (P = 0.1), which ranged from 134 ± 6 kg in the exDiet C treatment to 153 ± 4 kg in the Diet B + Actigen treatment (Figure 29). This would suggest that the compensatory growth exhibited by fish in the exDiet C treatment was not the result of a greatly increased food intake, however these fish were significantly smaller than those in the other treatments for most of the trial and therefore their intake on a percentage body weight basis may have been higher.

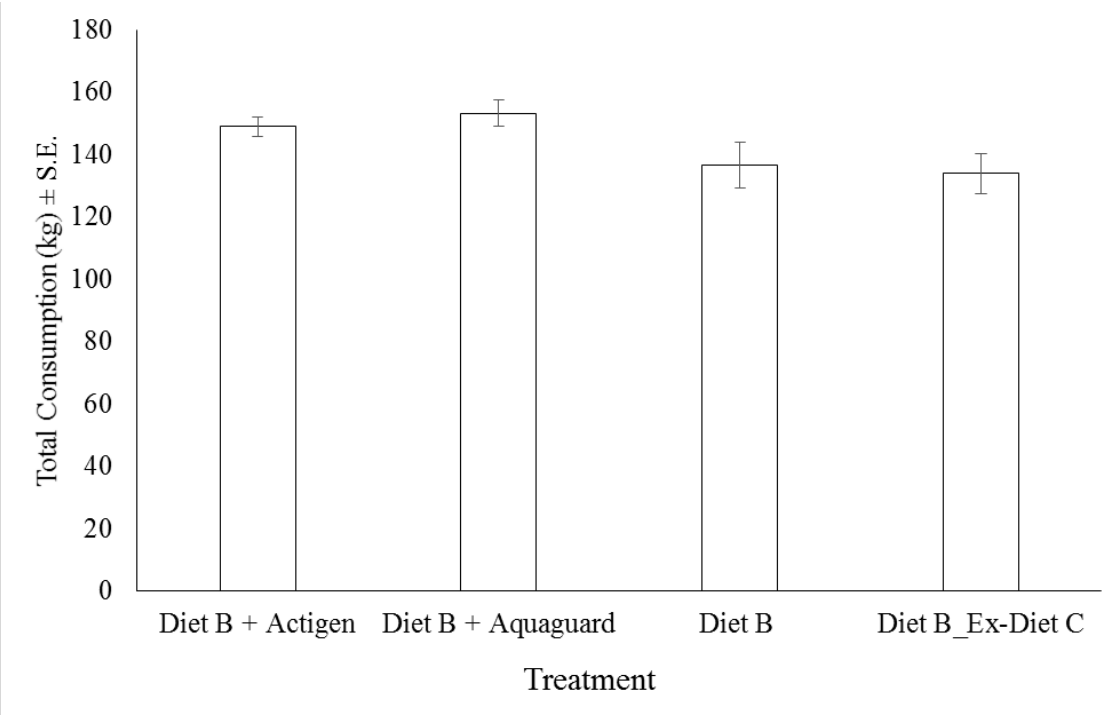

Figure 29: Average total consumption (kg) of each diet by YTK during Block 2.

The amount of food eaten on a %BW basis as a function of fish size is shown in Figure 30. Also shown in this figure are the high and low range values derived from the equation from Booth et al. (2010) described above.

This figure and the low R^2 values of the fitted curves demonstrate that intake was highly variable, much more so than was seen for the smaller fish in Block 1. Generally speaking, however, the lines of best fit fall within the range of intakes predicted by the Booth et al. (2010) model, except at the start and end of the trial when the average ration was slightly above and slightly below that predicted by the model, respectively. This feeding response whereby fish eat a lot one day, followed by a day of much lower intake is known as 'ratchet' or 'yo-yo' feeding and is responsible for the variable daily feed intake seen in Figure 30. Offering a fixed ration based on these lines of best fit may be worthy of investigation in order to prevent ratchet feeding and improve FCR. Fish in the exDiet C treatment appeared to be often above the line of best fit at the start of the trial and below the line towards the end of the trial, suggesting that there was some elevated compensatory intake at the start of this trial.

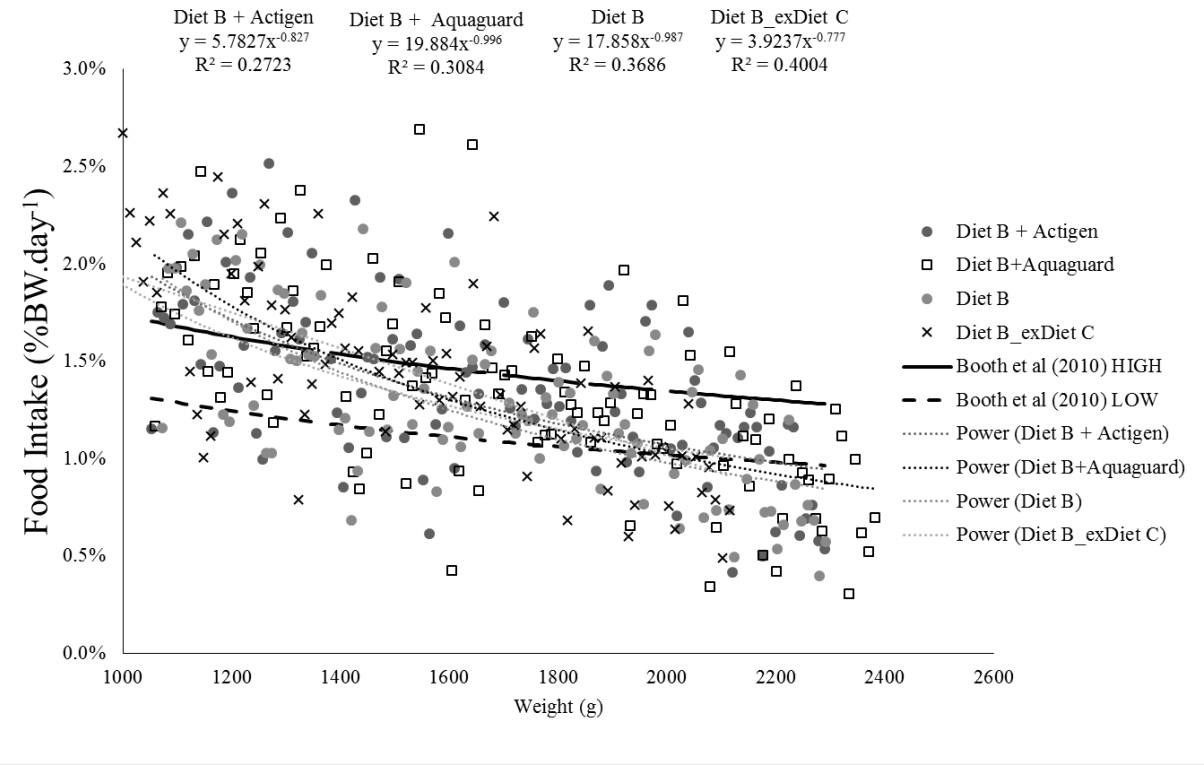

Figure 30: Relationships between fish weight (g) and relative food intake (%BW.day^{-1}) for YTK fed different diets during Block 2 and modelled food intake data from Booth et al. (2010).

Food conversion ratio (FCR) over the full four month period of Block 2 is shown in Figure 31. Whilst those fish in the exDiet C treatment had the lowest FCR of 1.58 ± 0.07, there was no significant effect of diet on FCR (P = 0.07).

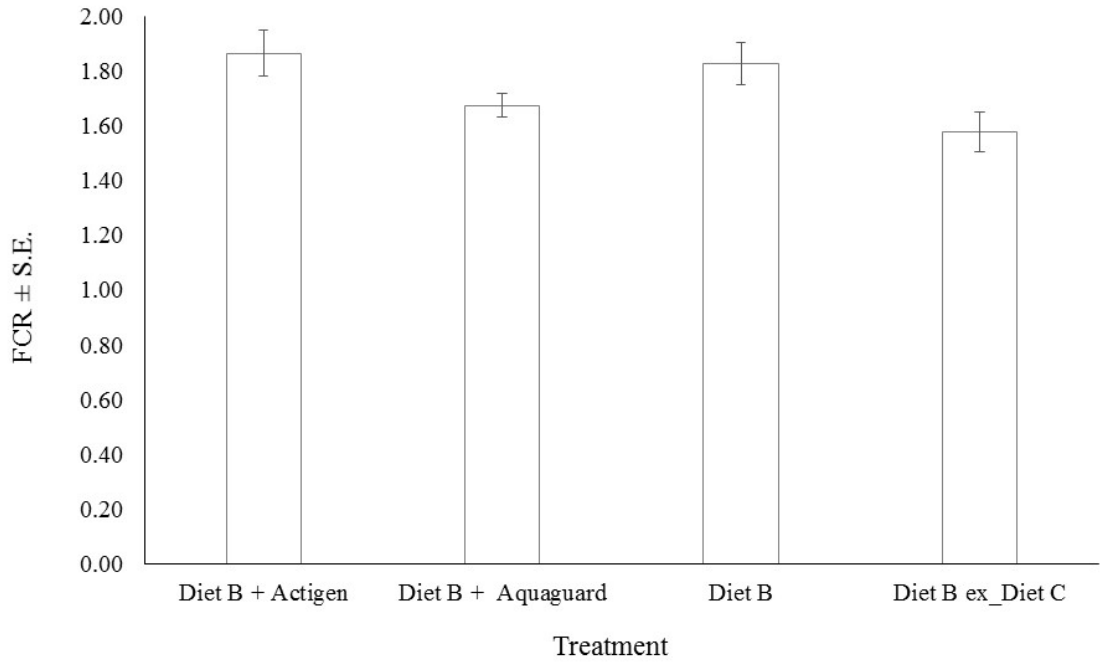

Figure 31: Average food conversion ratios (FCR) of YTK fed different diets during Block 2.

The change in FCR over time for each diet is shown in Figure 32. This figure demonstrates an increase in FCR over time, which is expected, with the largest increase in FCR occurring between months 3 and 4. The only significant difference that occurred in FCR between diets at any time point was during the first month when those fish in the exDiet C treatment had a significantly lower FCR (1.42 ± 0.05) than all other treatments, which did not differ from each other (pooled FCR = 1.64 ± 0.03). This lower FCR for the exDiet C fish is likely due to the compensatory growth they were experiencing during this time.

Two-way analysis of variance showed that diet had no effect on FCR ($P = 0.6$) but that time was significant ($P < 0.001$), with FCR in the final month (2.31) being significantly higher than all other months. This significant jump in FCR corresponded to a fish weight ranging from 2.0 to 2.2 kg. As noted above, feeding a fixed ration to prevent the ‘ratchet’ or ‘yo-yo’ feeding highlighted in Figure 26 may be effective at improving FCR and should be investigated.

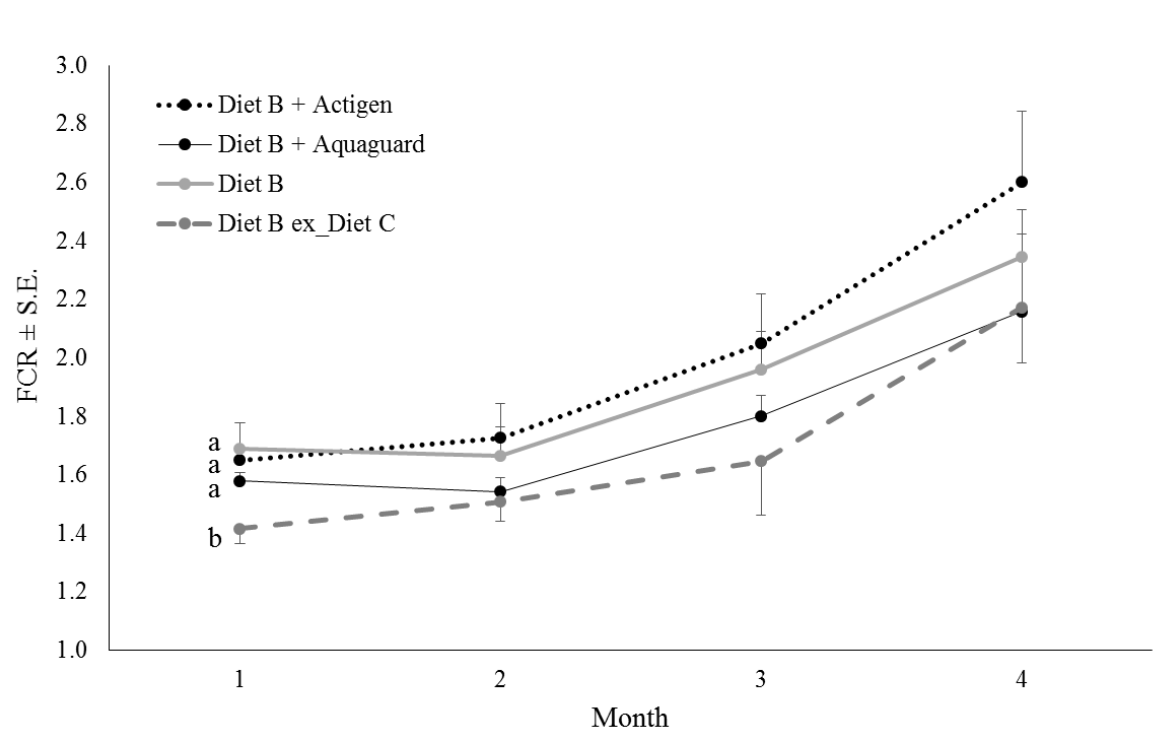

Figure 32: Change in FCR with time in YTK fed different diets during Block 2.

4.3.3. Block 3

There was a significant effect of dietary treatment on the total amount of food consumed (P = 0.0001), with those fish fed Diet D eating significantly more food (54.95 kg) than those fed Diet A (45.88 kg) and Diet B (48.56 kg), which did not differ from each other (Figure 33).

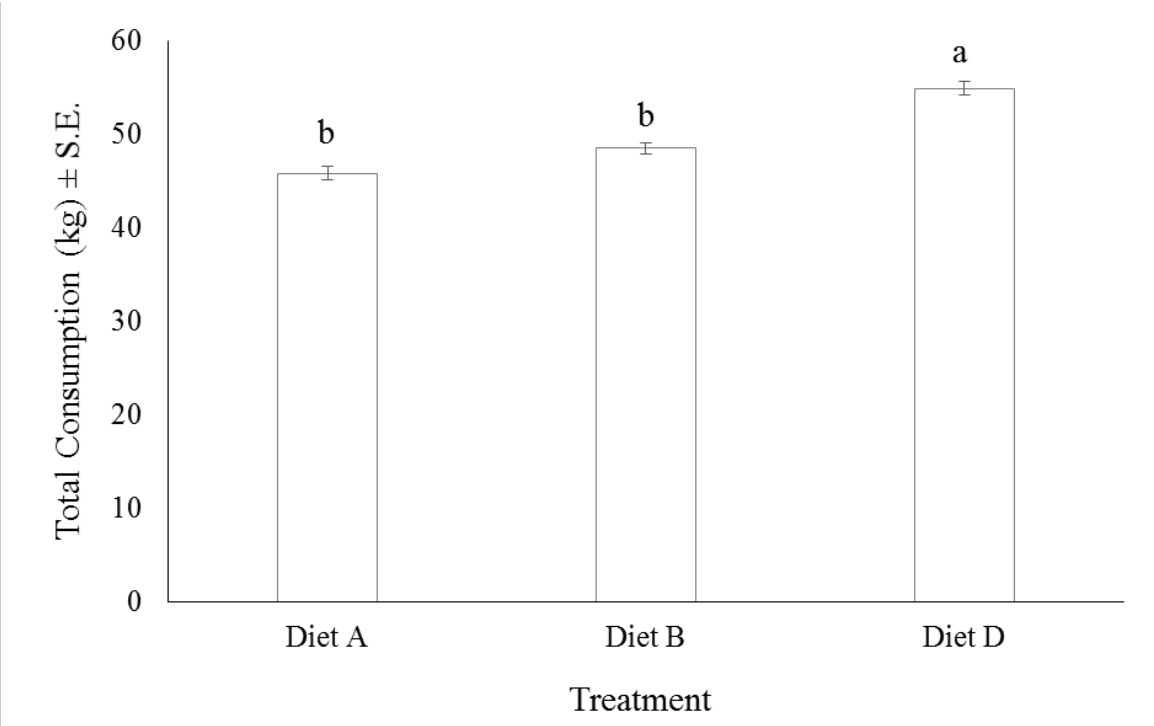

Figure 33: Average total consumption (kg) of each diet by YTK during Block 3.

The amount of food eaten on a %BW basis as a function of fish size is shown in Figure 34. Also shown in this figure are the high and low range values derived from the Booth et al. (2010) model previously described.

This figure and the low R^2 values of the fitted curves demonstrate that intake was highly variable and much lower than predicted by the Booth et al. (2010) model. As previously noted this model was developed using fish sizes up to 2 kg and therefore this demonstrates that the feed intake aspect of this model is inaccurate for fish of this size, or that our fish were being underfed during this block. In Block 3 water temperature averaged 18.7°C. At a water temperature of 19˚C, the feeding tables of Matsumoto et al (2002) for Japanese yellowtail (*Seriola quinqueradiata*), also suggest that fish between 2.5 and 3.5 kg should eat between 1.00 and 1.20% of their body weight per day, similar to the range in the Booth et al (2010) model. Feeding more slowly or more frequently may have enabled us to achieve a higher food intake and more rapid growth.

That the Booth model suggests our fish should be eating more, but we obtained growth rates in line with the bottom end of the model suggest that we achieved a superior FCR than would be forecast by this model.

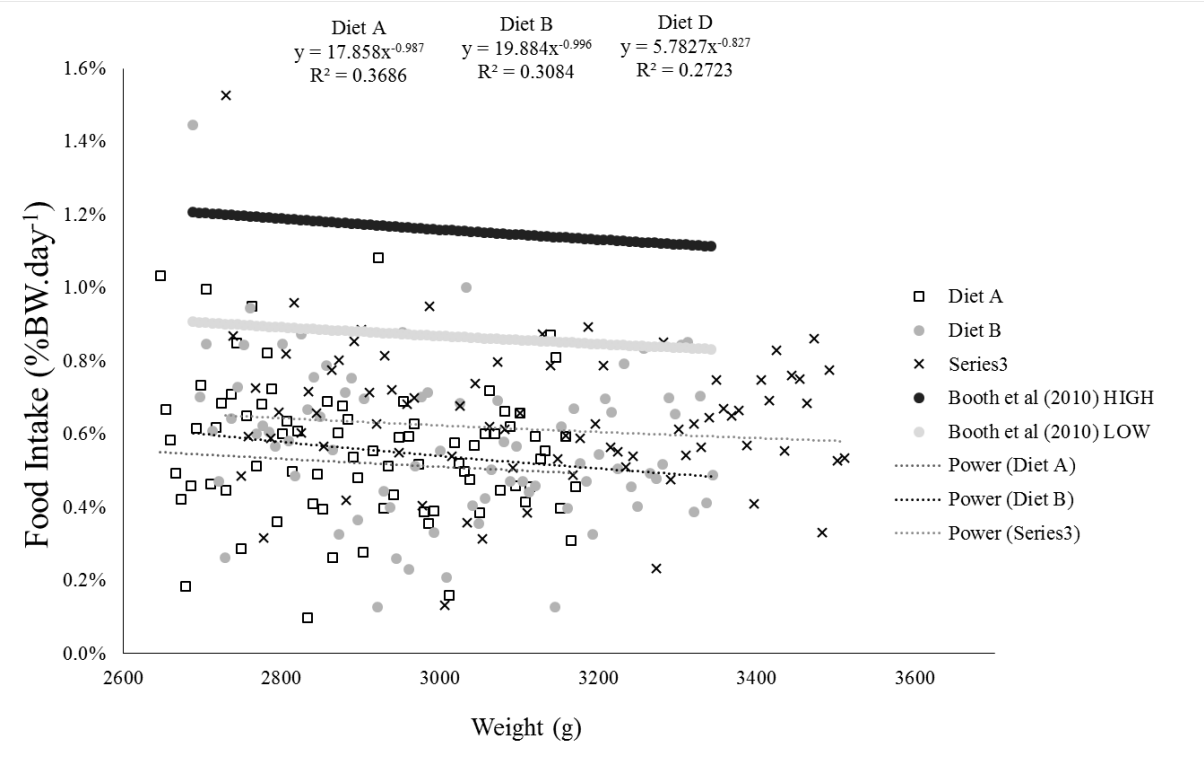

Figure 34: Relationships between fish weight (g) and relative food intake (%BW.day^{-1}) for YTK fed different diets during Block 3 and modelled food intake data from Booth et al. (2010).

FCR over the full three month Block 3 trial ranged from 1.90 ± 0.03 in fish fed Diet D to 2.27 ± 0.16 in fish Diet A, however there were no statistical differences in FCR between the three treatments (P = 0.06)(Figure 35).

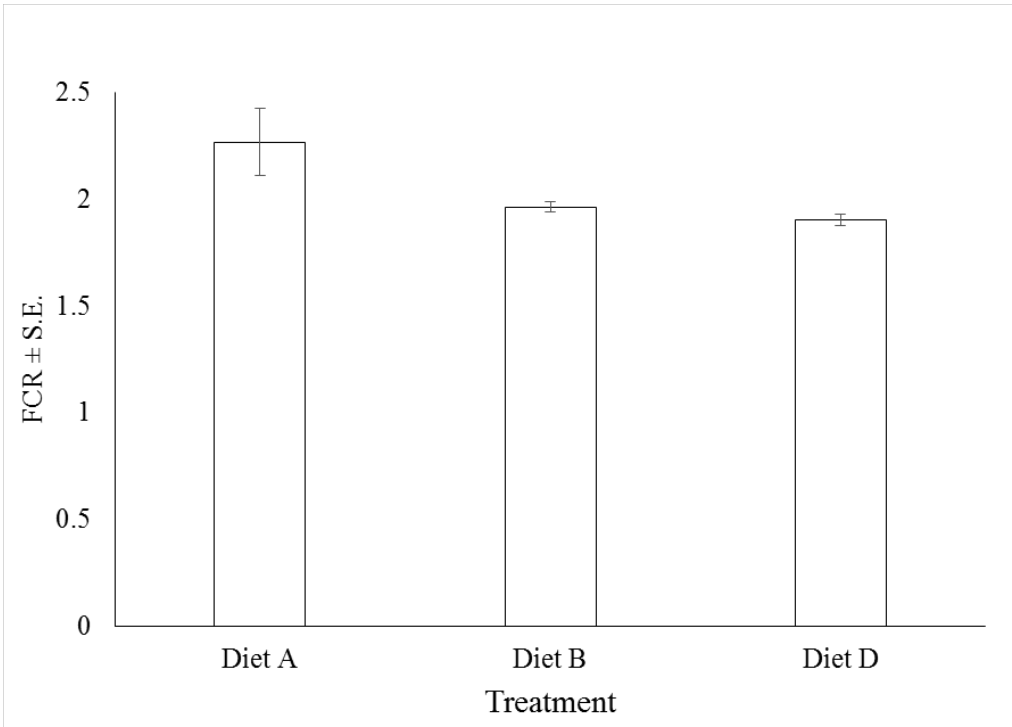

Figure 35: Average food conversion ratios (FCR) of YTK fed different diets during Block 3.

4.3.4. Block 4

Whilst fish in Block 4 again consumed more of Diet D (9562 grams) than Diet B (9121 grams), this difference was not significant (P = 0.27)(Figure 36). This is in contrast to the results of Block 3 where fish consumed significantly more of the same Diet D than Diet B.

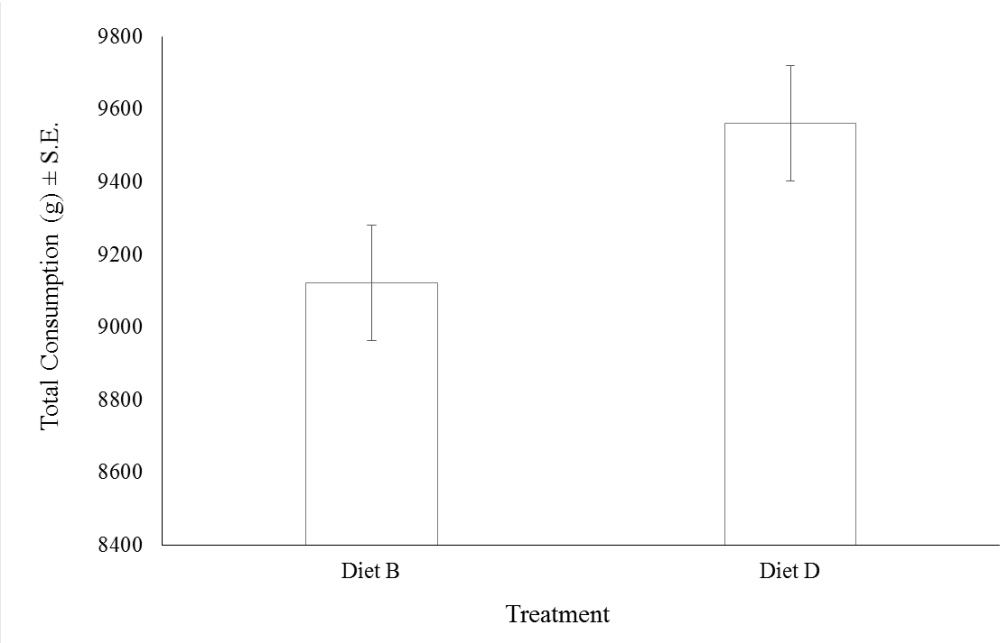

Figure 36: Average total consumption (kg) of each diet by YTK during Block 4.

Similarly to the results presented from Block 3, the average feed consumption was around 0.57%BW.day^{-1}. This was equal between treatments and less than the 0.9 to 1.0% suggested for Japanese yellowtail of this size by Matsumoto et al (2002) and by the Booth et al (2010) model.

Food conversion ratio did not differ between treatments (P = 0.42) and was considerably higher than during Block 4 than Block 3 (Figure 37) and was also higher than during the final month of Block 3, where FCR values were 1.98 and 2.03 for Diet D and Diet B, respectively.

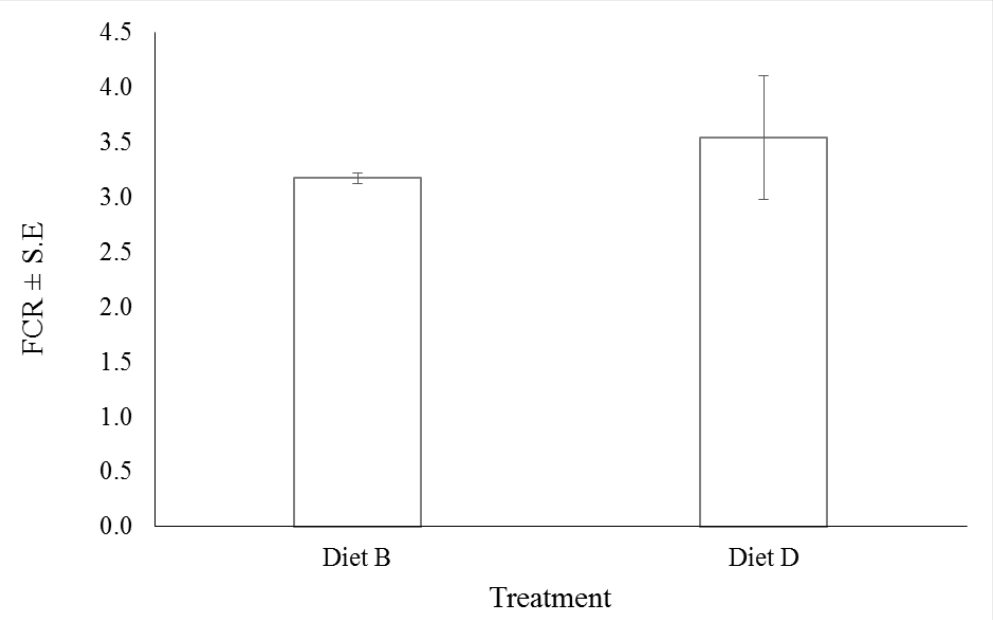

Figure 37: Average food conversion ratios (FCR) of YTK fed different diets during Block 4.

4.4. Survival

4.4.1. Block 1

There was considerable variation in mortality within and between treatments (Figure 38). As such, two-way ANOVA on arcsine transformed data showed no effect of fish strain ($P = 0.08$), diet ($P = 0.59$) or their interaction ($P = 0.58$) on the percentage mortality. Therefore, whilst the average mortality in Strain 2 (6 ± 2%) was double that of Strain 1 (3 ± 1%) (Figure 39), this difference was not significant. Similarly, whilst Diet A had the highest mortality (6 ± 2%) this was not significantly different to that of fish fed Diet B (3 ± 2%) or Diet C (4 ± 2%)(Figure 40). The effects of diet quality on survival are discussed in further detail below.

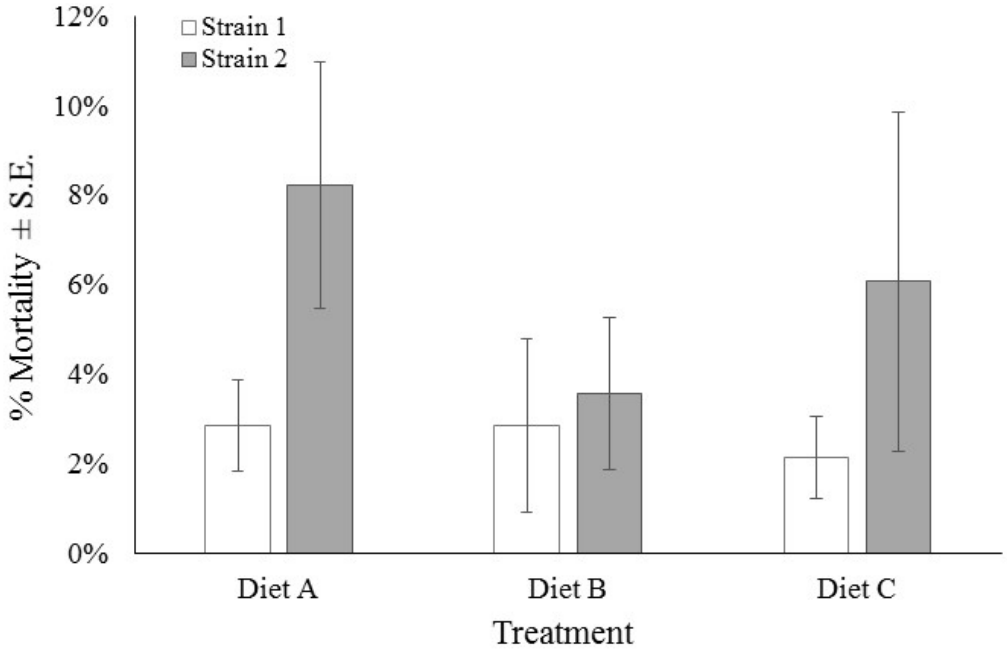

Figure 38: Mortality of YTK from different strains and fed different diets during Block 1.

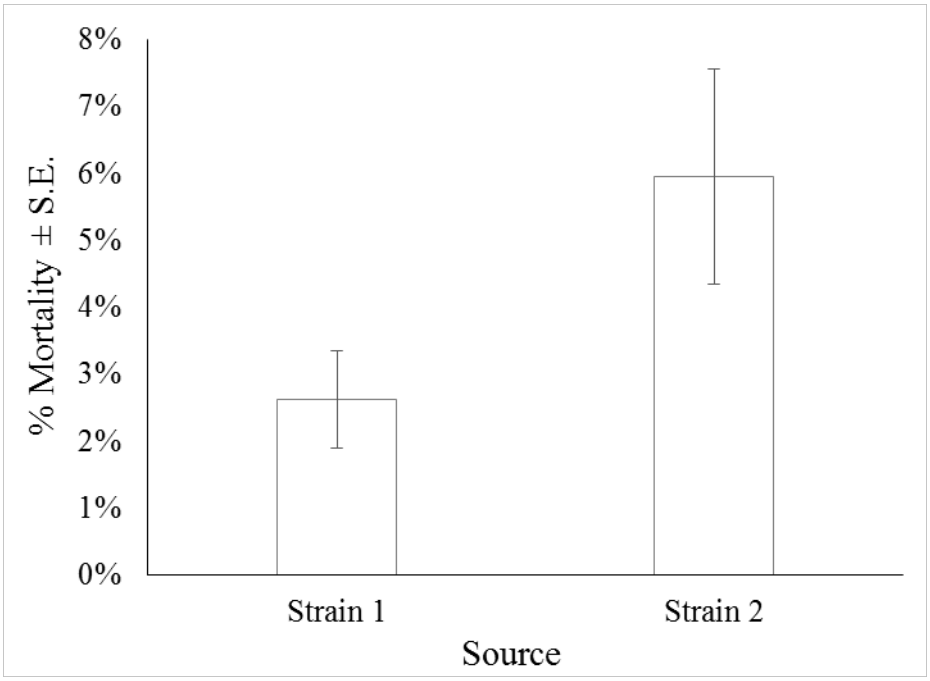

Figure 39: Mortality of YTK from different strains fed all diets during Block 1.

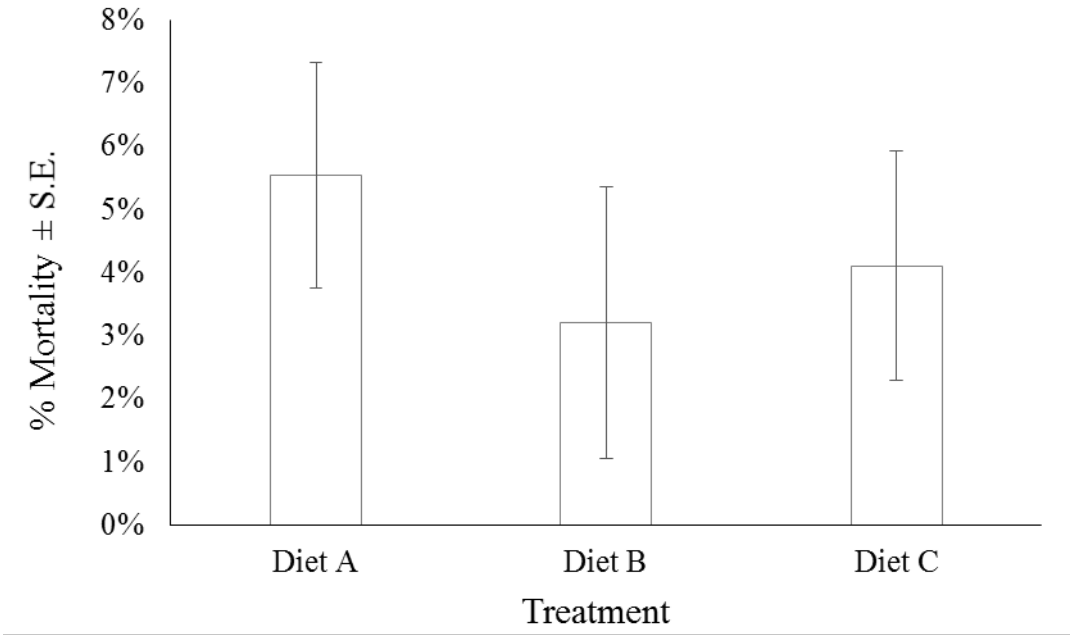

Figure 40: Mortality of YTK fed different diets regardless of strain during Block 1.

Figure 41 demonstrates that mortality increased over time, with most mortalities occurring in the final month of the trial. On the 22nd February 2016, following a relatively large mortality event the previous night (see Day 81 in Figure 41), a sample of nine fish were processed for health assessment. These included two seemingly healthy fish from each of two tanks which had experienced high mortality over the previous weeks and two fish from each of two tanks which had experienced no morality. One moribund fish was included in this submission. Swabs of the biofilm on the floor of each of these four tanks were also taken. A haemolytic strain of the bacterium *Photobacterium damselae* subsp *damselae* was isolated from the gut of all fish tested, including those which were seemingly healthy. In general, there was very little other bacterial flora in the gut of these fish. In addition to *P. damselae, Aliivibrio fisherii* was isolated from the gut of one seemingly healthy fish. The gut of the moribund fish also contained *Vibrio harveyi* (or *V. parahaemolyticus*) in addition to *P. damselae*. This moribund fish had a mottled red liver, a good PCV of 44% and severe diffuse hyperplasia and hypertrophy of the gill epithelium. Interestingly many of the mortalities in this trial also died with open mouths and flared opercula despite safe levels of dissolved oxygen being maintained at all times. *P. damselae* subsp *damselae* was isolated from the tank floor biofilm of three of four tanks tested, however the tank in which *P. damselae* subsp *damselae* was not isolated was a tank which had experienced high mortality. Of note is the fact that a bacterial

sample taken from one fish just prior to the commencement of the nutrition trial on the 19th November 2015 did not contain *P. damselae* subsp *damselae*, but did contain *Vibrio harveyi* in the gut and *Vibrio pelagius* and *Vibrio alginolyticus* in the coelmic cavity. One week later (26th November, 2015), three moribund and one healthy fish were submitted for bacteriology. The three moribund fish all contained *Vibrio harveyi* and the healthy fish contained only *P. damselae* subsp *damselae*. All three moribund fish submitted on 10th March 2016 contained *P. damselae* subsp *damselae*, showed nothing remarkable histologically and had high PCVs of ≥ 49%.

P. damselae subsp *damselae* is generally considered a secondary opportunistic pathogen. Whilst several of the biotypes isolated in these cases showed haemolytic properties on the agar plates on which they were cultured, none of the fish sampled, including moribund fish, showed any symptoms of anaemia. Furthermore, there was no histological evidence of bacterial infection and the majority of healthy fish sampled were shown to be carrying *P. damselae* subsp *damselae*. We cannot, therefore, attribute the trial mortalities to this bacterium alone and the theory of *Vibrio harveyi* contributing to mortality was investigated further in Block 2 (see below).

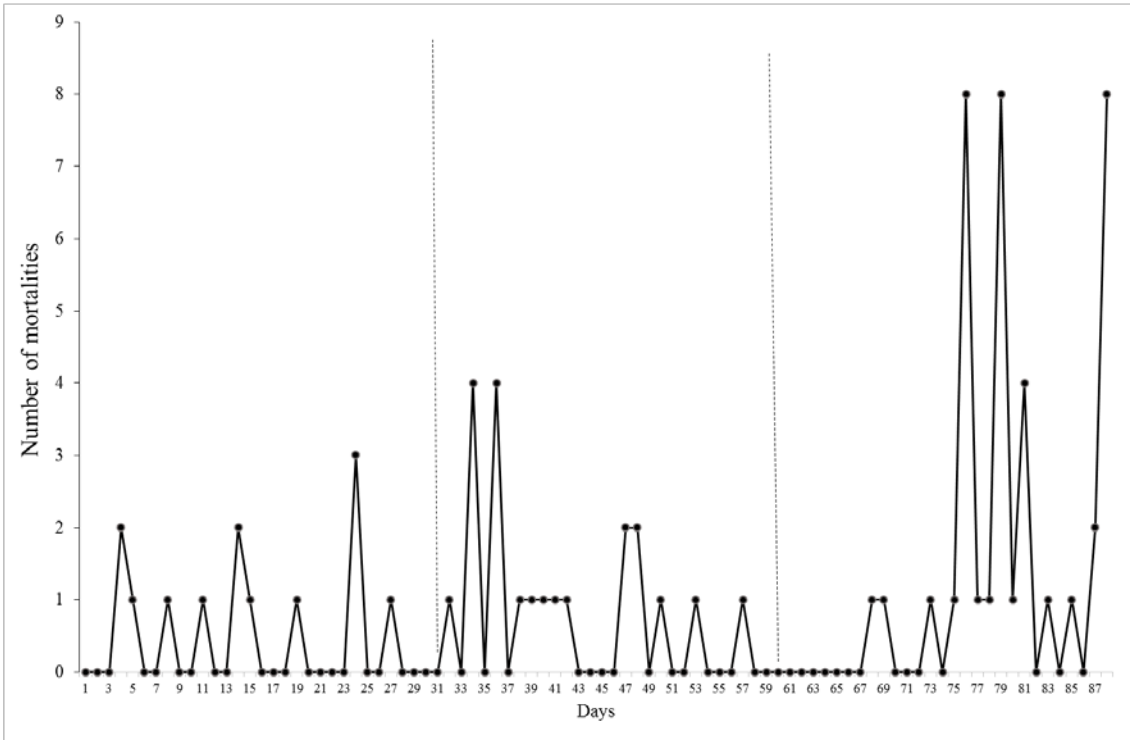

Figure 41: Total number of mortalities each day from all tanks over time during Block 1.

4.4.2. Block 2

Daily mortality data taken from all tanks during Block 2 demonstrates a spike in mortality during the first month of the trial (Figure 42). As we describe in detail below in the Feed Nutritional Composition section of this report, the nutritional composition of the 6 mm Diet B being fed to all tanks during this first month was out of specification for a number of key components and this may have been a contributing factor to the mortality which occurred during this time.

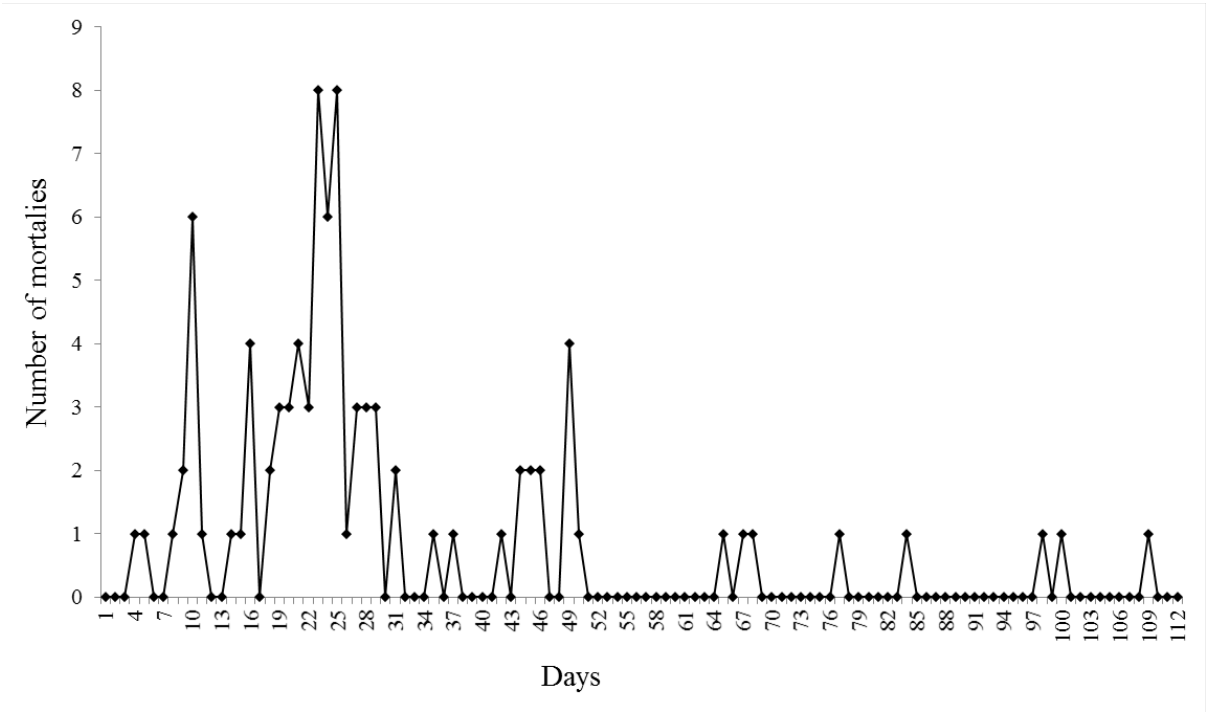

Figure 42: Total number of mortalities each day from all tanks during Block 2.

The generalised linear model showed a significant effect of diet on mortality (P = 0.006), but no effect of strain of fish (P= 0.37). Those fish fed Diet B supplemented with Aquaguard had significantly lower mortality (3.3 ± 1.1%) than all other treatments, which ranged from 9.2 ± 1.1% (Diet B + Actigen) to 13.8 ± 5.6% (Diet B)(Figure 39). These data suggest a benefit to the immune system of fish fed the diet supplemented with Aquaguard. This is consistent with other studies with beta glucans. Onarheim et al. (1992), for example, concluded that Atlantic salmon pre-smolts fed a diet containing beta glucans exhibited reduced mortality when challenged with *Aeromonas salmonicida*, compared to mortality of fish that were not fed beta glucans. We discuss in further detail below the effect of glucans and MOS on survival of challenged fish. We also demonstrate in further detail below, however that the extended feeding period of the beta glucan during this four month period resulted in a compromised immune system at the end of the study. Mortality of fish fed Actigen was also lower (9.2 ± 1.1%) than those fed the control diet (13.8 ± 5.6%), but this difference was not significant. In a study by Zhao (in press), catfish fingerlings fed a diet supplemented with Actigen, lived significantly longer when challenged with *Flavobacterium columnare* bacteria when compared to an unsupplemented diet.

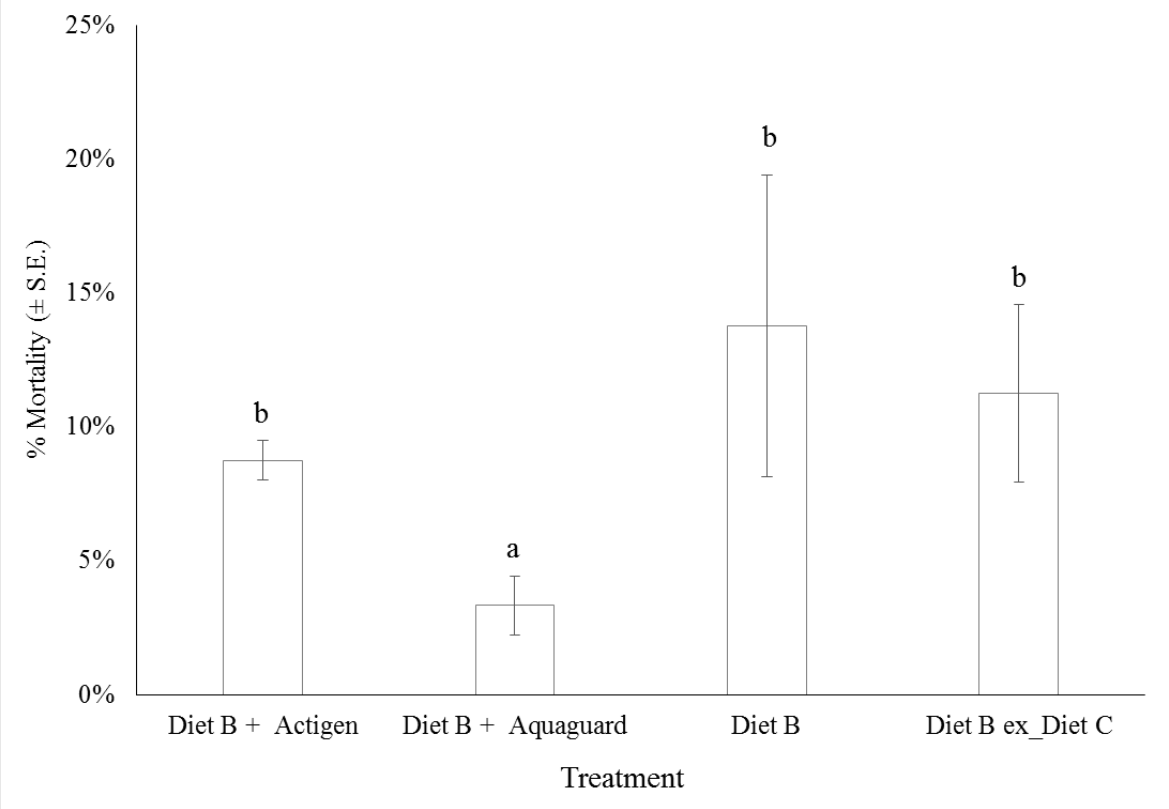

Figure 43: Effect of diet, pooled across strains, on mortality during Block 2.

The Kaplan-Meier analysis of survival time also demonstrated a significant effect of diet (P = 0.005). The mean survival time in the fish fed Diet B supplemented with Aquaguard was 95.8 ± 1.5 days compared with 45.4 ± 0.8 days for those fish from the Diet B_exDiet C treatment (Figure 44).

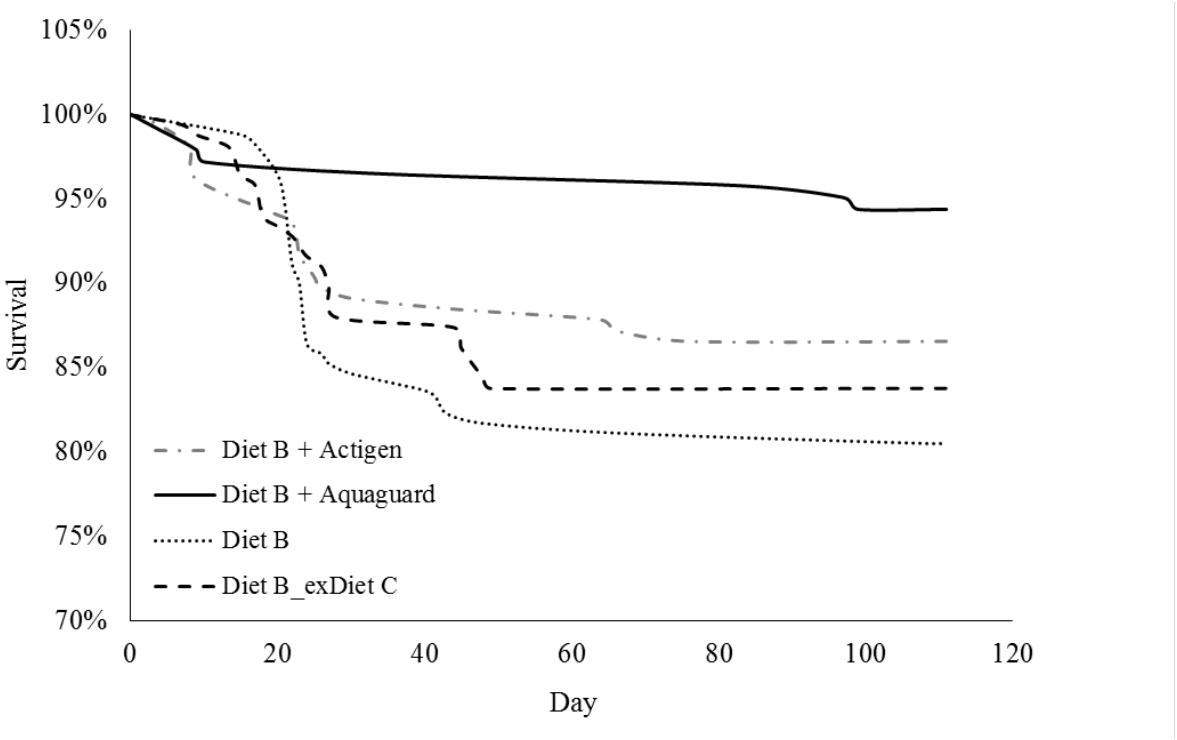

Figure 44: Modelled output of survival time during Block 2 from the Kaplan-Meier analysis

On March 29th, during the period of high mortality shown in Figure 42, samples of both moribund (n=7) and apparently healthy fish (n=2) were taken. Bacterial plating was conducted on blood, faeces and swabs of kidney and the mid-gut of these fish. This plating revealed that many fish (both moribund and healthy) contained *Photobacterium damselae* subspecies *damselae* and/or *Vibrio harveyi*. These were the only bacteria isolated and were detected in both organs sampled and the faeces, but never in blood. Of the organ and faecal samples analysed, 44% contained *P. damselae* and 56% contained *V. harveyi*. Neither of the two healthy fish sampled contained *V. harveyi* (Table 7).

A score ranging from 0 to 4 was assigned to the extent of growth of bacteria on each plate (Table 6).

Table 6: Scoring system applied to quantify bacterial abundance.

Description	Growth	Score
None	no growth	0
Scanty	5-10 colonies	1
Light	20-100 colonies	2
Moderate	colonies out to the third quadrant	3
Heavy	colonies out to the fourth quadrant	4

Healthy fish had an average *P. damselae* score of 2.0 and a *V. harveyi* score of 0.0. Sick fish, on the hand, had a lower *P. damselae* score of 1.1 but a higher *V. harveyi* score of 2.4. Whilst this was only a small data set with a low number of replicates, these data suggest that *V. harveyi* may be the more important bacteria responsible for the mortality than *P. damselae* and this observation requires further investigation. These findings are consistent with those of Gauger et al (2006) who found *V. harveyi* to cause mortality in summer flounder whilst *P. damselae* did not. There were an insufficient number of replicates and samples taken from fish across both diet and health status to determine whether there was an effect of diet on the abundance and score of these bacteria and how these compare between sick and healthy fish, however the available data are summarised in Table 7 .

Table 7: Incidence (%) and abundance (score) of *Photobacterium damselae* subspecies *damselae* (P.d.d) and *Vibrio harveyi* (V.h) from healthy and moribund fish during the mortality event of Block 2 shown in Figure 41.

			%		Score	
	(n)	Fish Status	P.d.d	V.h	P.d.d	V.h
Diet B + Actigen	1	Healthy	100%	0%	4.0	0.0
Diet B + Aquaguard	1	Healthy	0%	0%	0.0	0.0
Diet B	2	Moribund	100%	100%	2.0	3.0
Diet B_exDiet C	5	Moribund	20%	60%	0.8	2.2

Two days following this sampling event, swabs for bacterial analysis were taken of the biofilm on the tank floor from two tanks which had experienced high mortality and two tanks

which had experienced no mortality. Interestingly, *V. harveyi* was not cultured from any of these swabs, however *P. damselae* was isolated from both tanks from which there had been no mortality and from one of the two tanks which had experienced high mortality. *Vibrio pelagius* and *V. rotiferianus* were also isolated from the tanks which had experienced high mortality.

A further two fish were sampled early during this mortality event (10th March). One fish had no bacterial growth in the kidney and a light growth (score 2) of *P. damselae* in the gut. The second fish had scanty growth (score 1) and light growth (score 2) of *P. damselae* in the kidney and gut, respectively and light growth (score 2) of *V. harveyi* in both kidney and gut.

During the cull to reduce stocking density on the 11th May (after the mortality peak had subsided; Figure 42), twelve apparently healthy fish were again sampled for bacteriology of their mid-gut. Kidney was not sampled. Results (Table 8) demonstrate that no *V. harveyi* were detected in any of the samples and 67% of the samples contained *P. damselae*. The average score of *P. damselae* in these fish was 1.0, similar to the moribund fish sampled previously. The lack of *V. harveyi* and presence of *P. damselae* in these healthy fish supports our theory that *V. harveyi* was the greater contributor to the mortality. Whilst there was no *V. harveyi* in any of these healthy fish, 67% of the samples contained *Vibrio scophthalmi* with an average score of 1.5. This vibrio species demonstrates strain-dependant virulence in flounder (Qiao et al., 2013) but has not been reported to be pathogenic in other fish species (Buller, 2004). It is noteworthy that Thompson et al (2004) consider *V. scophthalmi* to be a potential probiotic and therefore it is worth considering that the proliferation of this bacteria in the fish may have been effective at outcompeting *V. harveyi*. In this set of samples there was no effect of diet on *P. damselae* incidence (P = 0.5) or score (P = 0.7) nor on *V. scophthalmi* incidence (P = 0.4) or score (P = 0.3)(Table 8).

Table 8: Incidence (%) and abundance (score) of *Photobacterium damselae* subspecies *damselae* (P.d.d) and *Vibrio scophthalmi* (V.s) from fish following recovery from the mortality event shown in Figure 41.

			%		Score	
	(n)	Fish Status	P.d.d	V.s	P.d.d	V.s
Diet B + Actigen	3	Healthy	33%	67%	0.7	1.0
Diet B + Aquaguard	3	Healthy	100%	100%	1.0	3.0
Diet B	3	Healthy	67%	33%	1.7	1.0
Diet B_exDiet C	3	Healthy	67%	67%	0.7	1.0

On the 23rd May, a single moribund fish with a large skin lesion was submitted for bacteriology. This fish had no *Photobacterium damselae* in the organs but a moderate growth (score 3) of *V. harveyi* and *V. scophthalmi* in the gut and a heavy growth (score 4) of *V. harveyi* on the skin lesion.

4.4.3. Block 3

During Block 3 only one fish died, yielding an overall survival rate of 99.7%

4.4.4. Block 4

Three fish died during Block 4, giving an overall survival of 96%. All three mortalities occurred in Strain 1 fish being fed Diet D, which resulted in an average survival in this treatment of 83 ± 6% and subsequently significant effects of both diet ($P = 0.004$), strain ($P = 0.004$) and the interaction of these terms ($P = 0.004$). Of the three fish which died, one was sampled as a moribund fish for histology and bacteriology. This particular fish had lost weight since the previous weight check. No bacterial growth was obtained from the kidney swab and nothing remarkable was found histologically in the organs sampled (liver, stomach, heart and spleen). Given that there was no effect of the same diets during the longer Block 3 trial and given the high overall survival we suggest that these mortalities were probably of minimal biological significance.

4.5. Feed Nutritional Composition

This section of the report follows the format of that of Stone (2013).

The proximate composition, vitamin and mineral composition of all diets used during the trials are show in Table 9.

Table 9: Proximate composition, vitamins and minerals of all diets tested. Parameters out of specification are highlighted in bold. Refer to Table 2 for manufacturers' specifications.

	Diet A				Diet B							Diet C			Diet D	
Block	1	1	1	3	1	1	1	2	2	3	3 & 4	1	1	1	3&4	Suggested
Diet Size	3 mm	4 mm	6 mm	9 mm	3 mm	4 mm	6 mm	6 mm	9 mm	9 mm	9 mm	3 mm	4 mm	6 mm	9 mm	Requirement[1]
Moisture (%)[a]	5.5	5.1	4.5	8.1	8.4	7.1	7.5	**13.8**	**11.6**	7.3	7.3	4.5	6.0	7.5	5.9	<10
Crude protein (%)[b]	50.6	47.5	50.0	44.4	50.0	50.6	**40.0**	**45.0**	**41.3**	46.3	44.4	50.6	51.9	43.1	43.8	45 - 55
Crude lipid (%)[a]	15.8	18.6	19.2	22.5	17.1	17.8	19.6	**15.6**	22.5	23.8	23.5	18.9	19.0	21.4	24.2	12 - 25
Nitrogen free extract (%)*	16.7	18.0	16.5	23.6	15.0	14.9	24.3	30.8	27.1	19.3	21.8	16.7	14.6	19.1	21.5	<20
Fibre (%)[a]	1.3	1.3	1.3	1.0	1.7	1.1	1.6	1.3	1.0	1.0	0.8	1.3	1.4	1.5	1.0	<5
Ash (%)[a]	11.4	10.8	9.8	9.5	9.5	9.6	8.6	8.6	9.1	10.6	10.3	9.3	8.5	8.9	10.5	<15
Cholesterol (mg/100g)	100	114	187	109	160	78	84	**36**	162	347	228	112	116	174	224	unknown
Vitamins (mg/kg)																
Vitamin C [a/d]	13 / <10	12 / <10	93 / <10	ND	19 / <10	16 / <10	27 / <10	ND	ND	ND	ND	<10 / <10	<10 / <10	<10 / <10	ND	ND
Vitamin E [c]	416	386	375	436	374	364	394	**752**	195	471	488	180	167	172	365	119
Macro-minerals (%) [b]																
Potassium	0.59	0.60	0.63	0.33	0.67	0.82	0.60	0.47	0.61	0.65	0.69	0.63	0.61	0.59	0.45	dispensable
Phosphorus	1.8	1.7	1.6	1.5	1.6	1.5	1.5	1.5	1.6	1.8	1.7	1.6	1.6	1.6	1.8	0.86
Calcium	3.1	2.8	2.7	2.3	2.4	2.4	2.2	2.1	2.1	2.2	2.2	2.7	2.5	2.7	2.9	dispensable
Magnesium	0.21	0.19	0.20	0.16	0.23	0.21	0.20	0.18	0.2	0.21	0.21	0.15	0.13	0.13	0.17	0.07
Sodium	0.51	0.44	0.47	0.63	0.72	0.67	0.59	0.46	0.57	0.86	0.82	0.47	0.42	0.40	0.36	dispensable
Sulphur	0.81	0.77	0.76	0.74	0.94	0.91	0.83	0.83	0.78	0.78	0.80	0.63	0.60	0.53	0.55	dispensable
Micro-minerals (mg/kg) [b]																
Boron	6.0	5.0	5.0	4.1	5.0	4.0	5.0	2.8	4.1	4.3	4.2	5.0	5.0	4.0	3.9	dispensable
Copper	13.0	12.0	12.0	12.0	11.0	9.2	11.0	14.0	9.1	8.9	8.5	10.0	10.0	9.7	12.0	10
Iron	370	600	340	320	570	370	500	450	380	390	140	370	320	320	450	199
Manganese	60	57	49	44	46	120	40	82	43	71	63	29	33	35	49	13
Molybdenum	0.8	0.8	0.8	<0.4	0.7	<0.4	0.6	0.5	0.5	<0.4	<0.4	0.7	0.7	0.5	1.0	dispensable
Zinc	350	370	310	340	170	150	150	320	150	150	150	170	160	150	150	80
Selenium	3.8	3.5	3.4	3.4	2.1	2.3	2.0	3.0	2.0	2.1	2.3	1.6	1.3	0.9	1.5	0.38

[a] Agrifood Technology (AGT)
[b] Murdoch University Marine and Freshwater Research Laboratory (MFRL)
[c] Department of Agriculture & Food, WA (DAFWA)
[d] National Measurements Institute (NMI)
* NFE = Nitrogen free extract = 100% - (protein % + lipid % + ash %)
[1] From Stone (2013)

The moisture content of all diets, with the exception of the 6 mm (13.8%) and 9 mm (11.6%) diets of Diet B used in Block 2 were within specification. Whilst the level of moisture measured in these two diets was out of specification, some doubt was raised over the accuracy of these values, as food with these levels of moisture would expected to be mouldy.

The crude protein content of all sizes of Diet A, C and D were within specification. The 6 mm Diet B used in Block 1 and 2 contained only 40% and 45% crude protein, respectively, compared with the 48% prescribed by the manufacturer. The 9 mm Diet B used in Block 2 also contained less protein (41%) than the specification (44%). Despite the large drop in protein between the 4 and 6 mm pellets of Diet B, growth and FCR was maintained in fish fed this diet in the third month of Block 1 when fish were fed this lower protein diet (see Figure 4, Figure 5 and Figure 28).

The crude lipid levels of all sizes of Diets A were close to those specified by the manufacturer. The 6 mm Diet B used in Block 2 had less lipid (15.6%) than specified by the manufacturer (20%). All other pellets sizes of this diet were close to specification for lipid. The questionable moisture content of these diets may have impacted on the lipid analysis, as water displaces lipid. Testing of the same batch of diets by the manufacturer suggested that they contained the correct amount of lipid. The 3 & 4 mm pellets of Diet C contained more lipid than specified by the manufacturer, whilst the 9 mm Diet D was within specification for lipid. As we describe below in the histology section, fish fed Diet C has fattier livers than those fed on Diet A and B in this block.

Cholesterol values of all diets were quite variable, and particularly low in the 4 mm and 6 mm pellets of Diet B. As is discussed in more detail below, fish fed the 6 mm diet in the first month of Block 2, which had a lower cholesterol in the diet (36 $mg.100g^{-1}$) had lower plasma cholesterol (5.5 mM) than those in month 3 of Block 1 (6.5 mM), which were fed the 6 mm diet with higher dietary cholesterol (84 $mg.100g^{-1}$). It is our understanding that diets typically are not fortified with cholesterol. Cholesterol issues are discussed further below in relation to dietary taurine. Based on the similar fatty acid composition between pellets sizes of the same diet described below, we subsequently surmised that the same lipid source was probably used between pellet sizes within diet. We would therefore expect that the cholesterol levels between pellet sizes within diets would be also similar, however this was not always the case

and therefore perhaps the differences are primarily due to differences in protein source rather than lipid source. We were aware that Diet B contained poultry oil and given the high cholesterol content of Diet C, this diet is also likely to contain poultry or other land animal oils. The relatively high level of cholesterol in Diet C was ineffective at increasing plasma cholesterol in fish fed this diet in Block 1 (see blood chemistry section below), which is likely due to the deficiency in taurine in these diets (see below) and probably the inclusion of soy products in these diets.

In addition to being out of specification in terms of protein and perhaps lipid and moisture, the 6 mm Diet B used in the first month of Block 2 also contained the lowest level of cholesterol and a much higher level of vitamin E than the specification of 500 $mg.kg^{-1}$. We also detail below that this diet had an inferior fatty acid profile compared to most other diets and other batches of the same product. As this was the only diet offered during Block 2, we cannot compare the performance of the fish on it, relative to a diet within specification. However, it is possible that this diet may have been attributable to the poor health and high mortality we observed during the first month of this trial (Figure 42). This mortality event subsided after the diet was changed to 9 mm, which was within specification and had a superior fatty acid profile.

Two laboratories measured the vitamin C content of Block 1 diets. It was below the National Measurements Institute's (NMI) detectable limit of 10 $mg.kg^{-1}$ in all diets, whilst Agrifood Technology measured levels above this same detection limit in all sizes of Diets A and B but not in Diet C. Vitamin C content of diets ranged from <10 to 93 $mg.kg^{-1}$, which is well below the levels of 500 $mg.kg^{-1}$ declared from the manufacturers of Diets A and B. The lack of measured vitamin C is likely due to the fact that aquaculture diets typically utilise heat stable ascorbyl-monophosphate which is probably not detected in the assays used by these two laboratories. These results are therefore likely to be meaningless and no further vitamin C analysis was conducted on other diets. We are currently investigating whether there are other laboratories in Australia who can measure vitamin C in this form.

The measured values of vitamin E in all sizes of Diet A were close to the target value of 400 $mg.kg^{-1}$. The values of vitamin E in Diet B were generally similar to Diet A, but lower than the value of 500 $mg.kg^{-1}$ specified by the manufacturer, with the exception of the 6 mm batch of Diet B used in Block 2, which was much higher than the target value at 752 $mg.kg^{-1}$.

This is the same batch of diet that was out of specification in a number of other areas described above. The level of 365 mg.kg^{-1} in Diet D was similar to Diet A and B. Diet C had the lowest level of vitamin E (167 – 180 mg.kg^{-1}), however all diets were adequate based on the recommended requirement of 119 mg.kg^{-1} for Japanese yellowtail according to Shimeno (1991). Furthermore, Le et al. (2013) demonstrated that whilst muscle myopathy occurred in juvenile YTK fed diets containing only 40 mg.kg^{-1} of vitamin E and with 3.3 mg.kg^{-1} of selenium, this myopathy was avoided by either increasing selenium to 4.3 mg.kg^{-1} or increasing vitamin E to 180 mg.kg^{-1}. Le et al. (2014b) also demonstrated that increasing vitamin E from 40 to 180 mg.kg^{-1} increased lysozyme activity in skin mucous. Ortuño et al. (2000) demonstrated increased immune function (measured as increased haemolytic complement activity and phagocytosis) in gilthead seabream when dietary vitamin E was increased to 1200 mg.kg^{-1}. On the basis that high levels of vitamin E can improve immune function, the manufacturers of Diets A and B fortified their diets to a higher level than the minimum level at our request.

The levels of macro-minerals were generally similar between pellet sizes of diets from the same manufacturer, with the exception of the 9 mm diet of Diet A which contained lower levels of potassium, phosphorus, calcium and magnesium than the smaller sizes. This is consistent with its lower ash content. Generally speaking, Diet D contained higher levels of phosphorus and calcium than the other diets, which is again consistent with its relatively high ash content. In terms of the micro-minerals, the most notable differences were the considerably higher levels of zinc in all sizes of Diet A, the lower level of manganese in all sizes of Diet C and the very low level of iron in the 9 mm pellets of Diet B. It is noteworthy that the haemoglobin levels of fish in Block 3 decreased significantly over time and this is likely due to this decrease in iron in the diet fed during this block and this is discussed in further detail in the blood chemistry section below.

Diet A contained the highest level of selenium (average 3.5 mg.kg^{-1}), followed by Diet B (2.3 mg.kg^{-1}). Diet C and D had similar levels of selenium (1.3 and 1.5 mg.kg^{-1}, respectively).

Whilst the impact of most of these differences is largely unknown, each is above the minimum requirement suggested by the NRC (2011) and outlined in Table 9, with the exception of the very low level of iron in the 9 mm pellet of Diet B (140 mg.kg^{-1}), which is below the recommended level of 199 mg.kg^{-1} and as noted above, the haemoglobin levels in

the fish decreased whilst they were feeding on this diet. It should be noted, however, that none of these minimum values are based on *Seriola* sp., but others including salmon, trout and red drum. The requirement for iron of 199 mg.kg^{-1} is based on red sea bream (Sakamoto, 1976). In addition to its role in transporting oxygen via haemoglobin, iron is also an important mineral for maintaining immune function. Whilst exact requirements for *Seriola* sp. have not been determined for most of these nutrients, the importance of dietary selenium to YTK has been demonstrated by Le and Fotedar (2014a) and it is much higher than the value reported by the NRC (2011) of 0.38 mg.kg^{-1}, based on rainbow trout. These authors demonstrated that the optimum dietary selenium level for maximum growth of juvenile (~20 g) YTK was 5.56 mg.kg^{-1}. On the basis of these findings, the manufacturer of Diet A added an additional 4.5 mg.kg^{-1} of organic selenium to all of their diets to supplement the predicted basal level of 1 mg.kg^{-1}. Whilst this addition resulted in a higher level of selenium in this diet (3.5 mg.kg^{-1}) than all other diets (Diet B = 2.3, Diet C = 1.3 and Diet D = 1.5 mg.kg^{-1}), the target value of 5.5 mg.kg^{-1} was not achieved. Had it been achieved, growth may have been improved in Diet A relative to Diet B in Block 1, as Le and Fotedar (2014a) demonstrated a significant improvement in growth of YTK fed diets containing 5.56 mg.kg^{-1} versus those fed 3.35 mg.kg^{-1} (the lowest tested). It is also noteworthy that an increase in dietary selenium increases immunity against bacterial infection. Le and Fotedar (2014b) demonstrated a significant improvement in the survival of those YTK fed diets that had been supplemented to contain either 5.39 or 7.37 mg.kg^{-1} of selenium compared with the control diet which contained 3.35 mg.kg^{-1} following a bacterial challenge with *Vibrio anguillarum*.

Most essential amino acids were present well in excess of their requirement (Table 10), with the exception that most diets were deficient in methionine compared to the required value of 1.11 g.100g^{-1} suggested by Ruchimat et al (1997) for Japanese yellowtail. However, methionine was not limiting on basis of the requirement calculated by Stone (2013) of 0.68 g.100g^{-1} using the ideal protein concept from NRC (2011). However, we outline in further detail below that a potential cause of the cataracts seen in fish maintained on the 6 mm pellet of Diet C may be the lower methionine content of this diet (0.75 g.100g^{-1}) compared with the same sized pellets of Diet A and B (1.05 and 1.14 g.100g^{-1}, respectively). It has recently been reported that the requirement for lysine by *Seriola dumerili* is 2.1 g.100g^{-1}. This level is much higher than the requirement shown in Table 10 (0.68 to 1.11 g.100g^{-1}). However, all diets used in this study contained more than 2.1 g.100g^{-1} of lysine.

The ratio of essential to non-essential amino acids was typically higher in Diet B than in the other diets. Stone (2013) points out that whilst the ideal ratio is 1.0, adequate growth should still occur at ratios down to 0.66 and therefore all diets should be adequate from this regard.

The equal growth of fish fed Diet A and Diet B during Block 1 suggests that the lower level of methionine and lower ratio of essential to non-essential amino acids in the former diet was not limiting the growth of fish fed this diet. Whilst the protein and amino acid contents of all diets appear suitable for YTK, it is difficult to draw firm conclusions on the suitability of the protein sources as their digestibilities are unknown.

The taurine content of Diet C (0.15 to 0.17 $g.100g^{-1}$) was considerably lower than Diet A (0.84 to 0.94 $g.100g^{-1}$) and Diet B (0.86 to 1.13 $g.100g^{-1}$). The 6 mm Diet B that was out of specification in the many parameters described above also had the lowest taurine content of all batches of Diet B. The low taurine content of Diet C may suggest a high degree of fishmeal replacement in this diet, as diets made only with fishmeal should contain approximately 0.2 to 0.3 $g.100g^{-1}$ taurine. The low taurine content of Diet D (0.17 $g.100g^{-1}$) was lower than expected given that this diet was reported to contain only marine sources of protein and lipid. Given that fish fed Diet D grew very well and had no health issues and that their plasma cholesterol was high supports the manufacturers claim that this diet contained no soy products and subsequently that this level of taurine is adequate for this sized fish in a diet without soy (see below for further details).

As taurine is not involved in protein synthesis, it is often considered to be a non-essential amino acid, however based on its many physiological roles and broad distribution throughout the body, taurine is certainly a critical nutrient (Ripps and Shen, 2012) and should certainly be considered essential for those species which lack the enzyme cysteinesulfinic acid decarboxylase (CSD) which produces taurine from its precursors, L-cysteine and methionine. Whilst the CSD activity of YTK has not been studied, Japanese yellowtail has been demonstrated to be completely lacking in this enzyme (Yokoyama et al., 2001) and it is probable that YTK are the same. Included in its many physiological roles, taurine acts as a broad-spectrum cytoprotective agent and antioxidant (Ripps and Shen, 2012) and has been found to improve gut development (Li et al., 2009). It is considered a growth promoter in many fish species due to its role in enhancing the absorption of lipids and lipid-soluble vitamins. The exact requirement for taurine by YTK has not been well defined and is

complicated by the fact that soy has been demonstrated to strip the body of taurine and therefore the requirement for taurine increases as the inclusion level of soy increases. Takagi et al (2008), for example demonstrated that Japanese yellowtail fed a non-fishmeal diet with a high inclusion level of soy protein concentrate require a very high level of taurine in the diet (4.5 $g.100g^{-1}$). Assuming a minimal quantity of soy in Diets A and B, their level of taurine should be adequate. The manufacturers of these diets added an additional 1.0 and 0.4 $g.kg^{-1}$ of synthetic taurine, respectively, however the results are not consistent with these claims with Diet B having a higher taurine level than Diet A and neither diet having as much taurine as would be expected based on these supplementation regimes and assuming a high and equal fish meal content in both diets. Diet C and Diet D contained similar and much lower levels of taurine. Fish fed Diet D performed very well, yet fish fed Diet C performed poorly and these fish displayed other symptoms of taurine deficiency, such as hypochlorestemia. These data would suggest that Diet C contains soy whilst Diet D does not. Furthermore, it suggests that the low taurine content of Diet D is sufficient for YTK of >2.5 kg when offered a diet without soy.

It has been reported that taurine deficient Japanese yellowtail are more susceptible to bacterial infection (Maita et al., 2006). However, the level of mortality in fish fed Diet C during Block 1 trial was equal to those fed Diets A and B, despite their diet being deficient in taurine. This may suggest that mortality in the current trials may not have been due primarily to the presence of *Photobacterium damselae* subsp *damselae* in most fish sampled.

Table 10: Essential and non-essential amino acids measured in all diets tested.

	Diet A				Diet B							Diet C			Diet D	Suggested requirement[c] analysed	Suggested requirement[c] Calculated[3]
Block	1	1	1	3	1	1	1	2	2	3	3 & 4	1	1	1	3&4		
Diet Size	3 mm	4 mm	6 mm	9 mm	3 mm	4 mm	6 mm	6 mm	9 mm	9 mm	9 mm	3 mm	4 mm	6 mm	9 mm		
Essential amino acids (EAA g/100 g)																	
Arginine	2.85	2.63	2.70	2.38	2.72	3.12	2.70	2.42	2.69	2.45	2.36	3.32	3.45	3.09	2.70	1.63	1.46
Histidine	1.09	0.90	1.02	0.97	1.47	1.23	1.31	1.22	0.77	0.95	1.07	1.13	1.22	1.01	0.86	0.85	0.62
Isoleucine	1.94	1.78	1.80	1.38	1.51	1.57	1.48	1.27	1.50	1.38	1.25	1.80	1.86	1.55	1.40	1.40	0.96
Leucine	3.68	3.54	3.54	2.89	3.84	3.77	3.43	3.23	2.85	2.85	2.85	3.62	3.75	3.11	2.98	1.40	1.25
Lysine	2.74	2.49	2.98	2.15	3.06	3.07	2.76	2.39	2.23	2.30	2.53	2.43	2.56	2.21	2.31	1.78	178
Methionine	1.08	1.00	1.05	1.07	1.32	1.38	1.14	1.07	1.03	1.05	0.97	0.83	0.88	0.75	1.09	1.11	0.68
Phenylalanine	1.95	1.89	1.88	1.60	2.11	2.03	1.89	1.75	1.53	1.57	1.57	2.02	2.07	1.71	1.59	0.70	0.98
Threonine	1.94	1.78	1.81	1.55	2.00	1.99	1.84	1.63	1.55	1.52	1.48	2.02	2.12	1.80	1.58	1.30	1.00
Valine	1.98	1.81	1.83	1.77	2.34	2.26	2.12	1.98	1.70	1.74	1.74	2.31	2.37	1.95	1.76	1.60	1.09
ΣEAA (g/100 g)[1]	***19.24***	***17.82***	***18.60***	***15.76***	***20.37***	***20.42***	***18.67***	***16.96***	***15.84***	***15.81***	***15.82***	***19.47***	***20.28***	***17.19***	***16.28***		-
Non-essential aa (NEAA g/100 g)																	
Alanine	2.72	2.54	2.56	2.36	2.87	2.87	2.62	2.48	2.30	2.31	2.28	2.72	2.83	2.46	2.44		-
Aspartic acid	3.89	3.50	3.63	3.29	4.11	4.24	3.72	3.47	3.31	3.31	3.24	3.92	4.14	3.47	3.47		-
Cysteine-cystine	0.62	0.67	0.61	0.53	0.56	0.59	0.61	0.53	0.59	0.51	0.49	1.01	0.98	0.83	0.54		-
Glutamic acid	7.32	7.51	7.32	5.30	5.70	6.24	5.48	5.05	6.06	5.52	5.13	6.40	6.54	5.79	5.72		-
Glycine	2.97	2.69	2.77	2.43	2.68	2.93	2.66	2.44	2.60	2.47	2.41	3.38	3.52	3.25	2.66		-
Proline	3.59	3.84	3.42	2.22	2.52	2.97	2.55	2.40	2.81	2.43	2.39	3.72	3.64	3.32	2.43		-
Serine	2.17	2.08	2.10	1.72	2.14	2.27	2.04	1.85	1.89	1.73	1.70	2.80	2.87	2.41	1.89		-
Tyrosine	1.55	1.49	1.49	1.22	1.42	1.48	1.35	1.21	1.27	1.21	1.12	1.59	1.67	1.41	1.25		-
ΣNEAA (g/100 g)	***24.83***	***24.31***	***23.89***	***19.07***	***22.01***	***23.60***	***21.03***	***19.43***	***20.83***	***19.49***	***18.75***	***25.52***	***26.21***	***22.94***	***20.42***		-
ΣAA(g/100 g)[1]	*44.07*	*42.13*	*42.49*	34.83	*42.38*	*44.02*	*39.70*	36.39	36.67	35.30	34.56	*44.99*	*46.48*	*40.12*	36.70		-
Σ EAA : Σ NEAA	*0.77*	*0.73*	*0.78*	0.83	*0.93*	*0.87*	*0.89*	0.87	0.76	0.81	0.84	*0.76*	*0.77*	*0.75*	0.80		*1:01*
Taurine (g/100 g)	0.97	0.87	0.93	0.84	1.13	1.07	1.02	0.86	0.96	0.98	0.91	0.17	0.17	0.15	0.17		-

1. Excluding tryptophan.

2. Refer to Appendix B for dietary requirement references.

3. The calculated values are based on the "ideal protein concept" from NRC (2011).

The individual fatty acids analysed in the diets and presented in Table 11 were selected for presentation here as they are important to marine fish and/or they are useful in estimating the degree of fish oil substitution in these diets (Stone, 2013).

These data demonstrate considerable differences in many fatty acids both between diets from different manufacturers and (in some cases) within and between diet sizes from the same manufacturer. Diet A and Diet C were both very consistent in their fatty acid profile between pellets sizes. However, the 6 mm batch of Diet B fed in Block 2 was considerably different from the other pellet sizes of this diet and also from the other batch of 6 mm Diet B used in Block 1. In particular, this batch of feed contained lower levels of all key omega 3s than the latter 6 mm diets and all other diet sizes of Diet B. As described above, this diet was also out of specification in a number of other criteria and these fish suffered high mortality in the period they were fed this diet. The manufacturer's fatty acid analysis was consistent with ours.

Diets A and D contained much higher percentages of docosahexanoic acid, total PUFA and total omega-3 than Diet B and Diet C. Similarly, Diet B generally contained much higher levels of these parameters than Diet C, with the exception of the 'out of specification' 6 mm diet described above, which was more similar to Diet C in terms of these parameters. In addition, the total PUFA content of most Diet B diets was more similar to Diet C than to Diet A and Diet D.

In addition to having considerably higher DHA than Diets B and C, Diet A also had a significantly lower EPA content than most other diets. The combination of these two factors in Diet A (i.e. low EPA and high DHA) led to a higher DHA to EPA ratio (3.6 to 4.2) in this diet than Diet B (1.1 to 1.9), Diet C (0.8 to 1.0) and Diet D (1.2).

Table 11: Key fatty acids and their relative abundances and ratios in all diets tested. Values in red differ considerably to other similar products from the same manufacturer

		Diet A				Diet B							Diet C			Diet D	
Block		1	1	1	3	1	1	1	2	2	3	3 & 4	1	1	1	3&4	Fish Oil
Diet Size		3 mm	4 mm	6 mm	9 mm	3 mm	4 mm	6 mm	6 mm	9 mm	9 mm	9 mm	3 mm	4 mm	6 mm	9 mm	
Myristic	C14:0	3%	3%	3%	3%	3%	2%	3%	2%	3%	3%	4%	4%	4%	4%	5%	6-9%
Palmitic	C16:0	19%	18%	19%	20%	18%	16%	18%	20%	20%	21%	21%	18%	18%	19%	16%	10-20%
Palmitoleic	C16:1n7	3%	3%	3%	3%	5%	4%	5%	5%	5%	5%	6%	5%	6%	6%	5%	6-12%
Oleic	C18:1	19%	19%	20%	23%	24%	29%	26%	31%	27%	27%	25%	28%	26%	27%	13%	9-16%
Gondoic	C20:1	1%	2%	2%	2%	1%	5%	1%	3%	1%	1%	2%	1%	0%	0%	3%	1-17%
Linoleic	C18:2n6	7%	7%	7%	7%	9%	10%	10%	11%	9%	9%	8%	11%	10%	10%	4%	0.7 - 2.2%
Arachidonic	C20:4n6	2%	2%	2%	2%	1%	1%	1%	1%	1%	1%	1%	1%	2%	2%	1%	
Eicosapentanoic	C20:5n3	6%	6%	6%	6%	10%	7%	9%	**5%**	8%	8%	9%	8%	9%	9%	17%	5-10%
Docosahexenoic	C22:6n3	24%	24%	23%	20%	15%	12%	12%	**9%**	11%	10%	10%	8%	9%	7%	21%	6-13%
DHA:EPA		4.1	4.2	3.8	3.6	1.5	1.9	1.4	1.8	1.4	1.2	1.1	1.0	0.9	0.8	1.2	
Total PUFA		46%	47%	45%	41%	41%	36%	39%	31%	34%	32%	33%	35%	36%	34%	51%	
LC n3 PUFA		30%	30%	29%	29%	25%	19%	21%	**12%**	20%	21%	21%	16%	18%	16%	41%	
Total omega-3		31%	32%	31%	31%	28%	22%	24%	**18%**	23%	22%	22%	20%	21%	19%	45%	
Total omega-6		15%	14%	15%	11%	14%	14%	15%	13%	11%	11%	11%	15%	0.1	14%	6%	
Omega-3:Omega-6		2.1	2.3	2.1	2.9	2.0	1.6	1.6	1.5	2.0	2.0	2.1	1.3	1.4	1.3	7.0	

Comparison of the fatty acids in the various diets from typical fish oil values suggests that fish oil substitution did not occur in Diet D, but did occur in Diets A, B and C and was probably highest in Diets B and C. This is despite the manufacturer of Diet A purporting to have used only marine fish oil in these diets. It was the high level of linoleic acid in all sizes of Diet A (7%) that suggest terrestrial oils were present in these diets as the vast majority of fish oils contain less than 2.2% linoleic acid. One exception is oil from farmed fish, for example the oil from the trimmings of farmed salmon has been demonstrated to contain up to 34% linoleic acid and therefore this could be an inclusion in these diets rather than terrestrial oils. The very high level of DHA in Diet A and Diet D suggests that an oil high in this fatty acid was utilised. The most likely candidate for this is tuna oil, which can contain up to 29% DHA. There is little published data available on the specific fatty acid requirements of YTK. Deshimaru et al. (1982) however, fed juvenile Japanese yellowtail (initial weight ~40 grams) on diets containing different marine fish oils at a 9% inclusion level for 4 weeks. Those fed on oils containing >20% of fatty acids as long-chain (>20 carbons) omega-3 HUFA (i.e. squid oil, sardine oil and skipjack oil) achieved more rapid growth and greater feed efficiency than those fed on pollack liver oil and herring oil, which both contained ca. 12% long-chain omega-3 HUFA. Growth and conversion efficiency were best in the diet containing skipjack oil, which had 16.7% DHA and a DHA:EPA ratio of 3.3. Whilst the level of inclusion of lipid in the diets used in the current studies were much higher than that used by Deshimaru et al (1982), Diet D and Diet A had the most similar EFA profile in terms of %DHA and DHA:EPA ratio and it is possible that the significantly improved FCR achieved on Diet A, relative to Diet B in Block 1 could have been attributable to this favourable fatty acid profile. Deshimaru et al. (1982) reported that the skipjack oil used in their study contained 1.9% linoleic acid. It would therefore appear that if Diet A does contain tuna or skipjack oil that it must also contain another oil high in this terrestrial fatty acid or salmon trimming oil.

Whilst Diet A performed equally to Diet B during Block 1 in terms of growth and had a significantly better FCR, the 9 mm version of this diet performed significantly worse than Diet B and Diet D in Block 3, despite having a similar fatty acid profile to the smaller diets of Diet A and B tested in Block 1. The 9 mm diets of Diet A and D both still had a high percentage of DHA (>20%), but Diet D also had a high level of EPA, which yielded a lower DHA:EPA ratio of 1.2, which was similar to that of Diet B (but which was derived from lower levels of EPA and DHA than Diet D). This may suggest that the larger fish in Block 3

benefit from the lower DHA:EPA seen in Diets B and D. Alternatively the relatively poor performance of the fish diet Diet A in Block 3 may have been result of a different dietary protein source or other dietary ingredient that was different to that used in the smaller pellet sizes of this diet.

4.6. Fillet Biochemical Composition

As noted above, samples of fillets from Block 1 fish were taken after the completion of this trial from fish that were maintained on their treatment diets. All parameters, with the exception of cholesterol, were measured in fillets taken from fish at the end of the live bacterial challenge trials in April, whilst cholesterol was measured in fillets from fish sampled in July following the challenge with dead *Streptococcus iniae*. Fillet data from fish fed Block 1 diets are shown in Table 12.

Table 12: Nutritional profile of fillets from fish fed Block 1 diets.

	P	Diet A	Diet B	Diet C
Moisture (%)[1]	0.08	70.8 ± 0.2	71.3 ± 0.5	72.3 ± 0.5
Crude lipid (%)[2]	0.16	12.5 ± 0.9	10.4 ± 2.5	7.2 ± 1.5
Cholesterol (mg/100g)[2]	0.77	90 ± 7	109 ± 27	104 ± 18
Vitamin E (mg/kg)[3]	<0.0001	31.6 ± 1.7[a]	32.6 ± 1.8[a]	13.9 ± 1.8[b]
Sum AA (g/100g)	0.3	71.9 ± 1.0	74.0 ± 2.1	75.4 ± 1.2
Taurine (g/100g)	<0.00001	0.55 ± 0.03[a]	0.46 ± 0.02[a]	0.17 ± 0.01[b]
Histidine (g/100g)	0.00001	3.69 ± 0.04[a]	4.48 ± 0.07[b]	4.32 ± 0.07[b]
Cysteine (g/100g)	0.03	0.93 ± 0.01[a]	0.95 ± 0.02[a,b]	1.01 ± 0.01[b]
Selenium (mg/kg)[a]	<0.0001	1.73 ± 0.03[a]	2.35 ± 0.09[b]	0.79 ± 0.02[c]
DHA	<0.00001	29 ± 1%[a]	17 ± 1%[b]	16 ± 1%[b]
EPA	0.14	6 ± 0%	9 ± 0%	10 ± 0%
DHA:EPA	<0.00001	4.70 ± 0.04[a]	1.94 ± 0.04[b]	1.52 ± 0.05[c]
LC n3 PUFA	0.0005	38 ± 1%[a]	29 ± 1%[b]	29 ± 1%[b]
Total omega-3	0.001	40 ± 0%a	31 ± 1%b	32 ± 1%b
Total omega-6	0.23	10 ± 0%	12 ± 0%	13 ± 0%
Omega-3:Omega-6	<0.00001	3.82 ± 0.12[a]	2.66 ± 0.09[b]	2.51 ± 0.06[b]

1 Murdoch University Marine and Freshwater Research Laboratory (MFRL)
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3 Department of Agriculture & Food (DAF)

Despite a wide range in fillet lipid content from 7.2% (Diet C) to 12.5% (Diet A), these differences were not statistically significant. Despite the lack of statistical difference between diets, the relationship between dietary lipid and fillet lipid content was strongly negatively correlated ($R^2 = 0.93$)(Figure 45). That is, the fish fed the diet with the highest lipid had the lowest fillet lipid content. We believe the low level of fillet lipid, despite high dietary lipid content of Diet C may be due to impaired lipid metabolism as a result of taurine deficiency.

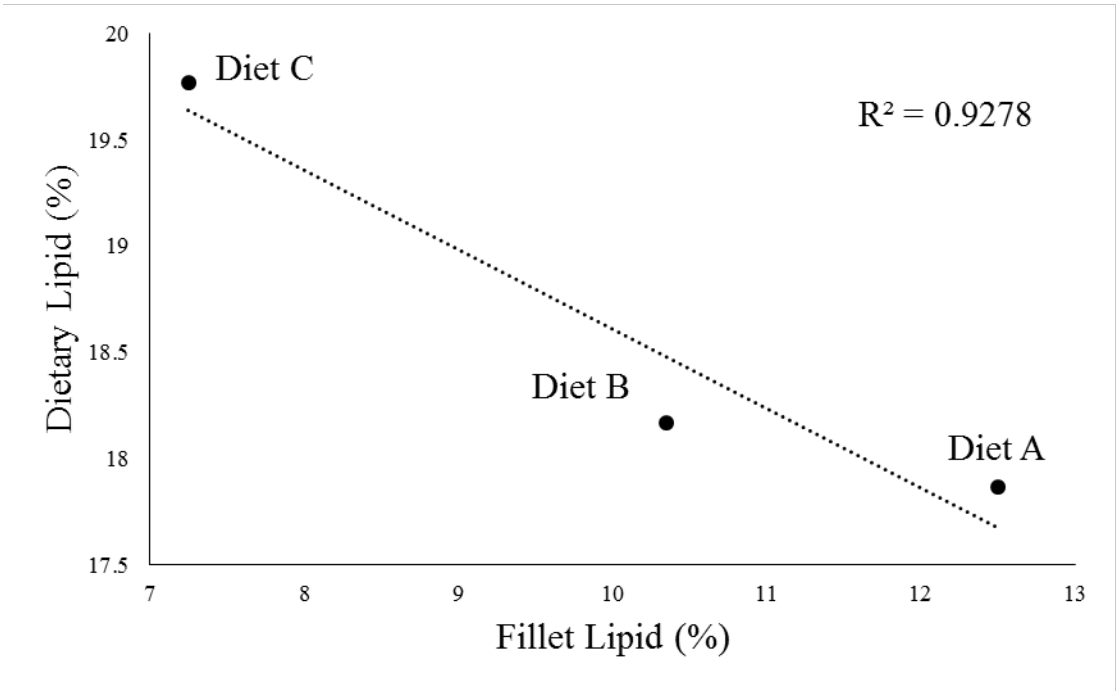

Figure 45: Relationship between dietary lipid and fillet lipid in fish from Block 1.

There were no differences in fillet cholesterol content in July and this is likely due to the recovery of the fish fed Diet C from the plasma hypocholesteremia they exhibited in the early stages of Block 1 (see blood biochemistry section of the report below). The vitamin E content of the fillets was significantly affected by diet and was strongly correlated with the vitamin E content of the diet ($R^2 = 0.99$)(Figure 46). Fillet selenium content was also significantly affected by diet, however the correlation was not as strong as expected ($R^2 = 0.22$) and it was not fish fed Diet A, which had the highest dietary selenium content, which had the highest selenium content in their fillets (Figure 47). This was unexpected and is unlikely to be due to

differences in the bioavailability of selenium, as the selenium added to Diet A was organic selenium, which is known to have a very high bioavailability. The implications for these differences in the antioxidants vitamin E and selenium on fillet quality and shelf life are discussed below.

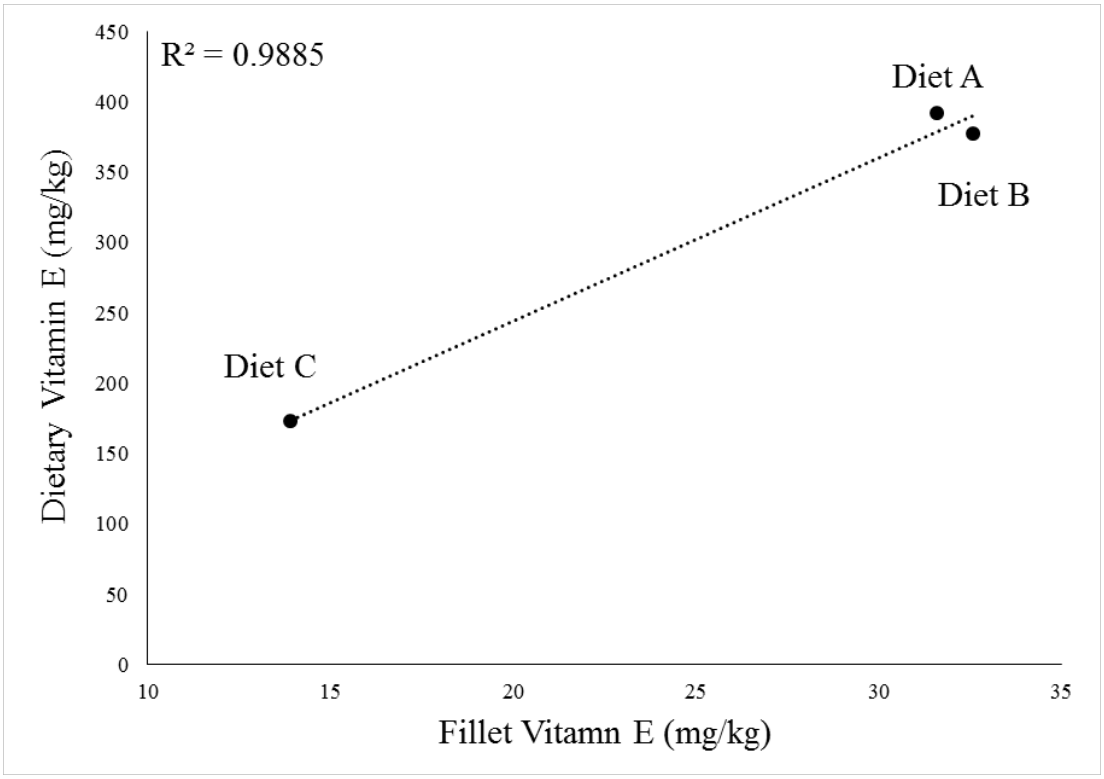

Figure 46: Relationship between dietary vitamin E and fillet vitamin E in fish from Block 1.

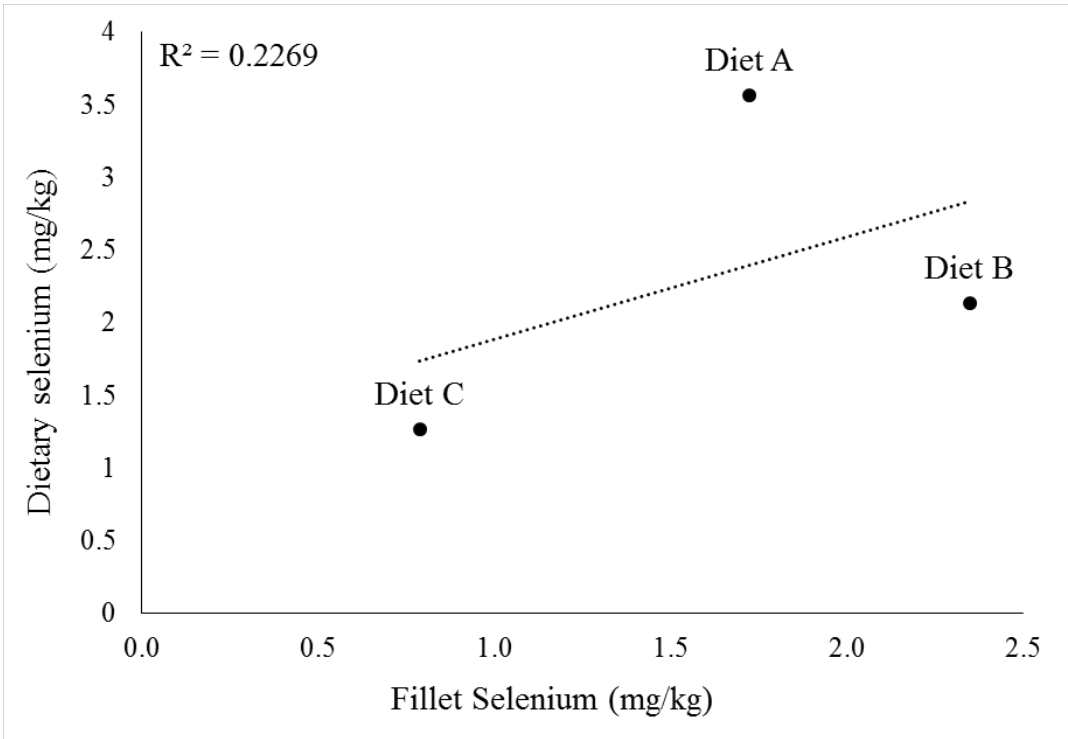

Figure 47: Relationship between dietary selenium and fillet selenium in fish from Block 1.

The sum of amino acids in the fillets was not affected by diet, but three individual amino acids were statistically different between diets; histidine, cysteine and taurine. There was a positive correlation the content in fillet and diet for taurine (R^2= 0.86) (Figure 48), histidine (R^2 = 076)(Figure 49) and cysteine (R^2 = 0.87)(Figure 50). There is probably little importance in the difference in cysteine between treatments, as this is non-essential amino acid. We discuss the differences in histidine in relation to cataracts in the histology section below and the differences in taurine are expected, given the assumption that YTK cannot synthesise taurine from its precursors and must therefore obtain it from the diet.

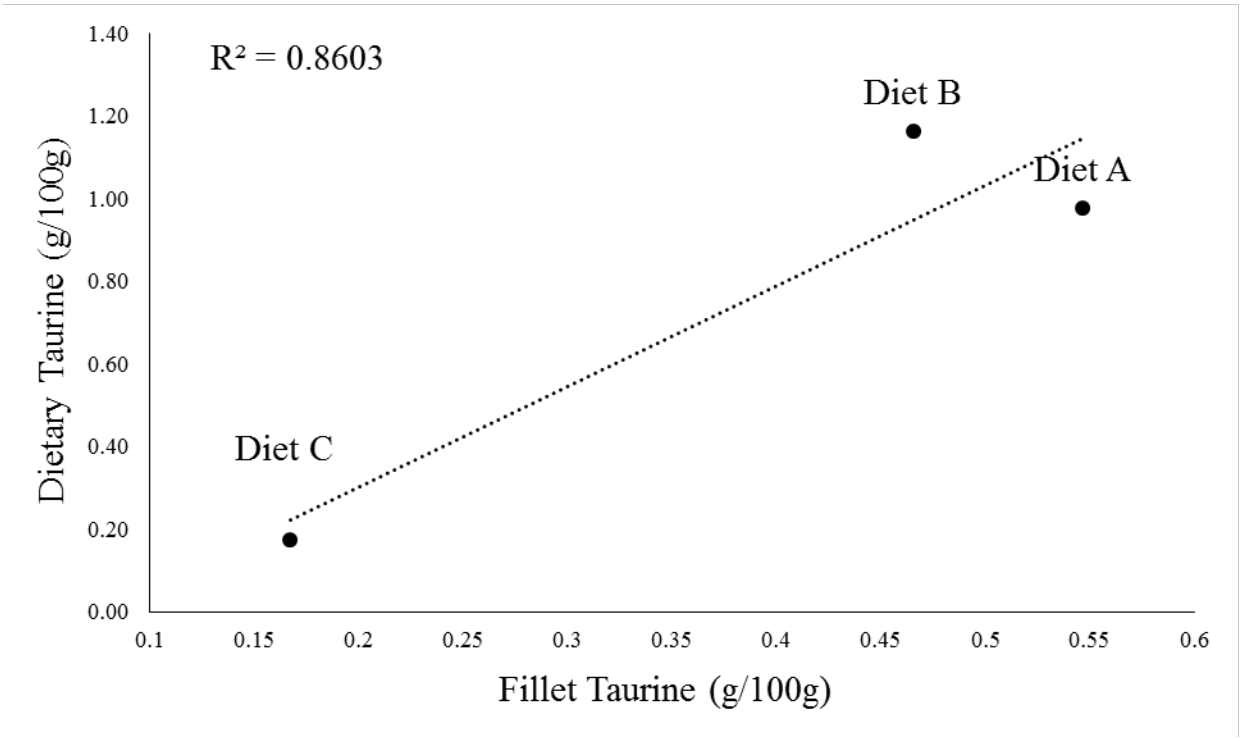

Figure 48: Relationship between dietary taurine and fillet taurine in fish from Block 1.

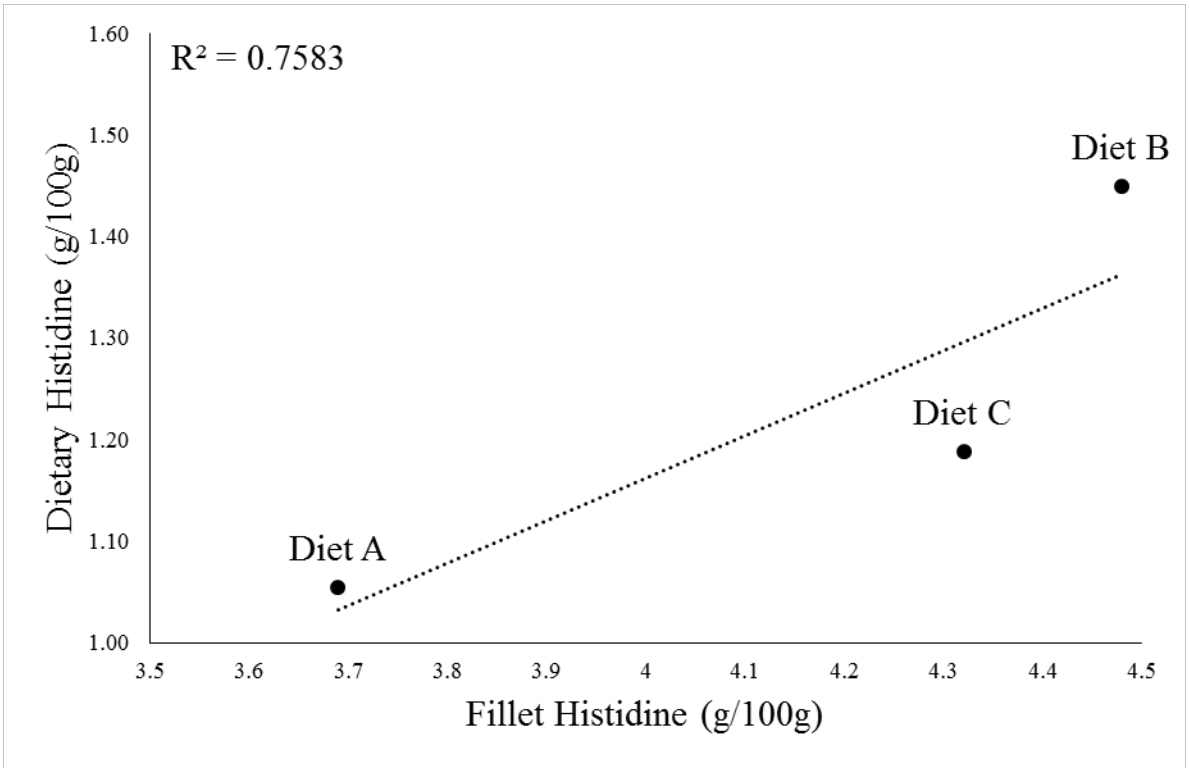

Figure 49: Relationship between dietary histidine and fillet histidine in fish from Block 1.

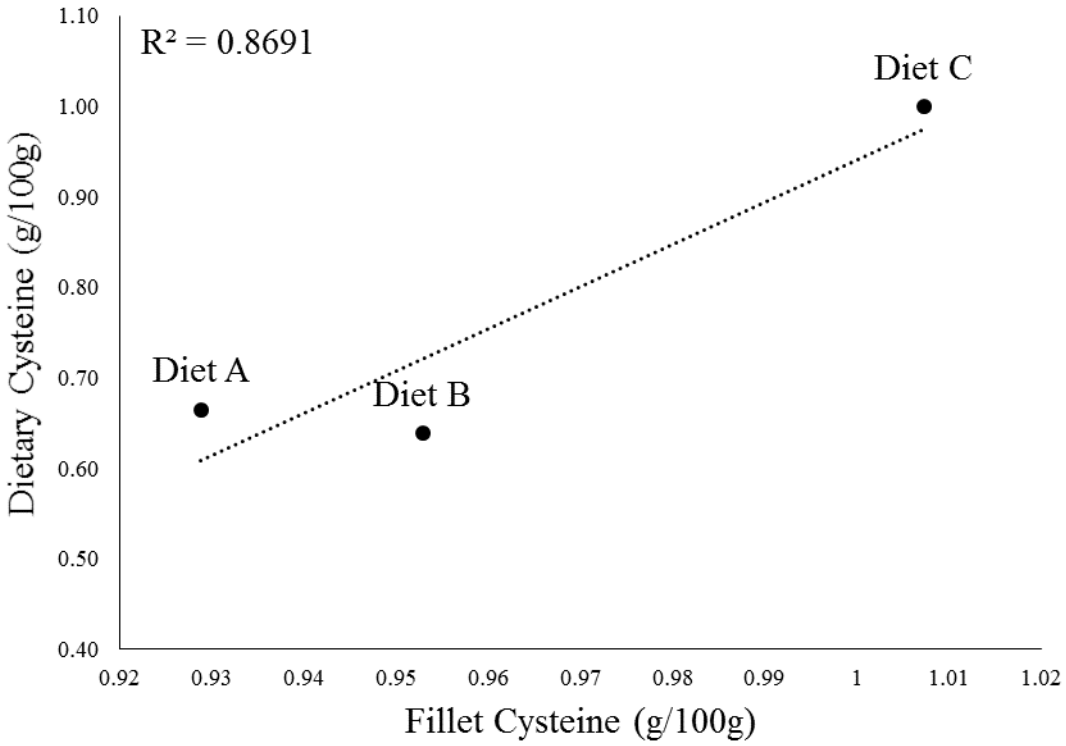

Figure 50: Relationship between dietary cysteine and fillet cysteine in fish from Block 1.

There were significant differences in the fatty acid profile of the fillets from fish fed the various diets (Table 12). Somewhat surprisingly, the fillet fatty acid profiles were very similar between fish fed Diets B and C, despite Diet B having a superior fatty acid profile to Diet C. Fish fed Diet A had significantly higher DHA, DHA:EPA ratio, long chain omega 3 PUFAs and a superior omega-3 to omega-6 ratio to those fed the other two diets. This can largely be attributed to the very high level of DHA in this diet.

Fillet data from Block 2 is shown in Table 13. There were no differences in any of the measured parameters between the various treatments. This was not unexpected given that the same diet was fed in all treatments and demonstrates that the inclusion of the immunostimulants did not affect the fillet profile. It also demonstrates that the fillet profile in those fish fed Diet C in Block 1 had 'recovered' to become equal to those fed Diet B. The taurine content of the fillets at the end of Block 2 averaged 0.28 $g.100g^{-1}$, which was a considerable decline compared with those measured in the Block 1 fillets from fish fed Diet B (0.46 $g.100g^{-1}$)(Table 12), despite no reduction in dietary taurine content. The reasons for this are therefore unclear but may represent the effect of fish size and a reduction in the taurine

uptake from the diet with increasing size or an increase in excretion rate. Alternatively, whilst there was no difference in the taurine content of the Diet B diets fed in Block 1 and 2, there could have been an increase in the soy content of the diet, which would account for the reduction in fillet taurine. Selenium or cholesterol were not analysed in these Block 2 fillet samples. The fatty acid profile of the fillets was also equal between treatments (Table 13) and were somewhat similar to the profiles observed in fish fed Diet B at the end of Block 1, with the exception of EPA. The reduction in fillet EPA was the result of the low EPA in the 6 mm Diet B pellets fed during Block 2, as previously discussed. Whilst this diet was also low in DHA, this did not reflect in the fillet composition.

Table 13: Nutritional profile of fillets from fish fed Block 2 diets.

	P	Diet B	Diet B + AG	Diet B + Act	Diet B_exDiet C
Moisture (%)[1]	0.09	70.7 ± 1.5	69.7 ± 1.4%	68.6 ± 1.4%	70.1 ± 1.2%
Crude lipid (%)[2]	0.36	12.7 ± 1.6%	17.3 ± 2.4%	17.7 ± 2.7%	14.8 ± 0.4%
Vitamin E (mg/kg)[3]		na	na	na	na
DHA	0.48	15 ± 1%	17 ± 0%	16 ± 1%	15 ± 0%
EPA	0.14	5 ± 0%	5 ± 0%	5 ± 0%	6 ± 0%
DHA:EPA	0.29	2.80 ± 0.34	3.19 ± 0.05	2.92 ± 0.16	2.54 ± 0.06
LC n3 PUFA	0.68	23 ± 1%	25 ± 0%	24 ± 1%	23 ± 1%
Total omega-3	0.68	25 ± 1%	26 ± 0%	26 ± 1%	25 ± 1%
Total omega-6	0.13	11 ± 0%	11 ± 0%	11 ± 0%	12 ± 0
Omega-3:Omega-6	0.49	2.23 ± 0.13	2.32 ± 0.04	2.27 ± 0.07	2.10 ± 0.11

1 Murdoch University Marine and Freshwater Research Laboratory (MFRL)
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Fillet biochemical composition from fillets sampled from Block 3 fish are shown in Table 14. Again, due to the high variation between replicates, there were no differences detected in total lipid content of the fillets, with values ranging from 24.2% in those fish fed Diet A to 27.7% in those fish fed Diet D. Similarly, cholesterol ranged from 158.7 mg.100g^{-1} in Diet A to 206.8 mg.100g^{-1} in Diet D but with no significant differences between treatments. There was no effect of diet on the vitamin E content of the fillets, which is expected given the similar levels of vitamin E in the diets. The only amino acid within fillets to show a significant

difference between dietary treatments was taurine (P = 0.04), with those fish fed Diet D having significantly lower taurine in their fillets (0.18 $g.100g^{-1}$) than those fed both other diets, which did not differ from each other. The level of taurine in the fillets of fish fed Diet B (0.27 $g.100g^{-1}$) in this block was equal to those fed the same diet at the end of Block 2 (0.28 $g.100g^{-1}$). The selenium content of fillets from fish fed Diet D was significantly lower (1.6 $mg.kg^{-1}$) than those from the other two diets, which did not differ. The lower level of the Diet D fillets was expected on basis of the lower level of selenium in this diet. The higher level of selenium in the 9 mm Diet A (3.4 $mg.kg^{-1}$) compared with Diet B (2.1 to 2.3 $mg.kg^{-1}$) did not result in a higher fillet selenium content in those fish fed the former diet.

Diet D resulted in a superior fatty acid profile of the fillets of fish fed this diet compared with the other two diets, with all key fatty acid parameters being significantly better than those in fillets from fish fed Diet A and Diet B. Diet D may therefore represent an excellent finishing diet which can improve the fillet fatty acid profile and therefore the marketability of these fillets.

Table 14: Nutritional profile of fillets from fish fed Block 3 diets

	P	Diet A	Diet B	Diet D
Crude lipid (%)[2]	*0.80*	24.2 ± 0.5	25.5 ± 3.0	27.7 ± 5.7
Cholesterol (mg/100g)[2]	*0.57*	158.7 ± 27.1	192.6 ± 31.5	206.8 ± 35.6
Vitamin E (mg/kg)[3]	*0.68*	83.7 ± 3.7	75.3 ± 9.8	80.4 ± 5.0
Taurine (g/100g)[3]	***0.04***	0.30 ± 0.04	0.27 ± 0.01	0.18 ± 0.01
Selenium (mg/kg)[1]	***0.002***	2.1 ± 0.1	2.2 ± 0.1	1.6 ± 0.1
DHA[3]	***0.019***	18 ± 1%	15 ± 1%	18 ± 1%
EPA	***0.001***	5 ± 0%	5 ± 0%	8 ± 0%
DHA:EPA	***0.001***	4.02 ± 0.09	2.70 ± 0.13	2.23 ± 0.28
LC n3 PUFA	***0.001***	26 ± 1%	23 ± 1%	30 ± 1%
Total omega-3	***0.000***	28 ± 1%	25 ± 0%	33 ± 1%
Total omega-6	***0.002***	12 ± 0%	12 ± 0%	10 ± 0%
Omega-3:Omega-6	***0.000***	2.38 ± 0.09	2.08 ± 0.05	3.39 ± 0.16

[1] Murdoch University Marine and Freshwater Research Laboratory (MFRL)

[2] Agrifood Technology (AGT)

[3] Department of Agriculture & Food (DAF)

4.7. Shelf Life

Fillet shelf life parameters were compared between fish fed Block 1 Diets A, B and C. There were no significant differences in muscle pH at harvest between diets (P = 0.9), an average pH of 7.45 was achieved at harvest. The sensory and taste assessment was completed by 5 assessors. Unfortunately, the method of fish slaughter using AQUI-S imparted a very distinct flavour taint into the cooked meat which compounded any meaningful differences in flesh flavour between the dietary treatments. Total plate counts on the fillets in the vacuum packs were initially very low at 5×10^4 CFU.g^{-1}, with no significant difference between diet treatment (P = 0.8) on day 1 and rose continuously reaching 1.6 X 10^7 CFU.g^{-1} on the 12th day of storage which negatively impacted on the quality index score of the flesh. These data suggest that the higher level of the antioxidants, selenium and vitamin E, in the fillets from fish fed Diet A and Diet B did not improve the shelf life of these fillets relative to those from fish fed Diet C.

4.8. Routine Health Samples

In Block 1, those fish fed Diet C had a significantly lower percentage of visceral fat (%VSI) than those fed Diets A and B (Figure 51). This is consistent with the lower fillet lipid content in fish fed this diet and despite the fact that Diet C contained more lipid than both other diets and further demonstrates impaired lipid metabolism in these fish as a result of taurine deficiency. After the first month of Block 2, the visceral fat in the exDiet C fish was still lower than those in the other treatments, however had increased to become equal by the end of the second month (Figure 52) and remained equal from thereon. There was no effect of diet on the percentage of visceral fat in fish in Block 3 and this was not measured in Block 4.

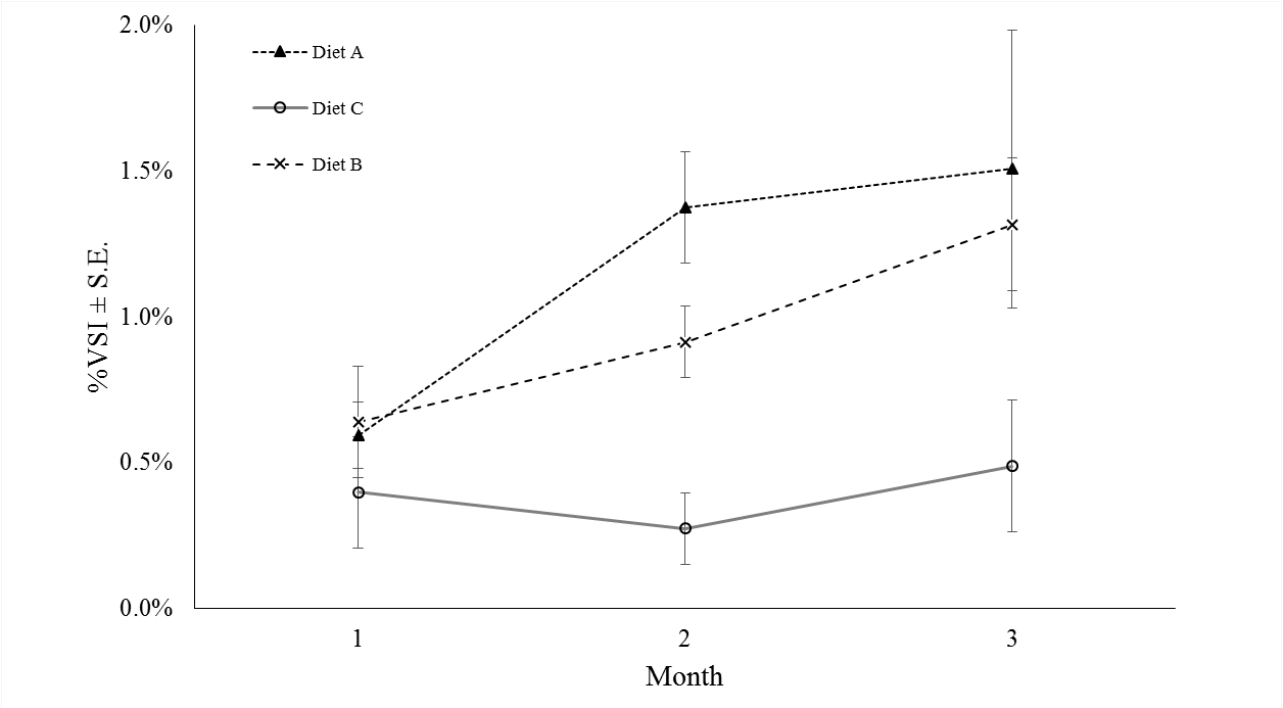

Figure 51: Effect of time on percentage visceral fat in fish fed different diets in Block 1.

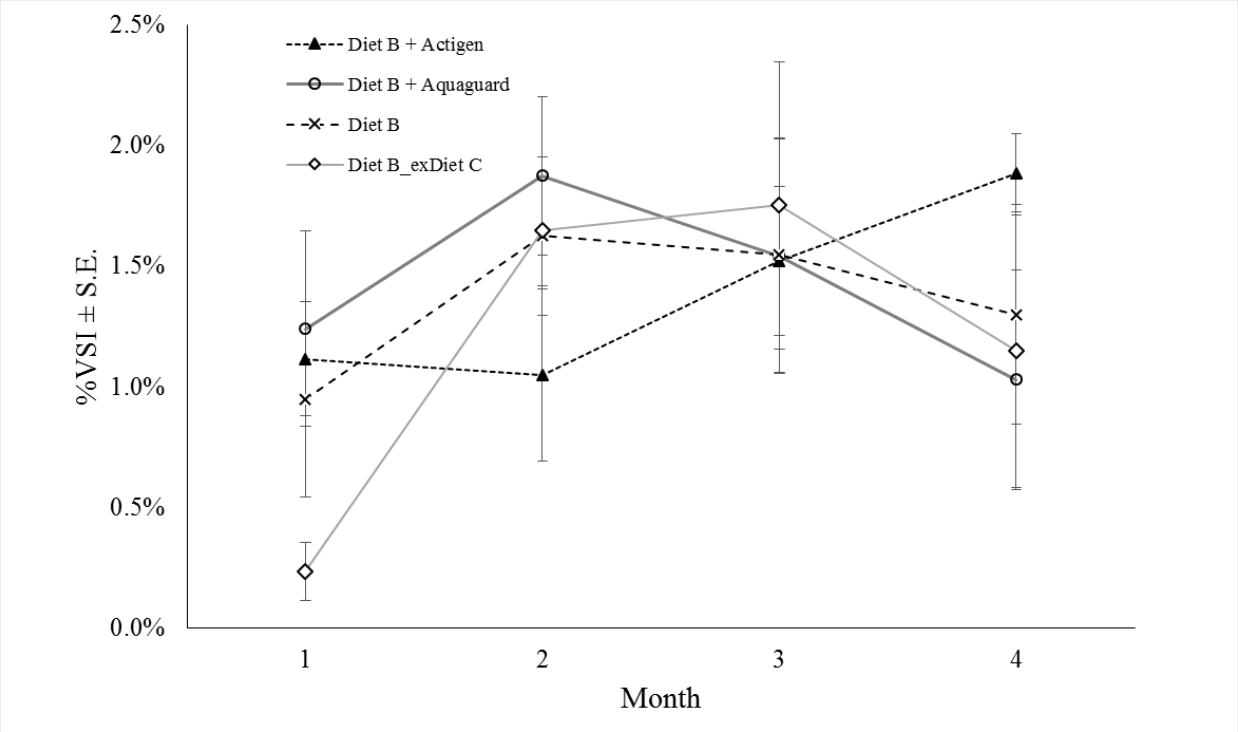

Figure 52: Effect of time on percentage visceral fat in fish fed different diets in Block 2.

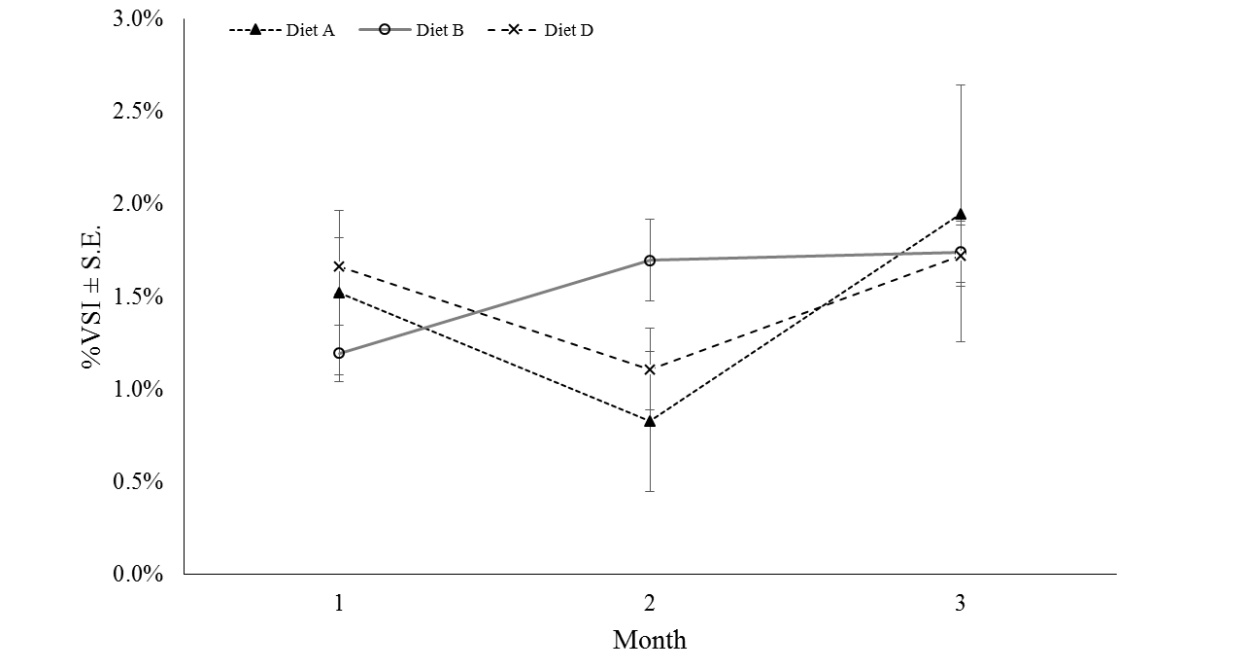

Figure 53: Effect of time on percentage visceral fat in fish fed different diets in Block 3.

The effect of body size on the percentage of visceral fat being carried by all fish sampled during the 56 week trial fish is shown in Figure 54. The positive correlation between these

two factors suggests that the increase in dietary lipid with increasing pellet size is not being used efficiently and that excess dietary lipid is being deposited in the body cavity. The amount of lipid being carried in the fillet also increased considerably with fish size, particularly between Block 2 and Block 3. Considering only those fish fed Diet B, fillet lipid content increased from 12.7 ± 1.6% at the end of Block 2, when fish averaged 2255 grams to 25.5 ± 3.0% at the end of Block 3, when these fish averaged 3351 grams. This increase in fillet lipid is a desirable from a marketing point of view, but there is little benefit in increased visceral fat and it may be detrimental to fish health and lead to high FCR. Minimising visceral fat whilst optimising fillet fat requires further research.

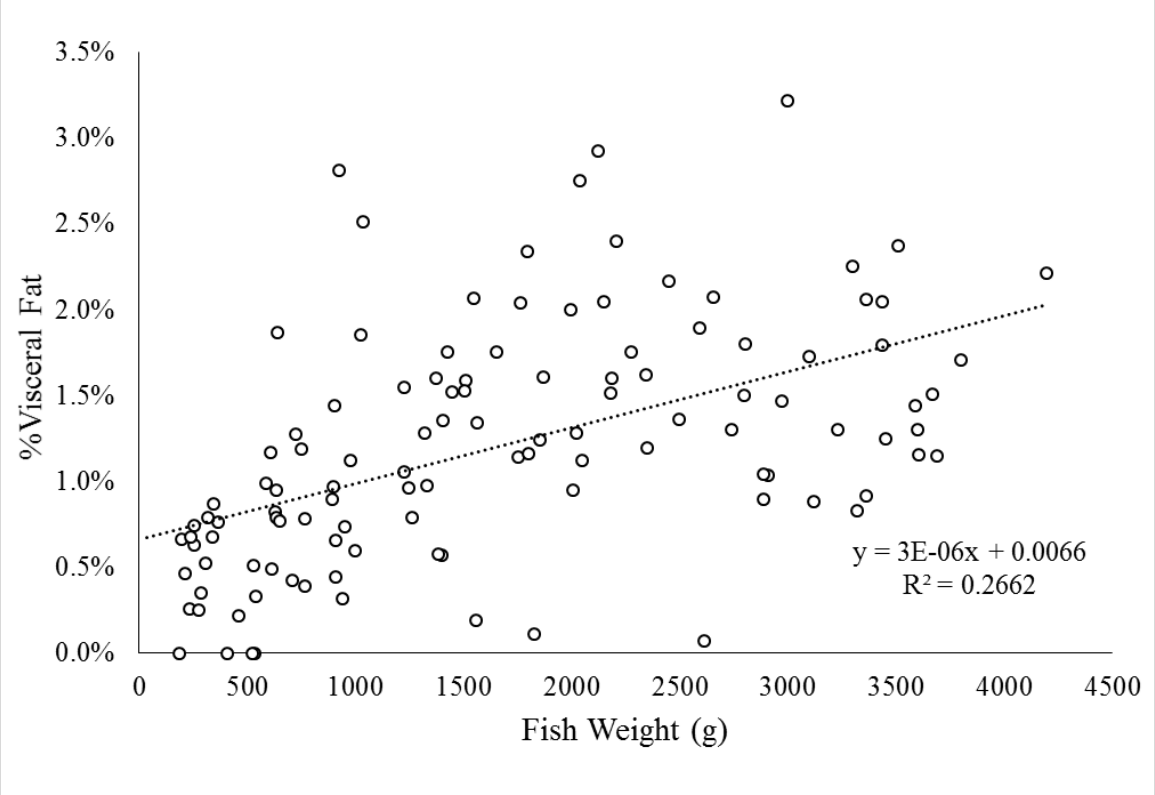

Figure 54: Relationship between fish size and percentage visceral fat across the whole data set.

The effect of diet on the hepatosomatic index (%HSI) of fish in Block 1 is shown in Figure 55. Fish fed Diet C had higher %HSI in all months compared with fish fed Diets A and B. As is described below, Diet C fish were observed to have fattier livers histologically compared with the other two treatments, which may be the reason for their higher %HSI. In Block 2, the ex_Diet C fish recovered in terms of their %HSI (Figure 56). In Block 3 there was no effect of diet on %HSI, however there was a clear trend of decreasing %HSI with time in this block

(Figure 57). This is also apparent from the slight negative correlation between fish size and %HSI for all fish sampled across the 56 week trial (Figure 58).

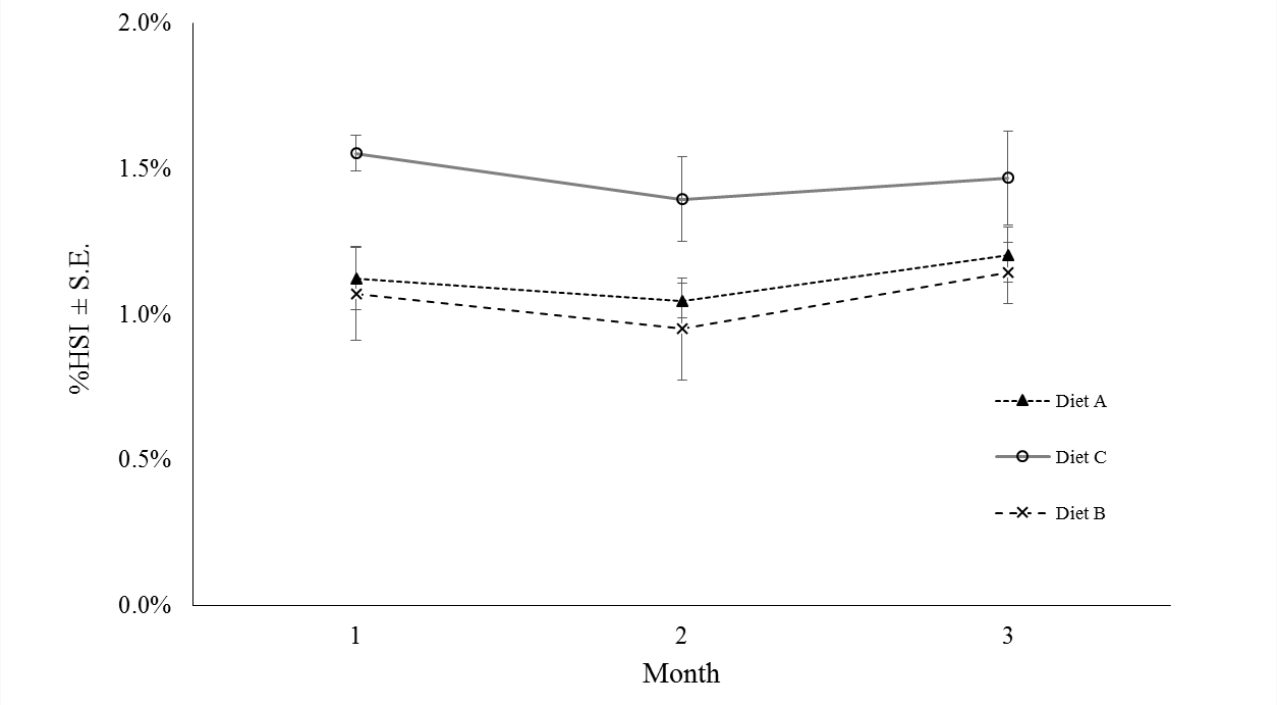

Figure 55: Effect of time on the hepatosomatic index in fish fed different diets in Block 1.

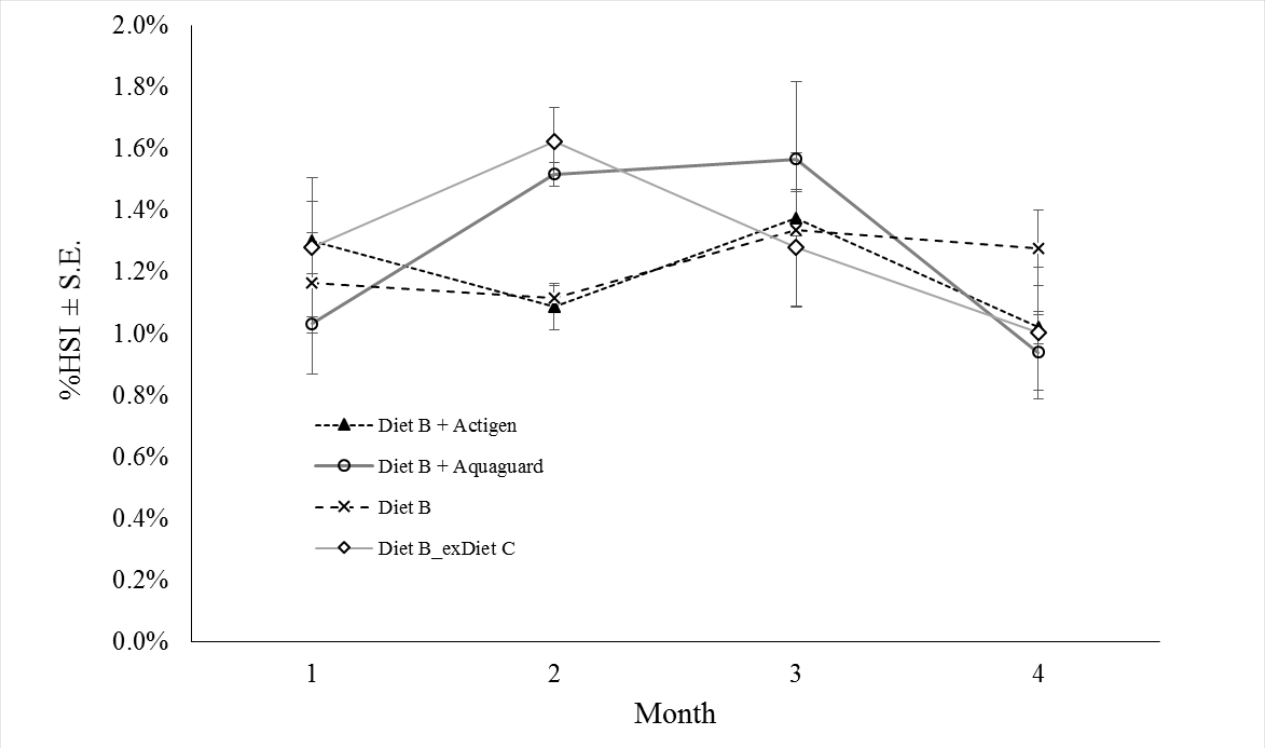

Figure 56: Effect of time on the hepatosomatic index in fish fed different diets in Block 2.

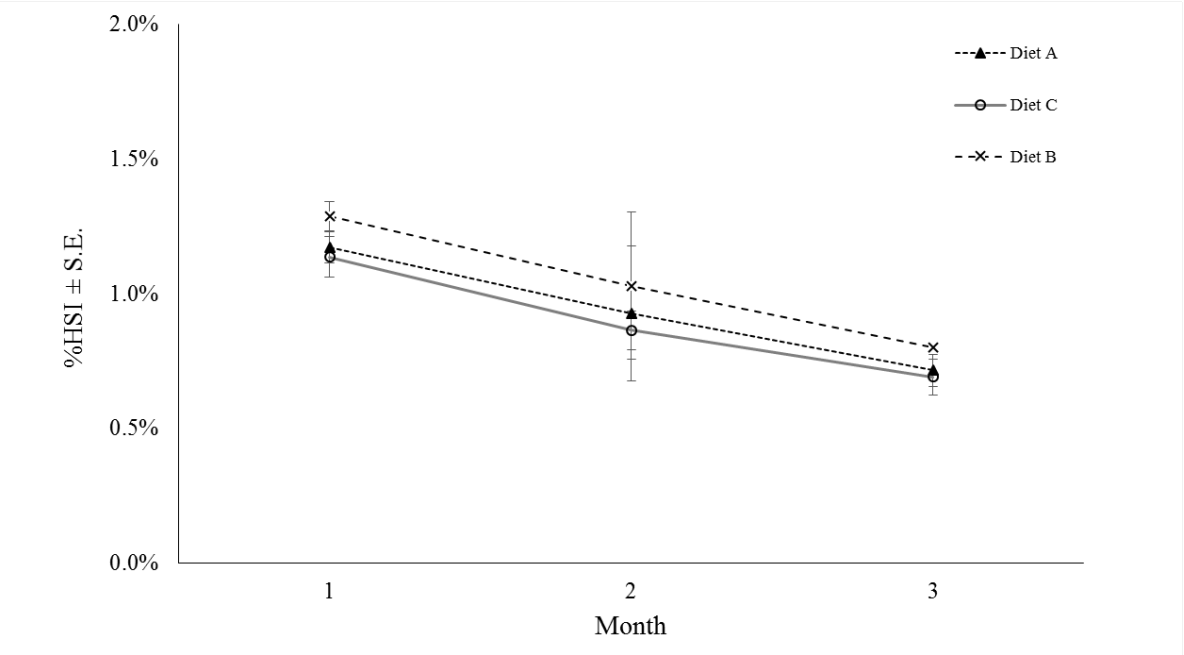

Figure 57: Effect of time on the hepatosomatic index in fish fed different diets in Block 3.

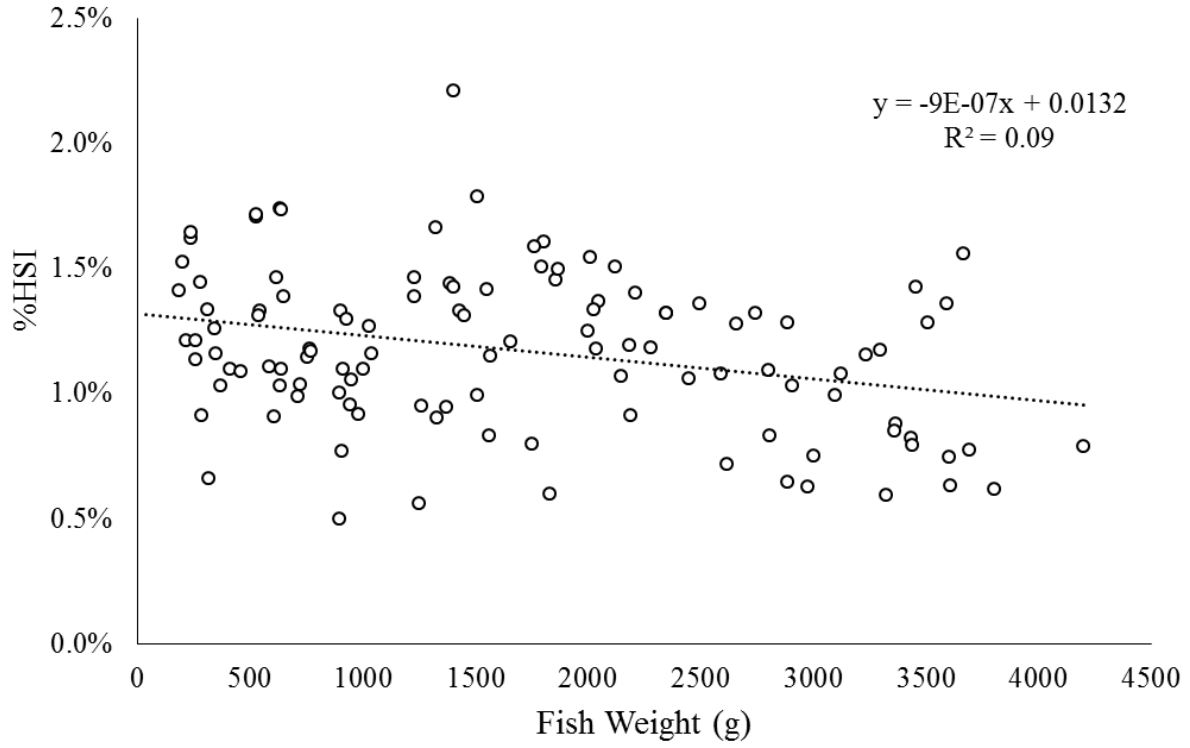

Figure 58: Relationship between fish size and hepatosomatic index across the whole data set.

Of those fish which had identifiable gonads during the samplings in Block 2 and 3, their GSI and sex are plotted against fish weight in Figure 59. This demonstrates that gonads are beginning to develop at 1.25 kg but remain immature and indeterminate in sex until approximately 2.5 kg. At this time GSI increases and some males were observed to be expressing milt (‘running ripe’) at this size, but no mature females were found. These values of GSI are small compared with older maturing fish. Mature adult male YTK have GSI values ranging from 2 to 5% during the spawning season and mature females from 4 to 10% (Poortenaar et al., 2001). The low GSI values here suggest that the amount of energy being invested into maturation is likely to be relatively minor, however it is noteworthy that we observed a jump in FCR once fish reached approximately 2.5 kg (Figure 32), which appears to correlate with the increase in GSI.

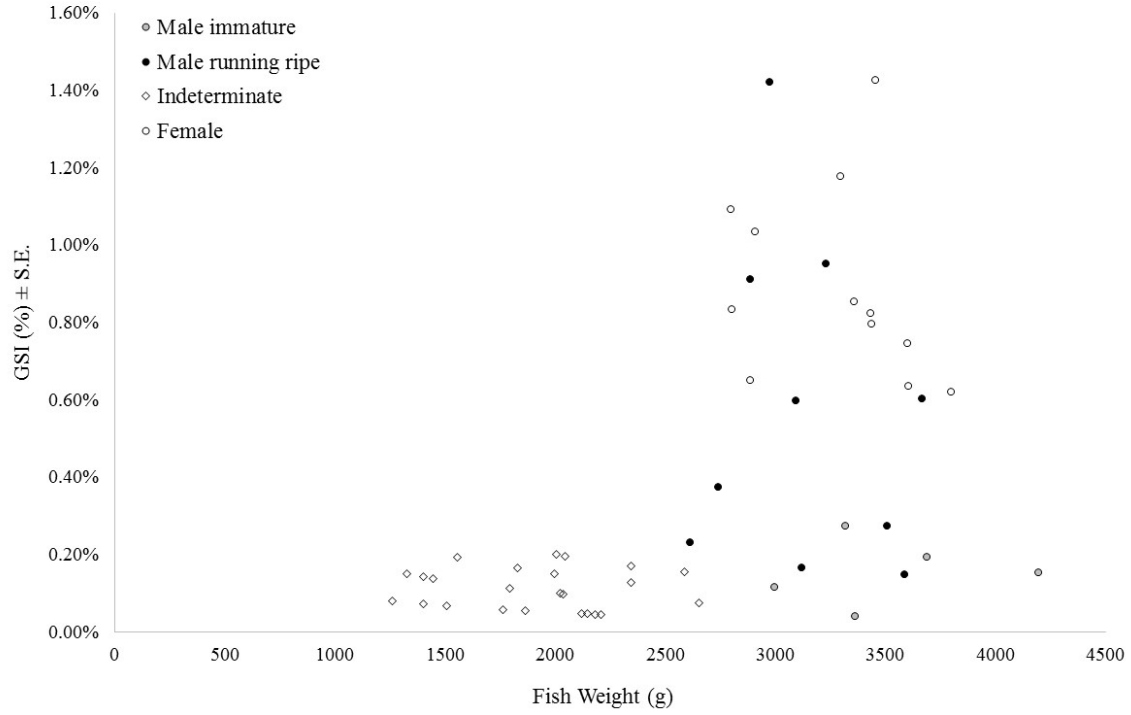

Figure 59: Relationship between fish size and gonadosomatic index (GSI) of fish with gonads from Block 2 and Block 3 and the sex and stage of maturation of these fish.

Of the 195 fish sampled at the end of Block 3, 47% were male and 53% were female. There were no significant differences between the weight of male and female fish pooled across strains and diets (Figure 60).

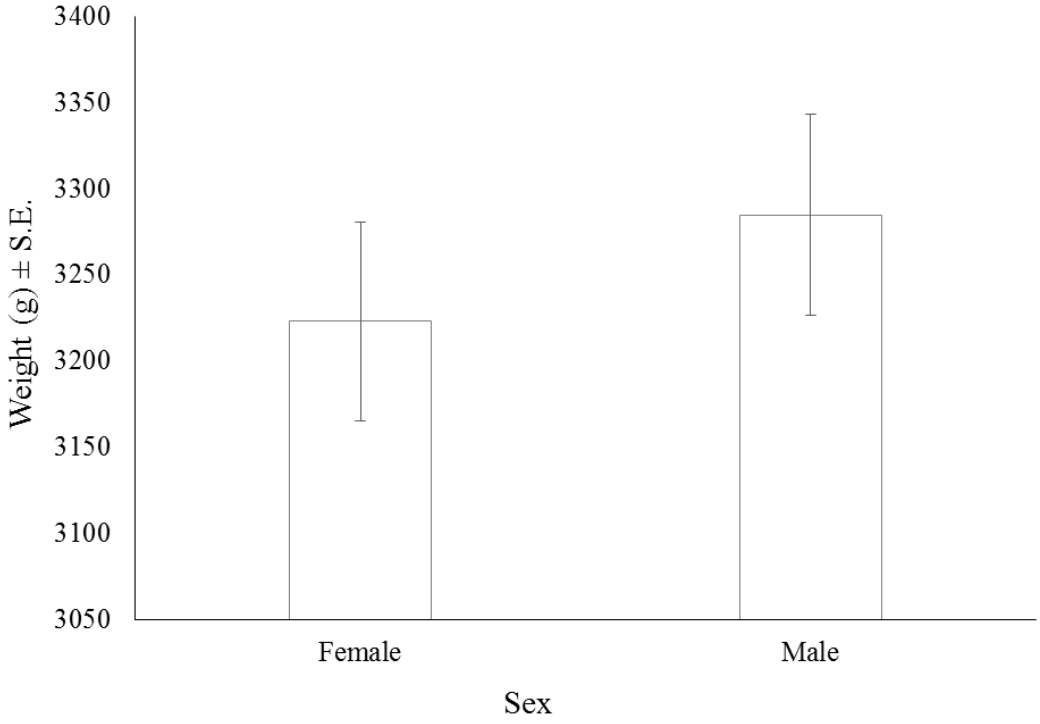

Figure 60: Weight of female and male fish sampled at the end of Block 3.

4.9. Histology

At the end of each month the majority of organs examined from all blocks had no significant issues histologically, regardless of dietary treatment or fish strain, with the exception of liver in Block 1. A summary of all of the histological findings from all four blocks is given in Appendix 1.

The condition of the livers of Block 1 fish was typically worse in those fish fed Diet C with a greater extent of fatty vacuolation than those fed Diets A and B (Figure 61). All pellet sizes of Diet C contained the highest level of fat of the three diets, which is likely to have contributed to this difference, however other differences such as the dietary taurine level and blood plasma cholesterol levels are likely to influence fat metabolism and the extent of the fatty vacuolation. Despite having fattier livers, these fish had less fat in their fillet and in their body cavity.

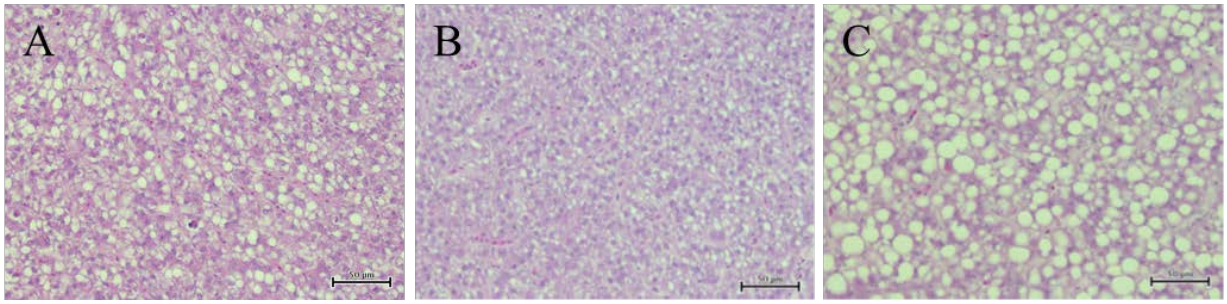

Figure 61: Typical livers of fish fed Diet A (A), Diet B (B) and Diet C (C) during Block 1.

Whilst no issues were seen in the eyes in the fish at the end of Block 1, 100% of those fish which continued to be fed on Diet C for a further 20 weeks (29 Feb to 14 July) had developed cataracts (Figure 62), whilst no cataracts were found in those fish maintained on Diet A and B.

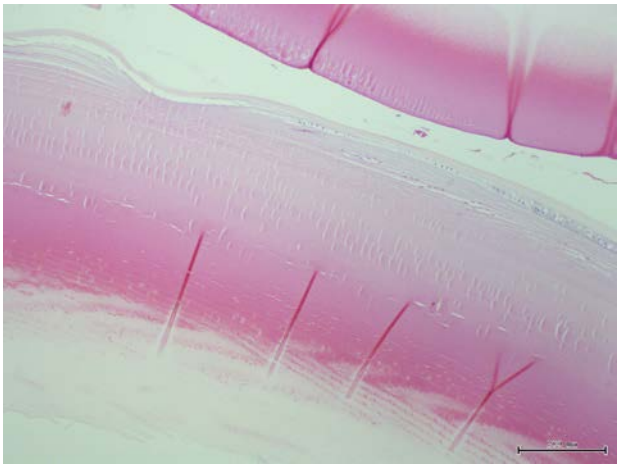

Figure 62: Histological section showing cataracts in the lens of fish fed Diet C for a further 20 weeks past the end of Block 1.

Cataracts have been associated with a number of nutritional components of the diet. A lack of dietary histidine has been found to be a cause of cataracts in rapidly growing salmon smolts and the dietary inclusion level required to prevent cataracts is 14.4 $g.kg^{-1}$ (Remø et al., 2014). None of the diets in this study contained this level of histidine, suggesting the requirement by YTK is lower than salmon smolt. As previously demonstrated (Table 10 and Table 12), the level of histidine in both Diet C itself and in the fillets of fish fed Diet C were both higher than those in Diet A, yet fish fed Diet A did not develop cataracts. Available dietary zinc has

also been implicated in cataracts in fish, with fish fed high levels of animal protein being more susceptible to cataracts, with the zinc from such sources being less bioavailable (Hardy, 2012). Whilst the total amount of zinc in Diet C was similar to Diet B, fish fed Diet B did not develop cataracts. This may suggest that Diet C contained a higher percentage of animal meal such as blood meal, which may have rendered this zinc less bioavailable. Lenticular cataracts have also been associated with methionine and tryptophan deficiency (Hardy, 2012). Whilst we did not measure tryptophan in the diets, the level of methionine in the 6 mm pellets of Diet C (0.75 $g.100g^{-1}$) (i.e. those on which these fish fed for the longest) was much lower than the same sized pellets of Diet A (1.05 $g.100g^{-1}$) and Diet B (1.14 g. 100 g). Furthermore, it is our opinion that the cataracts seen in these fish were most likely to be lenticular cataracts, as they don't involve the capsule but are mostly just beneath it in one part of the lens.

In all months of Block 1, many fish had a shredded appearance in skeletal muscle known as flocculation degeneration. Despite not growing as fast as fish fed Diets A and B, those fish fed Diet C had a similar incidence and severity of this feature, suggesting that it is not a function of the fish's rapid growth. In Block 2, flocculation degeneration was still seen, however its incidence and severity was noted to be declining and this may be the result of the fish's slowing relative growth. This lesion may be a function of the thrashing that occurs in some fish as they become anaesthetised and this is discussed further below in the plasma biochemical data.

At the end of the first month of Block 1, three of the four fish examined from the Diet C treatment had moderate numbers of eosinophilic globules in the tubular epithelium of the kidney, whereas only one fish from each of the Diet A and Diet B showed this effect. At the end of the second month, however, there was a great deal of variation in the presence of eosinophilic droplets in the tubule kidney epithelium in all fish examined, ranging from none to very high and with no clear pattern between dietary treatments.

There was no effect of diet or time on the number of melanomacrophage centres seen in the spleens of fish in Block 1.

The effect of treatment on skin thickness at the end of Block 2 is shown in Figure 63. Whilst the thickness of the skin in the two treatments supplemented with immunostimulants was slightly greater than those fed just Diet B, these differences were not significant (P = 0.16)

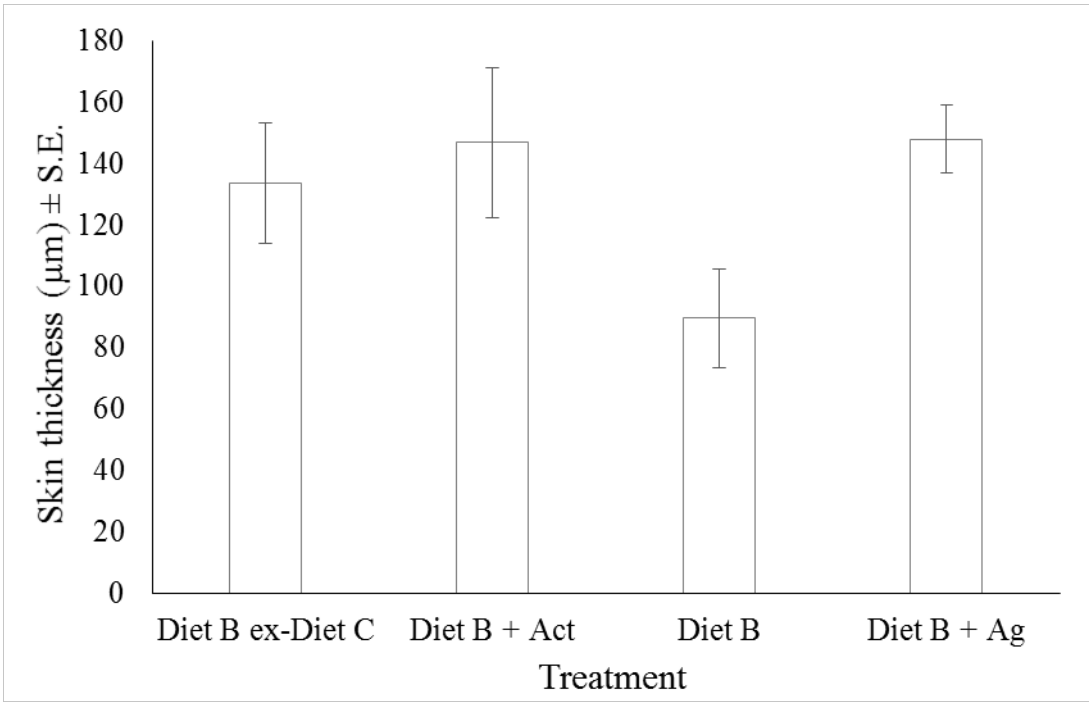

Figure 63: Thickness of the skin of fish fed different diets during Block 2.

The effect of immunostimulant addition to Diet B during Block 2 on the height of villi in each gut section is shown in Figure 64.

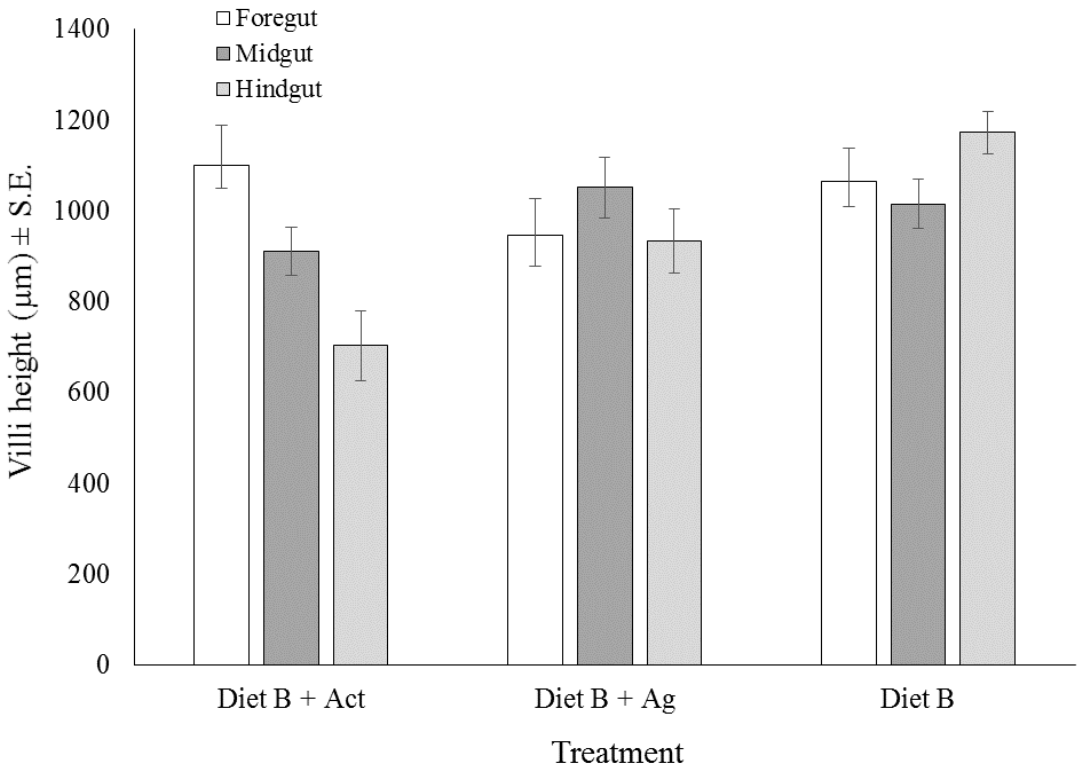

Figure 64: Height of villi in different gut sections of fish fed different diets during Block 2.

Two-way ANOVA revealed no effect of treatment diet (P= 0.05), gut section (P = 0.33) or the interaction of these terms (P= 0.18) on villi height (P= 0.18).

Figure 65 shows the average number of mucous cells per villi by treatment diet and gut section. Two way ANOVA demonstrated a significant effect of gut section (P = 0.005) on the number of mucous cells per villi with the hindgut section have significantly fewer mucous cells than the other two sections. There was no effect of diet (P = 0.67) or the interaction term (P = 0.36) on mucous cell count.

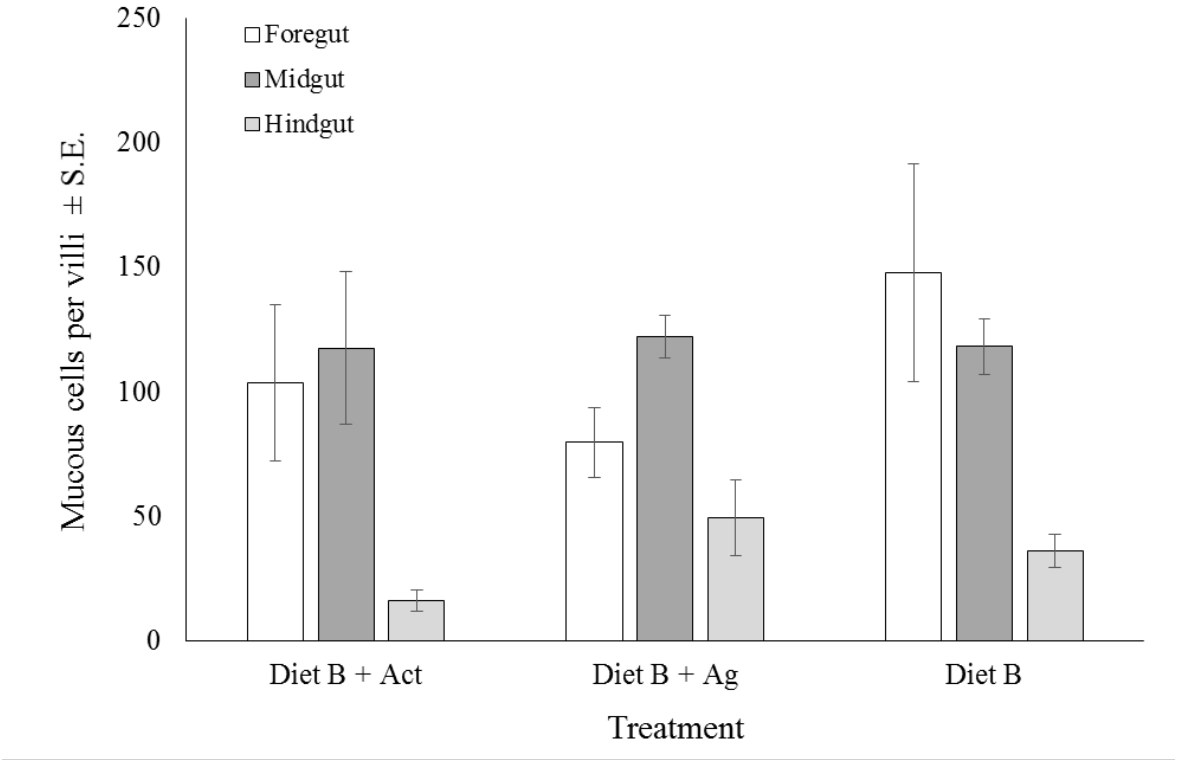

Figure 65: Number of mucous cells in different gut sections of fish fed different diets during Block 2.

At the end of Block 3 there was no effect of diet on the thickness of the fish's skin (P = 0.80) (Figure 66) or the number of mucous cells in the skin (P = 0.96)(Figure 67).

There were no significant histological changes, regardless of dietary treatment or strain in Block 3. At the end of Block 3, flocculation degeneration in the skeletal muscle was evident in only two samples and at very low incidence. The mild depletion of spleen tissue and the moderate melanomacrophages seen in Block 2 occurred across all treatment diets through Block 3. Those fish fed Diet B, had higher incidence of thickening of the lamina propria in the mid- and hind-gut and some evidence of chronic low grade enteritis at the end of the Block.

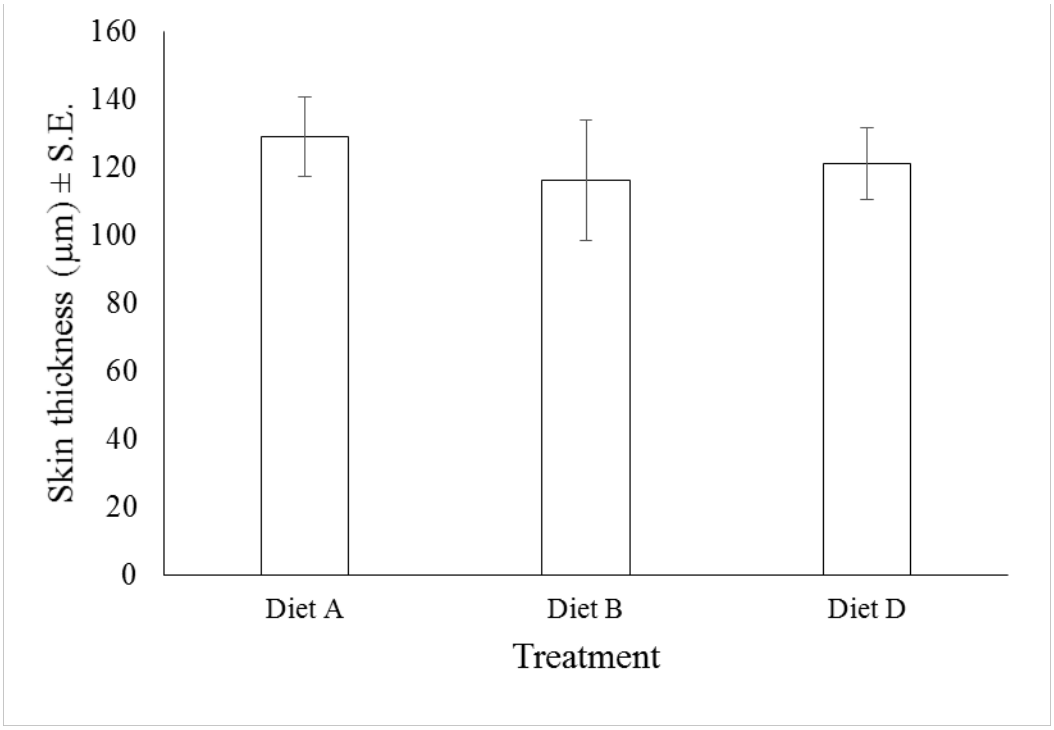

Figure 66: Thickness of the skin of fish fed different diets during Block 3.

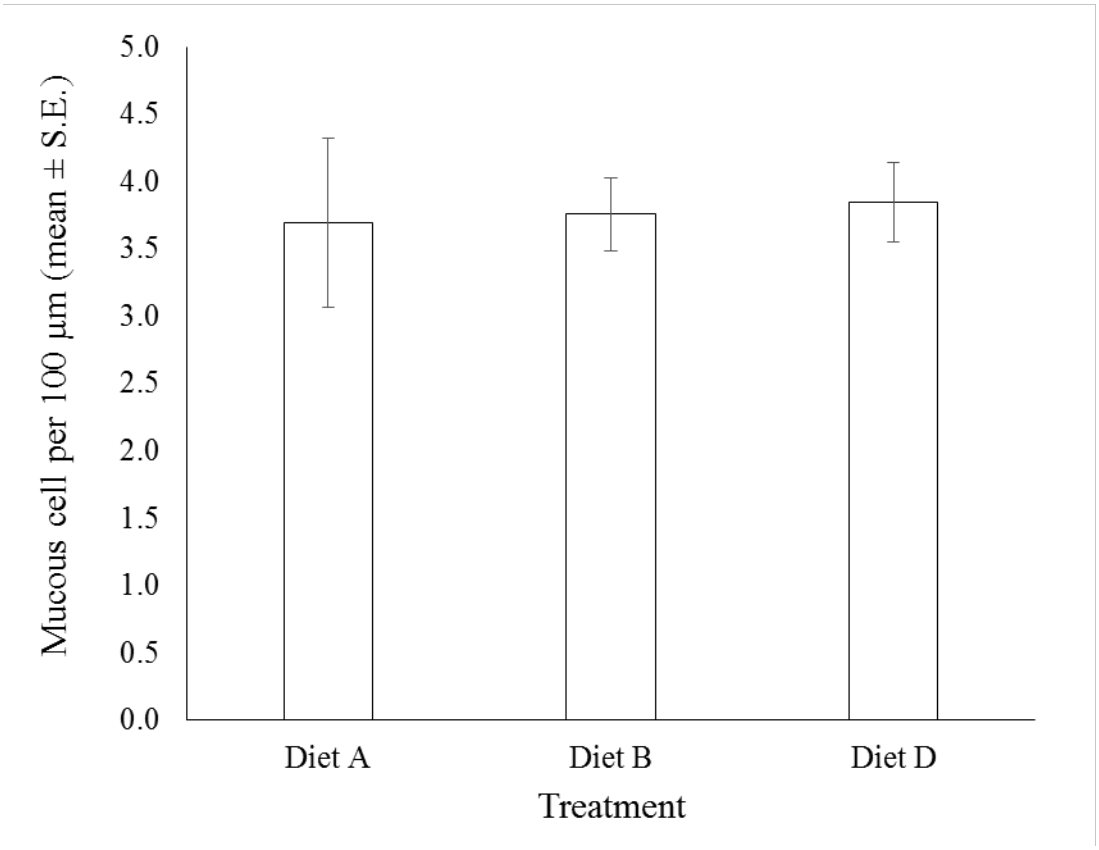

Figure 67: Number of mucous cells in the skin of fish fed different diets during Block 3.

4.10. Blood biochemistry

Blood biochemistry data at the end of each month of Block 1 are shown in Table 15. In this table we have included data by fish strain, as well as diet and time. In all subsequent blocks, insufficient replicates from each strain were obtained for statistical analysis between strains and therefore data were pooled between strains and presented only by diet and month.

For the vast majority of parameters in Block 2 (Table 16) and all of the parameters in Block 3 (Table 17), there was no effect of diet on blood biochemistry parameters and therefore the data have also been presented in more simple formats in Table 18 and Table 19 in which the data were pooled across diets and presented by month. As Block 4 only ran for one month, the data were analysed only by diet and these data are presented in Table 20

Finally, overall relationships between fish size and the various biochemical parameters were plotted for the entire data set of fish sizes ranging from ~70 grams to >4000 grams to determine if there was an effect of size on the various biochemical parameters. Only data for seemingly healthy fish are included in this data set. The blood biochemistry of moribund fish are described separately below. The majority of the parameters did not show a correlation with fish size and all of the plots are shown in Appendix 2. Those parameters which did show a correlation with fish size are discussed in the text below. A summary table of the various parameters from this full data are given in Table 21.

Table 15: Average values (n=2) of blood parameters measured monthly in YTK of different strains fed on different diets during Block 1.

		Month 1						Month 2						Month 3					
		Diet A		Diet B		Diet C		Diet A		Diet B		Diet C		Diet A		Diet B		Diet C	
		Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2	Strain 1	Strain 2
PCV	%	56	43	46	43	48.5	nd	52	48	55	46	43	44	47	47	54	53	45	47
CK	U/L	527	1698	533	1148	739	509	273	2607	1250	831	1752	2226	806	1230	3197	1038	3681	682
ALT	U/L	10	29	17	20	23	20	12	55.5	24	30	19	27	7	17	39	19	20	15
GGT	U/L	<1	<1	<1	<1	<1	<1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
GLDH	U/L	30.3	28.6	31.6	24.5	46.0	35.0	37.3	64.5	62.1	34.4	24.6	42.4	50.0	31.5	70.4	22.8	52.5	26.8
BT	µmol/L	<2	<2	<2	<2	<2	<2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
UREA	mmol/L	6.1	6.6	6.4	5.7	10.5	10.8	5.4	6.9	6.4	8.5	8.3	9.0	7.6	6.8	5.8	7.3	9.6	9.3
CREAT	µmol/L	15.5	18.0	18.5	17.0	21.0	20.5	14.5	16.5	24.5	16.5	20.0	31.0	36.0	21.0	116.0	21.7	50.0	30.5
BOHB	mmol/L	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
CHOL	mmol/L	5.7	6.3	6.0	5.8	2.6	2.6	6.1	6.2	5.7	5.5	4.0	3.8	6.4	6.7	6.2	6.9	6.3	4.0
Na	mmol/L	195	195	194	193	196	194	189	189	195	191	182	192	192	190	191	194	203	187
K	mmol/L	4.5	6.4	4.4	5.7	5.1	3.8	5.5	6.1	6.8	5.5	6.8	5.6	4.0	6.7	6.7	6.2	4.4	8.0
Cl	mmol/L	165	169	164	166	166	164	158	159	160	161	151	158	162	159	156	164	166	157
PROT	g/L	34	36	38	36	41	36	35	35	39	33	39	39	41	47	43	43	50	44
ALB	g/L	9.5	10.5	10.5	9.5	10.5	9.5	9.5	9.5	11.0	9.0	10.0	10.0	12.0	12.5	12.5	13.3	13.7	11.5
GLOB	g/L	24.0	25.5	27.5	26.5	30.0	26.0	25.5	25.0	27.5	24.0	28.5	29.0	28.5	34.5	30.0	30.0	36.0	32.5
Ca	mmol/L	3.64	3.70	3.77	3.61	3.78	3.58	3.49	3.54	3.72	3.49	3.34	3.81	3.52	3.89	3.70	3.70	4.34	3.65
P	mmol/L	4.11	5.16	4.65	4.38	4.585	4.38	4.06	4.66	4.57	4.08	3.26	4.56	3.98	4.59	4.92	4.75	6.05	4.64
Mg	mmol/L	1.19	1.54	1.28	1.52	1.99	1.82	1.16	1.28	1.43	1.22	1.37	1.49	1.55	1.39	1.78	2.25	2.01	1.64
GPx	U/g of Hb	85.5	130.5	82	63.5	58.5	68	67.5	71.5	57	58.5	38.5	38.5	73.5	66.5	67.5	87.3	49.7	52.5
$Hb_{(red)}$	g/L	214	206	251	226	193	178	294	278	291	295	250	252	271	242	209	230	228	248
$Hb_{(w)}$	g/L	95	120	135	136	99	nd	142	144	130	161	143	142	143	129	97	109	119	135

Table 16: Blood biochemical parameters by diet and month in Block 2.

					Month 1				Month 2			
		P_{diet}	P_{month}	P_{int}	Diet B + ACT	Diet B + AG	Diet B	Diet B_exC	Diet B + ACT	Diet B + AG	Diet B	Diet B_exC
PCV	%	0.16	**0.0015**	0.76	44	47	50	48	45	46	46	48
CK	U/L	0.08	**<0.0001**	0.25	501	1515	486	517	8626	7928	7854	1845
ALT	U/L	0.88	**0.01**	0.07	8	16	19	10	22	54	38	23
GLDH	U/L	**0.0004**	**0.0002**	**0.02**	19.6	22.0	33.6	29.9	28.3	40.6	70.2	107.0
BT	µmol/L	na	na	na	1.13	0.09	<2	0.92	<2	<2	<2	<2
UREA	mmol/L	0.53	**<0.0001**	0.11	5.8	5.8	5.6	4.9	2.7	4.5	4.0	3.8
CREAT	µmol/L	**0.02**	**<0.0001**	**0.01**	29.6	20.7	56.0	35.0	31.0	34.0	24.7	34.7
CHOL	mmol/L	0.38	**0.009**	0.7	5.0	6.0	5.6	5.3	5.2	7.2	6.9	6.7
Na	mmol/L	0.24	**0.002**	0.48	199	191	194	192	209	186	189	183
K	mmol/L	0.81	0.14	0.27	4.3	4.9	6.3	4.0	6.4	6.3	6.3	5.1
Cl	mmol/L	0.64	**<0.0001**	0.89	168	164	167	162	166	157	161	154
PROT	g/L	0.57	**0.12**	0.27	44	39	37	45	41	44	44	42
ALB	g/L	0.52	**0.01**	0.32	12	11	11	13	12	14	13	12
GLOB	g/L	0.47	0.36	0	32	28	26	32	28	30	31	30
Ca	mmol/L	0.54	**0.040**	0.45	3.6	3.4	3.6	3.5	3.2	3.4	3.4	3.3
P	mmol/L	0.21	**0.02**	0.7	4.4	4.0	4.9	3.5	6.2	4.2	4.2	3.7
Mg	mmol/L	0.78	0.91	0.5	1.9	1.7	2.1	1.5	2.1	1.8	1.7	1.3
GPx	U/g of Hb	0.34	**0.0009**	0.31	75	83	63	63	75	70	63	71
$Hb_{(red)}$	g/L	0.66	0.06	0.5	273	263	280	240	276	321	265	261
$Hb_{(w)}$	g/L	0.15	**0.005**	1	nd	141	140	124	152	174	143	137

Table 16 (continued): Blood biochemical parameters by diet and month in Block 2.

					Month 3				Month 4			
		P_{diet}	P_{month}	P_{int}	Diet B + ACT	Diet B + AG	Diet B	Diet B_exC	Diet B + ACT	Diet B + AG	Diet B	Diet B_exC
PCV	%	0.16	**0.0015**	0.76	54	49	56	59	46	48	47	51
CK	U/L	0.08	**<0.0001**	0.25	704	991	110	318	866	1301	638	331
ALT	U/L	0.88	**0.01**	0.07	67	21	23	63	18	41	23	25
GLDH	U/L	**0.0004**	**0.0002**	**0.02**	50.5	23.8	21.6	101.1	14.7	15.5	18.0	28.4
BT	µmol/L	na	na	na	<2	1.25	1.40	<2	0.29	0.31	0.13	0.01
UREA	mmol/L	0.53	**<0.0001**	0.11	3.9	3.2	4.1	5.8	2.2	2.2	3.1	3.0
CREAT	µmol/L	**0.02**	**<0.0001**	**0.01**	49.0	25.8	54.0	88.7	23.0	22.3	22.3	21.7
CHOL	mmol/L	0.38	**0.009**	0.7	5.9	5.6	5.4	5.3	6.6	6.8	6.4	6.6
Na	mmol/L	0.24	**0.002**	0.48	189	186	190	188	206	207	202	203
K	mmol/L	0.81	0.14	0.27	6.5	5.6	5.0	5.9	6.0	4.6	4.8	6.0
Cl	mmol/L	0.64	**<0.0001**	0.89	159	159	158	157	176	176	172	176
PROT	g/L	0.57	**0.12**	0.27	45	40	45	45	46	43	42	43
ALB	g/L	0.52	**0.01**	0.32	14	12	13	14	15	14	13	13
GLOB	g/L	0.47	0.36	0	30	29	32	31	31	30	29	30
Ca	mmol/L	0.54	**0.040**	0.45	3.4	3.3	3.5	3.5	3.8	3.5	3.4	3.7
P	mmol/L	0.21	**0.02**	0.7	4.3	3.7	3.8	4.2	3.3	2.5	3.2	3.2
Mg	mmol/L	0.78	0.91	0.5	1.7	1.4	1.6	1.9	1.9	1.6	1.5	2.3
GPx	U/g of Hb	0.34	**0.0009**	0.31	81	77	86	80	57	70	60	61
$Hb_{(red)}$	g/L	0.66	0.06	0.5	219	227	254	248	279	229	250	226
$Hb_{(w)}$	g/L	0.15	**0.005**	1	100	116	111	100	193	153	166	113

Table 17: Blood biochemical parameters by diet and month in Block 3

					Month 1			Month 2			Month 3		
		P_{diet}	P_{month}	P_{int}	Diet A	Diet B	Diet D	Diet A	Diet B	Diet D	Diet A	Diet B	Diet D
CK	U/L	0.92	**0.02**	0.94	1559 ± 806	1287 ± 644	1360 ± 638	363 ± 335	112 ± 36	482 ± 203	861 ± 353	506 ± 312	310 ± 244
ALT	U/L	0.27	0.22	0.64	22 ± 4	62 ± 5	78 ± 60	24 ± 5	22 ± 2	34 ± 16	63 ± 23	70 ± 9	47 ± 11
GLDH	U/L	0.36	0.71	0.62	48 ± 33	20 ± 7	17 ±	20 ± 5	20 ± 2	22 ± 10	32 ± 12	17 ± 2	14 ± 3
UREA	mmol/L	0.45	**0.01**	0.97	1.8 ± 0.1	1.8 ± 0.2	2.3 ± 0.3	0.52 ± 0.1	1.9 ± 0.5	2.6 ± 0.4	2.7 ± 0.5	2.6 ± 0.4	3.4 ± 0.3
CREAT	µmol/L	0.06	**0.0004**	0.39	42.3 ± 18.9	90.0 ± 32.4	83.0 ± 19.0	1.20 ± 2.0	22.0 ± 1.2	21.3 ± 1.5	21.7 ± 1.5	21.3 ± 1.5	25.7 ± 0.9
CHOL	mmol/L	0.14	**0.0003**	0.16	9.1 ± 0.9	8.1 ± 0.7	7.3 ± 0.6	0.55 ± 1.0	9.2 ± 0.5	9.0 ± 0.5	8.2 ± 0.2	8.3 ± 0.1	8.4 ± 0.0
TRIG	mmol/L	0.25	**0.05**	0.48	1.4 ± 0.2	2.4 ± 0.9	2.4 ± 0.5	0.14 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.4	1.5 ± 0.4	1.0 ± 0.1
LIPASE	mmol/L	0.51	0.96	0.71	10.5 ± 0.5	12.7 ± 3.2	10.7 ±	0.63 ± 1.3	10.0 ± 0.6	11.0 ± 0.6	10.0 ± 0.6	10.7 ± 0.3	11.3 ± 1.3
Na	mmol/L	0.66	**0.0003**	0.72	194 ± 2	189 ± 6	190 ± 4	32 ± 3	210 ± 3	206 ± 7	203 ± 3	201 ± 4	207 ± 6
K	mmol/L	0.59	0.65	0.74	5.4 ± 0.5	6.6 ± 1.9	5.4 ± 1.2	0.69 ± 0.5	4.5 ± 0.6	5.8 ± 0.8	4.9 ± 0.4	5.7 ± 0.9	5.5 ± 0.6
Cl	mmol/L	0.97	0.82	0.46	160 ± 2	160 ± 5	157 ± 2	12 ± 4	162 ± 1	160 ± 3	168 ± 3	166 ± 4	133 ± 32
PROT	g/L	0.74	0.06	0.49	41 ± 1	44 ± 6	41 ± 4	33 ± 2	47 ± 3	47 ± 0	43 ± 4	43 ± 4	47 ± 1
ALB	g/L	0.42	0.53	0.54	12.0 ± 0.6	15.0 ± 4.0	12.7 ± 1.8	0.93 ± 1.5	13.3 ± 0.9	13.3 ± 0.7	12.0 ± 1.2	12.0 ± 0.0	13.3 ± 0.3
GLOB	g/L	0.97	**0.013**	0.64	29 ± 1	29 ± 2	29 ± 2	27 ± 1	33 ± 2	34 ± 1	31 ± 3	31 ± 4	34 ± 1
Ca	mmol/L	0.66	**0.04**	0.24	3.5 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	0.38 ± 0.1	3.8 ± 0.3	3.6 ± 0.2	3.6 ± 0.1	3.4 ± 0.2	3.9 ± 0.2
P	mmol/L	0.87	**0.03**	0.35	3.7 ± 0.1	3.1 ± 0.5	3.6 ± 0.5	0.27 ± 2.0	3.5 ± 0.2	4.6 ± 0.9	3.2 ± 0.1	3.1 ± 0.1	3.4 ± 0.2
Mg	mmol/L	0.90	0.09	0.16	1.5 ± 0.1	1.5 ± 0.2	1.6 ± 0.3	0.16 ± 0.6	1.6 ± 0.1	1.9 ± 0.3	1.6 ± 0.1	2.0 ± 0.4	1.8 ± 0.0
GPx	U/g of Hb	0.40	0.09	0.52	63 ± 5	79 ± 3	80 ± 2	62 ± 8	76 ± 6	87 ± 10	78 ± 11	83 ± 9	79 ± 6

Table 18: Average blood parameters by month, pooled across diets in YTK fed various diets during Block 2. Values within rows sharing the same letter are not significantly different.

		Month			
		1	2	3	4
PCV	%	47^{b}	46^{b}	54^{a}	47^{b}
CK	U/L	823^{b}	6563^{a}	530^{b}	784^{b}
ALT	U/L	14^{b}	34^{ab}	43^{a}	26^{ab}
GLDH	U/L	27^{bc}	62^{a}	49^{ab}	19^{c}
UREA	mmol/L	5.7^{a}	3.8^{bc}	4.3^{b}	2.6^{c}
CREAT	µmol/L	36.6^{b}	31.1^{b}	54.3^{a}	22.3^{b}
CHOL	mmol/L	5.4^{b}	6.5^{ab}	5.6^{ab}	6.6^{a}
Na	mmol/L	193^{a}	192^{a}	188^{a}	204^{b}
K	mmol/L	4.8	6.0	5.8	5.3
Cl	mmol/L	164^{a}	160^{a}	158^{a}	175^{b}
PROT	g/L	40	43	44	44
ALB	g/L	11.6^{b}	12.8^{ab}	13.2^{ab}	13.7^{a}
GLOB	g/L	29	30	30	30
Ca	mmol/L	3.5^{ab}	3.3^{b}	3.4^{ab}	3.6^{a}
P	mmol/L	4.2^{ab}	4.6^{a}	4.0^{ab}	3.0^{b}
Mg	mmol/L	1.8	1.7	1.6	1.8
GPx	U/g of Hb	71^{ab}	70^{ab}	81^{a}	62^{b}
$Hb_{(red)}$	g/L	264	281	237	245
$Hb_{(w)}$	g/L	140^{ab}	152^{a}	107^{b}	156^{a}

Table 19: Average blood parameters by month, pooled across diets in YTK fed various diets during Block 3. Values within rows sharing the same letter are not significantly different.

		Month		
		1	2	3
CK	U/L	1382 ± 336[a]	319 ± 126[b]	559 ± 173[b]
ALT	U/L	54 ± 21	26 ± 5	60 ± 8
GLDH	U/L	34 ± 16	17 ± 4	21 ± 5
UREA	mmol/L	1.9 ± 0.1[b]	2.2 ± 0.2[a,b]	2.9 ± 0.2[a]
CREAT	µmol/L	71.8 ± 14.2[a]	21.4 ± 0.8[b]	22.9 ± 0.9[b]
CHOL	mmol/L	8.2 ± 0.4[b]	9.9 ± 0.5[a]	8.3 ± 0.1[b]
TRIG	mmol/L	2.1 ± 0.4[a]	1.2 ± 0.1[b]	1.2 ± 0.2[b]
LIPASE	mmol/L	11.5 ± 1.5	10.8 ± 0.5	10.7 ± 0.5
Na	mmol/L	191 ± 2[a]	209 ± 3[b]	204 ± 3[b]
K	mmol/L	5.8 ± 0.7	5.1 ± 0.4	5.4 ± 0.4
Cl	mmol/L	159 ± 2	161 ± 2	156 ± 10
PROT	g/L	42 ± 2[b]	49 ± 2[a]	44 ± 2[a,b]
ALB	g/L	13.2 ± 1.4	14.0 ± 0.6	12.4 ± 0.4
GLOB	g/L	29 ± 1[b]	35 ± 1[a]	32 ± 2[a,b]
Ca	mmol/L	3.4 ± 0.1	3.7 ± 0.1	3.6 ± 0.1
P	mmol/L	3.5 ± 0.2	4.9 ± 0.8	3.2 ± 0.1
Mg	mmol/L	1.5 ± 0.1	2.0 ± 0.2	1.8 ± 0.1
GPx	U/g of Hb	74 ± 3	81 ± 4	80 ± 4

Table 20: Average blood parameters in YTK fed various diets at the end of Block 4.

			Diet	
		P	B	D
CK	U/L	0.59	4505 ± 2715	2870 ± 1522
ALT	U/L	0.86	118 ± 45	135 ± 70
GLDH	U/L	0.79	21.4 ± 3.9	24.2 ± 9.0
BT	µmol/L	-	1.5 ± 0.4	1.5 ± 0.1
UREA	mmol/L	0.06	5.1 ± 0.4	3.1 ± 0.3
CREAT	**µmol/L**	**0.002**	**39.5 ± 0.4**	**25.8 ± 1.6**
CHOL	mmol/L	0.47	7.4 ± 0.4	7.1 ± 0.2
TRIG	mmol/L	0.54	2.1 ± 0.4	2.4 ± 0.4
LIPASE	mmol/L	0.91	12.8 ± 0.4	12.6 ± 0.9
Na	mmol/L	0.56	191 ± 0.4	194 ± 5
K	mmol/L	0.83	3.6 ± 0.4	3.4 ± 0.7
Cl	mmol/L	0.37	149 ± 0.4	153 ± 4
PROT	g/L	0.38	48 ± 0.4	45 ± 2
ALB	g/L	0.68	13 ± 0.4	13 ± 0
GLOB	g/L	0.32	35 ± 0.4	32 ± 1
Ca	mmol/L	0.10	3.7 ± 0.4	3.4 ± 0.1
P	mmol/L	0.34	3.5 ± 0.4	3.2 ± 0.2
Mg	mmol/L	0.67	1.8 ± 0.4	1.7 ± 0.1

Table 21: Statisical parameters for blood biochemical parameters for the full data set collected in the current study, across all fish sizes ranging from 47 grams to 4199 grams.

Parameter	Average	Standard Deviation	Maximum	Minimum	n	Standard Error
CK	1663	2624	13314	8	133	228
ALT	39	47	394	4	135	4
GLDH	33	27	177.4	0.5	120	2
UREA	4.7	2.5	12	1	134	0.2
CREAT	40	35	201.0	13.0	128	3
CHOL	6.5	1.8	13.5	2.3	130	0.2
Na	194.9	10.4	237.0	169.0	135	0.9
K	5.3	1.8	11.8	2.0	135	0.2
Cl	161.0	11.7	196.0	70.0	135	1.0
SPROT	41.8	5.6	58.0	27.0	135	0.5
ALB	12.1	2.4	23.0	5.0	135	0.2
GLOB	29.7	3.8	42.0	21.0	135	0.3
Ca	3.6	0.2	4.4	2.8	135	0.0
P	4.1	1.2	11.3	1.8	135	0.1
Mg	1.7	0.5	4.1	1.0	134	0.0
GPx	74.4	18.7	183	33	126	1.7

Values of GGT and BOHB measured in all months of Block 1 were below detectable limits and these parameters therefore appear to be unsuitable for assessing health in YTK. These parameters were therefore not measured in subsequent blocks.

Values of total bilirubin (BT) were also below the detectable level of 2 $\mu mol.L^{-1}$ at each sampling point in Block 1. Hirazawa et al (2016) reported values of total bilirubin in *Seriola dumerili* of 0.20 to 0.25 $mg.dL^{-1}$, which equates to 3.42 to 4.28 $\mu mol.L^{-1}$, much greater than the current trial. We believe that the method used by Hirazawa was one in which bilirubin is firstly oxidised to biliverdin. As mammals possess virtually no biliverdin in blood then it is valid to report the results of this method as just bilirubin in mammals. Fish, however, have much higher levels of biliverdin than mammals and therefore the values reported by Hirazawa et al (2016) should be more accurately reported as bilirubin + biliverdin. We were interested in measuring bilirubin in YTK due to the fact that it has been demonstrated that taurine plays an important role in the conjugation of bilibrubin in Japanese yellowtail (Sakai et al., 1987). This is unlike mammals where bilirubin conjugates only with glucuronic acid and not taurine. We therefore considered that a taurine deficiency may limit bilirubin conjugation, thereby leading to an increase in bilirubin in the plasma of taurine deficient fish, however this appeared not to be the case, with all fish in this study showing normal (low) levels of bilirubin. Whilst Japanese yellowtail have been demonstrated to conjugate bilirubin with taurine, they can also conjugate with glucuronic acid and this pathway may be upregulated when taurine is deficient. Given the lack of ability to detect BT, a new method was trialled during Block 2 and some lower values became detectable, however we were unable to conduct statistical comparisons between diets in this block and all subsequent blocks as most samples remained below the detectable limit.

PCV values were similar in Blocks 1 and 2 and ranged from 43 to 60%. PCV data from Block 3 are presented in the haematology section below, but were also similar (62 to 63%). No PCV data was obtained for Block 4. There was never any detectable effect of diet or strain on PCV and there was no correlation between fish size and PCV across the full data set. All PCV values measured in this study are higher than recorded for most teleosts, however it is known that pelagic fish have higher PCVs than less active fish. Whilst it is generally considered that PCVs in fish greater than 45% indicate dehydration, there was no evidence of osmotic imbalance or gill damage that would support this and we conclude that these high PCV values are normal for healthy YTK. This is supported by data from Maita et al (2006), who found

PCV values of 47% in Japanese yellowtail fed a high fish meal diet. In the same study, those fish fed a non-fish meal diet had PCVs as low as 33%, indicating anaemia. Hirazawa et al (2016) also found PCV values of 41 to 45% in healthy *Seriola dumerili*.

Values of creatine kinase (CK) were highly variable in all blocks, ranging from 8 to 13314 $U.L^{-1}$ (Table 21). Given this variability it was not surprising that there was a poor correlation between fish size and CK across the whole data set (Appendix 3). As CK can be an indicator of muscle degeneration, we attempted to correlate the level of CK with the degree of flocculation degeneration of muscle seen histologically at the end of the first month of Block 1. This was achieved by assigning scores to the degree of muscular flocculation degeneration of: 0 = none, 1 = mild, 2 = moderate and 3 = severe. The correlation is shown in Figure 68 and whilst it is positive, the R^2 value is poor. Given that YTK exhibit varying degrees of reaction to the anaesthetic bath in terms of thrashing, the large variation in this enzyme may be related to this varying degree of reactivity, as extreme exercise is known to rapidly elevate plasma CK. Creatine kinase therefore appears to be of little use in assessing skeletal damage in YTK.

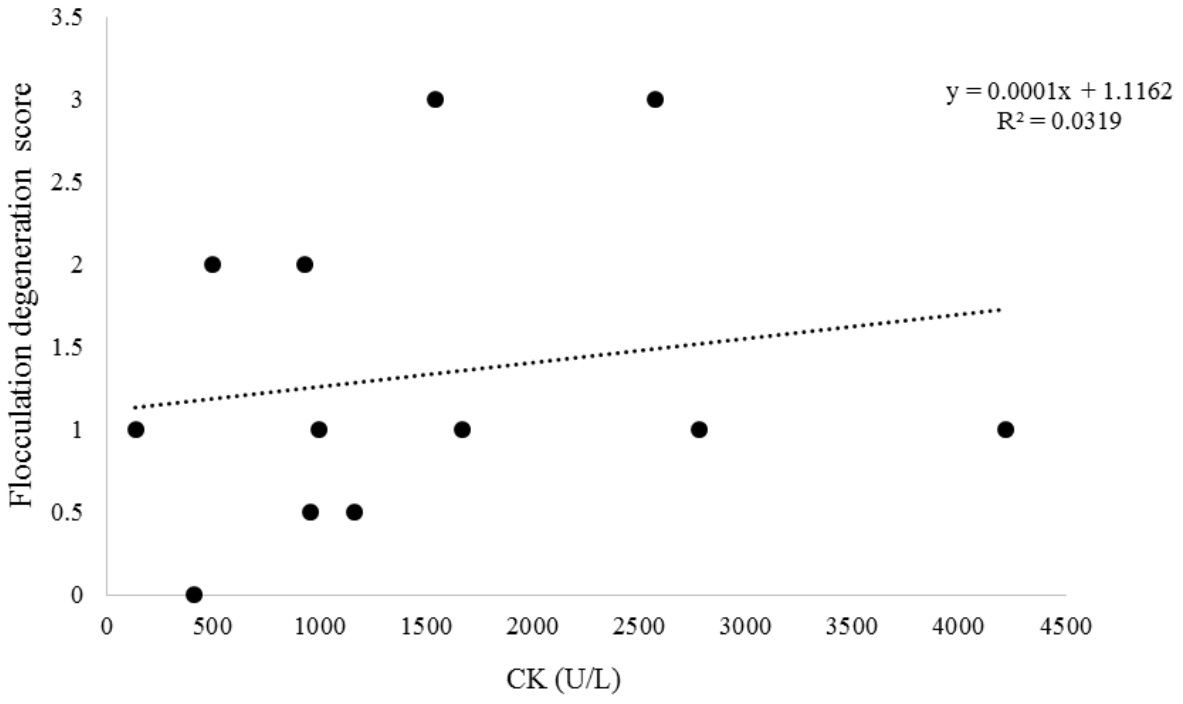

Figure 68: The relationship between plasma creatine kinase (CK) and floccular degeneration in fish from Block 1.

After the first month of Block 1, ALT was found to be affected by strain (P = 0.03) and the interaction of diet and strain (P = 0.02) but not diet alone (P = 0.55). Strain 1 fish had significantly lower ALT (16.3 $U.L^{-1}$) than Strain 2 fish (22.8 $U.L^{-1}$). In terms of the interaction, Strain 2 fish fed Diet A had significantly higher ALT than Strain 1 fish fed the same diet, with no other significant differences between treatments. At the end of the second month, fish from Strain 1 again had significantly lower ALT (18.3 $U.L^{-1}$) than those from Strain 2 (37.5 $U.L^{-1}$). Unlike month 1 there was no effect of diet on plasma ALT. By the end of month 3 there was no effect of strain or diet on plasma ALT. In all other blocks, no effect of diet on ALT was found, but there was some differences between sampling points and values ranged from 14 to 135 $U.L^{-1}$. In Block 2, ALT was affected by month (P = 0.01) but not diet (P = 0.88), however the relationship between month and ALT was not clear, with levels at the end of month 1 (14) being significantly lower than month 3 (43) but no differences between the other months. In Blocks 3 and 4 there was no effect of diet or month on ALT and the correlation between ALT and fish size across the full data set was weak (R^2 = 0.13). The overall average value of ALT across the full data set was 39, with values in seemingly healthy fish ranging from 4 to 394 $U.L^{-1}$ (Table 21).

Hirazawa et al (2016) measured ALT in *Seriola dumerili* between 56 and 410 grams and found levels ranging between 9.6 and 19.1 $U.L^{-1}$, somewhat lower than our overall average of 39 $U.L^{-1}$. Campbell (2012) suggested that interpretation of liver enzymes such as ALT may not be as straightforward in fish as it is in mammals. Elevated ALT in mammals is an indicator of liver disease, however there was no histological evidence in Block 1 that Strain 1 fish had greater liver damage than those from Strain 2. Indeed, the livers from fish fed Diet C were in worse condition than those fed Diets A and B, yet this was not evident in the ALT values of fish fed this diet. Campbell (2012) suggested that the high ammonia levels in fish are likely to lead to changes in the activity of transamine enzymes such as ALT and therefore differences may be due to differences in plasma ammonia concentrations rather than due to liver disease. Strain 2 fish may have had greater plasma concentrations of ammonia during month 1 and 2 of Block 1 due to their greater food intake and more rapid growth which would have resulted in greater protein deamination and ammonia production. However, the slightly positive correlation between fish size and ALT is inconsistent with this, as relative food intake and growth decreases with fish size and therefore ALT would be expected to decrease with fish size if its activity is primarily regulated by ammonia excretion.

There was no effect of diet, strain or time on GLDH in Block 1, however in Block 2, GLDH was only one of two parameters which were found to be effected by diet. Two-way analysis of variance revealed that GLDH was significantly higher in fish from the exDiet C treatment (66 $U.L^{-1}$) than all other treatments (range 25 to 36 $U.L^{-1}$). This parameter was also effected by month, however the relationship between month and GLDH did not follow a consistent pattern. There was no effect of diet or month on GLDH in Block 3, nor any effect of diet in Block 4. There appeared to be no correlation between fish size and GLDH in the full data set, with an overall average of 33 ± 2 (S.E) $U.L^{-1}$, with values ranging from 0.5 to 177 $U.L^{-1}$ (Table 21).

At the end of the first month of Block 1, plasma urea was significantly affected only by diet (P = 0.0005), with those fish fed Diet C having a significantly higher urea (10.6 mM) than Diets A (6.35 mM) and Diet B (6.05 mM), which did not differ from each other. Plasma urea in Diet C fish at the end of the second month had decreased to 8.6 mM and whilst this was still higher than Diets A (6.15 mM) and B (7.45 mM), this difference was no longer significant (P = 0.09). Similarly, at the end of month 3 the plasma urea of fish fed Diet C (9.4 mM) was again the highest of the three diets, but again no longer significantly different to the other two diets (P = 0.3). There was no effect of diet on plasma urea in Blocks 2, 3 and 4 but some differences between sampling points within these blocks, with values ranging from 1 to 12 mM (Table 21). Across the whole data set there was a strong negative correlation between fish size and plasma urea, which is probably due to the rate of protein turnover in smaller fish (Figure 69).

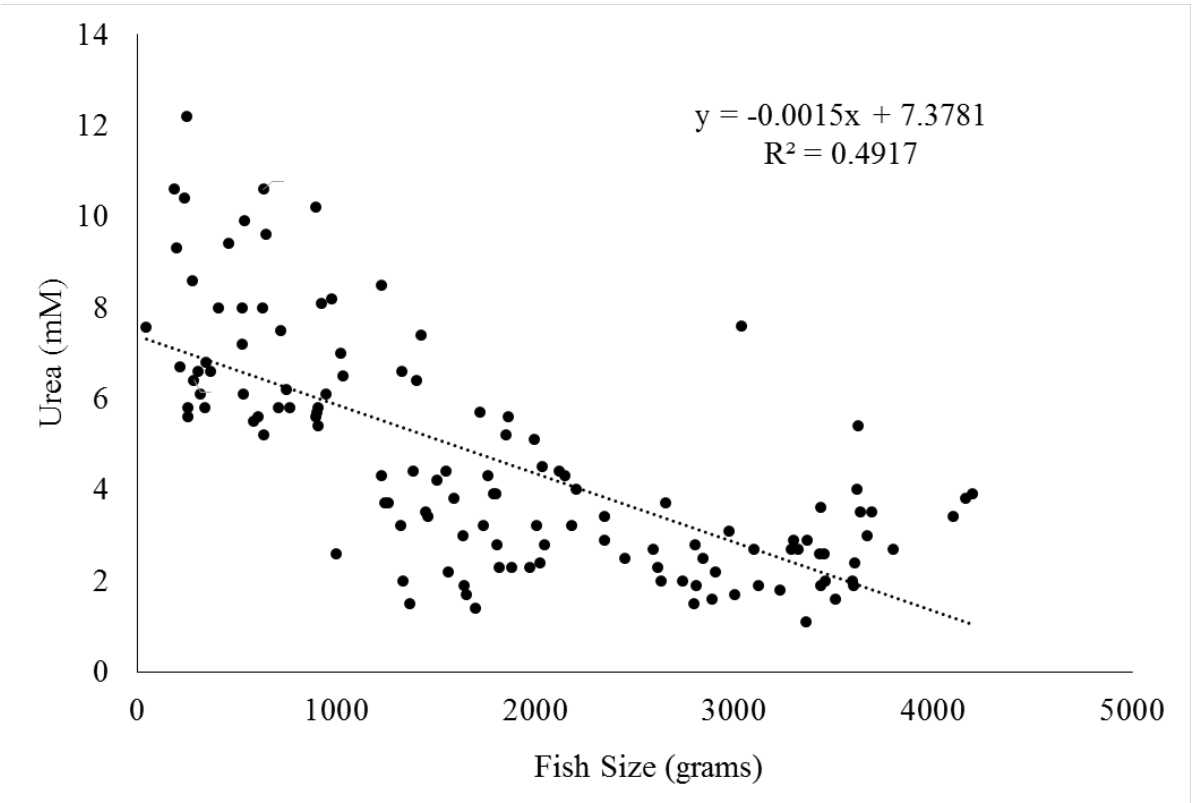

Figure 69: Correlation between fish size and plasma urea in seemlingly healthy fish across the whole data set.

Plasma urea content is used as an indicator of renal disease in mammals, however as fish kidneys play little role in processing nitrogenous waste, the differences in blood plasma urea between dietary treatments are unlikely related to an issue in kidneys. Most urea is excreted by the gills, however there was no histological or other evidence of gill problems with those fish in Diet C which contained significantly elevated urea compared to the other two diets. Ruchimat et al (1997) utilised serum urea nitrogen measurements in the assessment of different protein sources for Japanese yellowtail and found different responses depending on the protein source and inclusion level, but acknowledged that the biochemical significance or mechanism responsible for the differences were not understood. The urea concentrations obtained by Ruchimat et al (1997) were as high as 5 mM in fish fed the highest protein diets (52% crude protein), somewhat lower than obtained in the current study. Hirazawa et al (2016) also measured plasma urea in amberjack *Seriola dumerili* and found control levels ranging from 2.82 to 3.70 mM, again much lower than the current trial. The high levels of urea seen in fish fed Diet C in Block 1 are likely attributable to the high level of arginine in the diets used, as urea is a catabolite of arginine metabolism. Kaushik et al (1988), for

example, reported that the urea-producing pathway in rainbow trout was stimulated when dietary arginine increased above 1.0% of the diet. Given that YTK appear to have a similar requirement for arginine (1.46%) as rainbow trout (see Table 10) then the high levels of arginine in the current diets are likely to be the cause of the high plasma levels of urea. Supporting this theory is the fact that Diet C has the highest level of arginine of the three diets (Table 10) and the highest plasma urea content. The relationship between the dietary arginine content of the nine diets used in Block 1 against the plasma urea content of the fish fed these diets is shown in Figure 70.

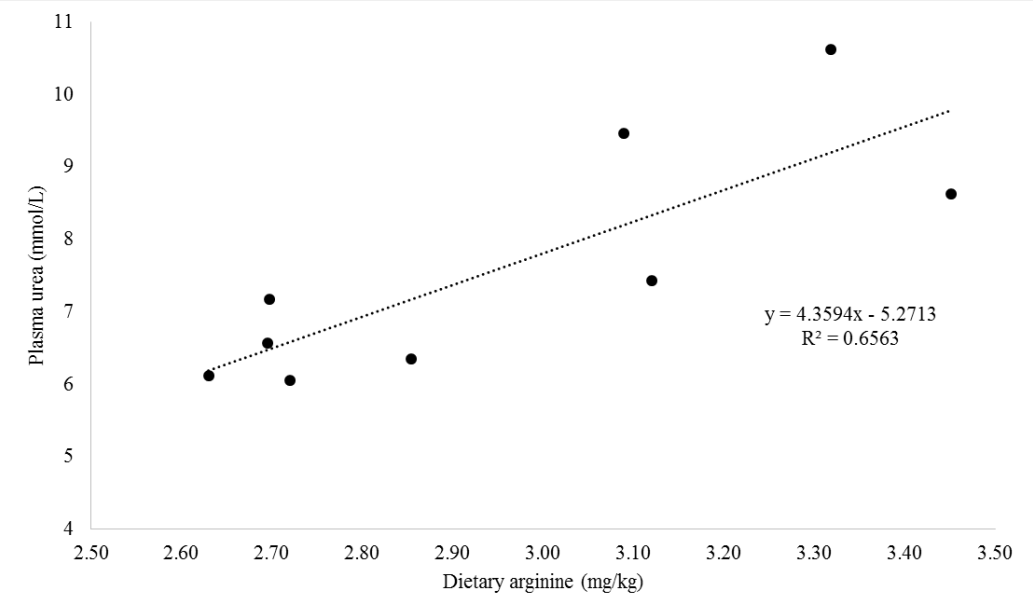

Figure 70: Relationship between dietary arginine and plasma urea in fish from Block 1.

Creatinine was the only other parameter (in addition to GLDH) which was found to be affected by diet in Block 2, with fish fed the diet supplemented with Aquaguard (26 µmM) having significantly lower creatinine than those in the exDiet C treatment (45 µmM). The reasons for these differences are unknown. There was also a significant effect of month on creatinine levels in Block 2 and Block 3, however the patterns were not consistent. The overall correlation between fish size and creatinine in the whole data set was poor.

Plasma cholesterol was significantly affected by diet type ($P<0.0001$) only at the end of months 1 and 2, but not at the end of month 3 ($P = 0.4$) in Block 1. At the end of month 1, fish

fed Diet C had significantly lower cholesterol (2.60 mM) than those fed Diets A (5.95 mM) and B (5.90 mM), which did not differ from each other. At the end of month 2, plasma cholesterol in fish fed Diet C had increased to 3.85 mM, but was still significantly lower than those fed Diets A (6.15 mM) and B (5.58 mM), which again did not differ. At the end of month 3 the plasma cholesterol of fish fed Diet C had increased to 5.1 mM and was no longer significantly different to those in Diet A and B which both had 6.6 mM. As described above, the cholesterol content of the 4 mm and 6 mm pellets of Diet B were much lower than the same pellets sizes in Diet A, yet fish fed these diets all had similar levels of cholesterol in plasma. In Diet C, however, there was an increase in dietary cholesterol with increasing pellet size and this resulted in an increase in plasma cholesterol in fish fed those diets (Figure 71). The low level of plasma cholesterol in fish fed Diet C is likely due to the low level of taurine in Diet C. Taurine plays two important roles in cholesterol metabolism. Firstly it stimulates bile acid production through enhancement of the enzyme cholesterol 7α-hydroxylase and secondly it increases *de novo* cholesterol synthesis by the enhancement of hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA). It therefore appears that increasing dietary cholesterol may assist in increasing plasma cholesterol when the diet is lacking in taurine, however if dietary taurine is adequate then dietary cholesterol does not closely correlate with plasma cholesterol. In Blocks 2, 3 and 4 there was no effect of diet on plasma cholesterol, but some differences between months. There was a positive correlation between fish size and plasma cholesterol across the whole data set, with plasma cholesterol increasing with fish size as shown in Figure 72 . This is the opposite of what is seen in mammalian species, with juveniles typically having higher cholesterol values.

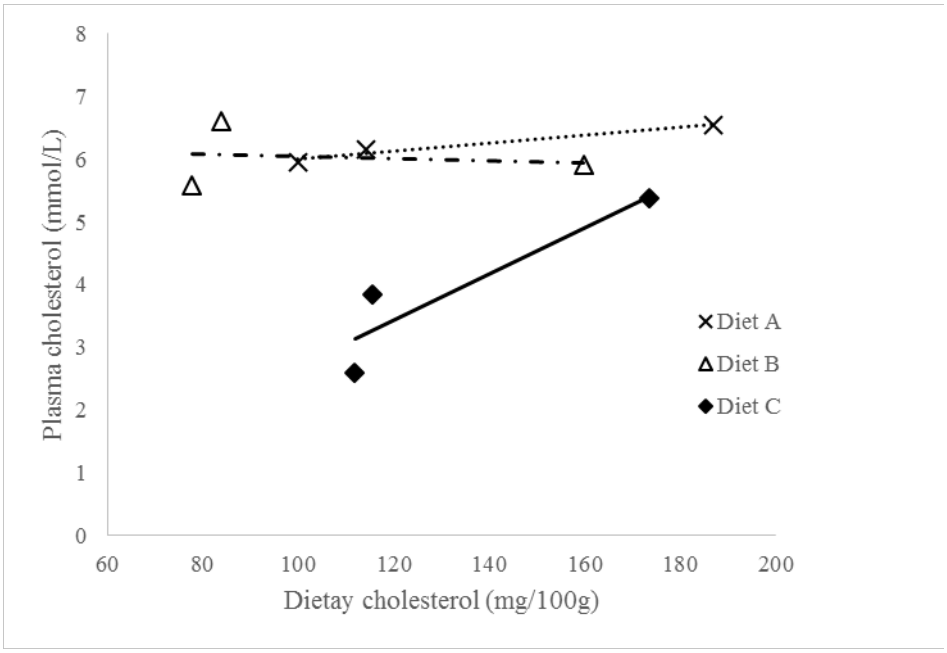

Figure 71: Relationship between dietary cholesterol and plasma cholesterol in fish from Block 1.

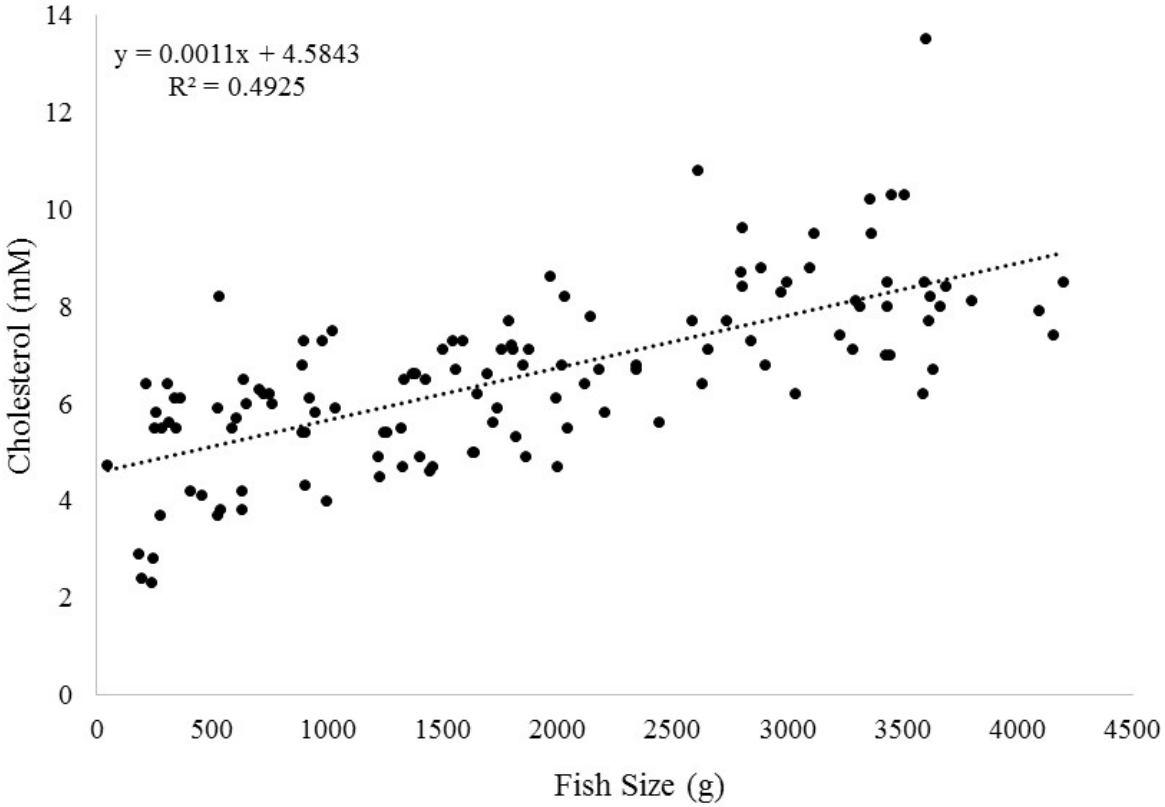

Figure 72: Correlation between fish size and plasma urea in seemlingly healthy fish across the whole data set.

As previously described, the level of cholesterol in fillets measured after maintaining fish on the Block 1 treatment diets for a further 20 weeks was not significantly different between the treatment diets. This is consistent with the improvement in plasma cholesterol over time in fish fed Diet C shown above as a result of an increasing level of dietary cholesterol.

Hypocholesterolemic Japanese yellowtail (i.e. those with a low level of plasma cholesterol) have been demonstrated to be more susceptible to bacterial infections than those with normal levels of cholesterol. Maita (2007) reported that Japanese yellowtail fed a diet containing spoiled sardines and/or oxidised fish oil had significant reductions in plasma cholesterol (3.7 mM) relative to those fed fresh sardines and fresh oil (5.6 mM) and that fish in the former group had significantly higher mortality following bacterial challenge with *Lactococcus garvieae* compared with the latter. Furthermore, Maita et al (1998) reported that Japanese yellowtail challenged with *L. garvieae* exhibited significantly greater mortality if their plasma cholesterol level was <250 $mg.100mL^{-1}$ compared with those which had cholesterol values >275 $mg.100mL^{-1}$. These levels of 250 and 275 $mg.100mL^{-1}$ equate to 6.5 and 7.1 mM, respectively.

D'Antignana et al (2013) reported cholesterol values in wild YTK of 6.56 ± 1.45 mM, very similar to the values obtained in fish feds Diets A and B in Block 1, suggesting that this level should be adequate for cultured YTK. Cholesterol values reported by Maita et al (2006) for Japanese yellowtail fed a 100% fish-meal diet were 6.1 mM, also very similar to that achieved on Diets A and B in the current trial. Those fish fed a non-fishmeal diet with no taurine or cholesterol supplementation for 60 days by Maita et al (2006) exhibited hypocholesterolemia, having a plasma cholesterol level of 2.97 mM. This value is higher than those fed Diet C at the end of month 1. Supplementation of the non-fishmeal diet by Maita et al (2006) to 0.8% taurine (from 0.07% in the non-fishmeal diet) increased plasma cholesterol to 3.78 mM. Given that Diet C had a taurine content of 0.17% suggests that it is neither a 100% fishmeal diet, nor a fishmeal free diet but one with a high level of fishmeal substitution. The very low level of plasma cholesterol in these fish (i.e. lower than Japanese yellowtail fed a non-fishmeal diet) was probably due to their small size relative to those used in the study by Maita et al (2006), as we demonstrated above in Figure 72 that plasma cholesterol increases with increasing fish size. The excretion and reabsorption of bile acids is critical for the digestion and absorption of lipids. Given the role of cholesterol in the formation of bile acids, the hypocholesterolemia seen in these fish would have had impaired lipid metabolism, which would be a major

contributing factor to their poor growth, fatty livers and low fillet lipid content. It should also have led to these fish being much more susceptible to bacterial infection, yet this did not seem to be the case.

The levels of protein, albumin and globulins were never affected by diet. Whilst some differences were seen between months, there were no clear trends in each block, however there was a slight positive correlation between size for all of these parameters as shown in Figure 73. The increase in globulins (and by default total protein, as total protein = globulins + albumin) with fish size may be associated with the transition from immunological naivety, as the fish are exposed to different antigenic challenges and develop a more competent immune system. Further work on smaller fish is warranted to investigate this. The lack of effect of diet on globulin levels in Block 2 is noteworthy as globulins are immune molecules produced by the liver and the immune system and include the complement proteins described below and immunoglobulins. These data therefore suggest no immune benefit of these supplemented diets and this is described in further detail below. A more detailed investigation into globulin types between sick and healthy fish was also conducted and is described in the section below titled ‘Moribund Fish’.

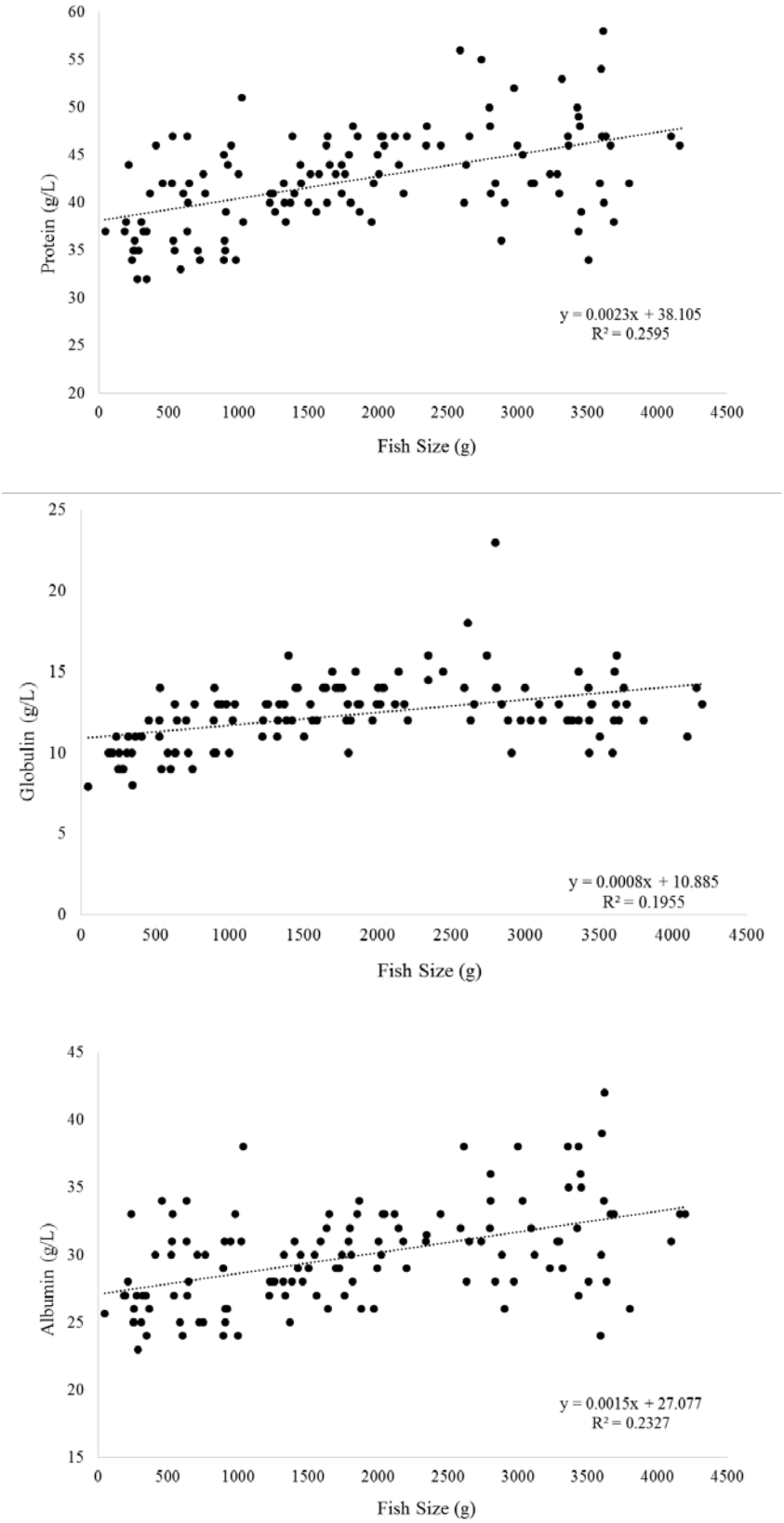

Figure 73: Correlation between fish size and plasma protein, albumin and globulin in seemlingly healthy fish across the whole data set.

The level of electrolytes in the fish was not effected by diet. In Block 2 there was a significant increase in sodium and chloride in the last month of the block. That FCR also increased significantly in this final month may indicate that the fish were investing more energy into osmoregulation than in previous months, however there was no indication of any gill damage at this time. Furthermore the values had dropped back to similar levels in the first month of Block 3 and there was no correlation between sodium and chloride and fish size in the full data set. This suggests that the significantly higher level of blood sodium and chloride at the end of Block 2 was not the result of the fish being larger than in previous months, but some other unknown factor.

Calcium and magnesium were fairly constant throughout the 56 week trial, with no correlation to fish size. When four outlying phosphorus values (>6 mM) were removed from the data set of 126 values, there was a negative correlation between size with an R^2 of 0.36 (Figure 74). This decrease in phosphorus with size is consistent with mammalian species and corresponds with skeletal maturity. In mammalian species, there would also be a similar trend with calcium, however this does not appear to be the case in YTK.

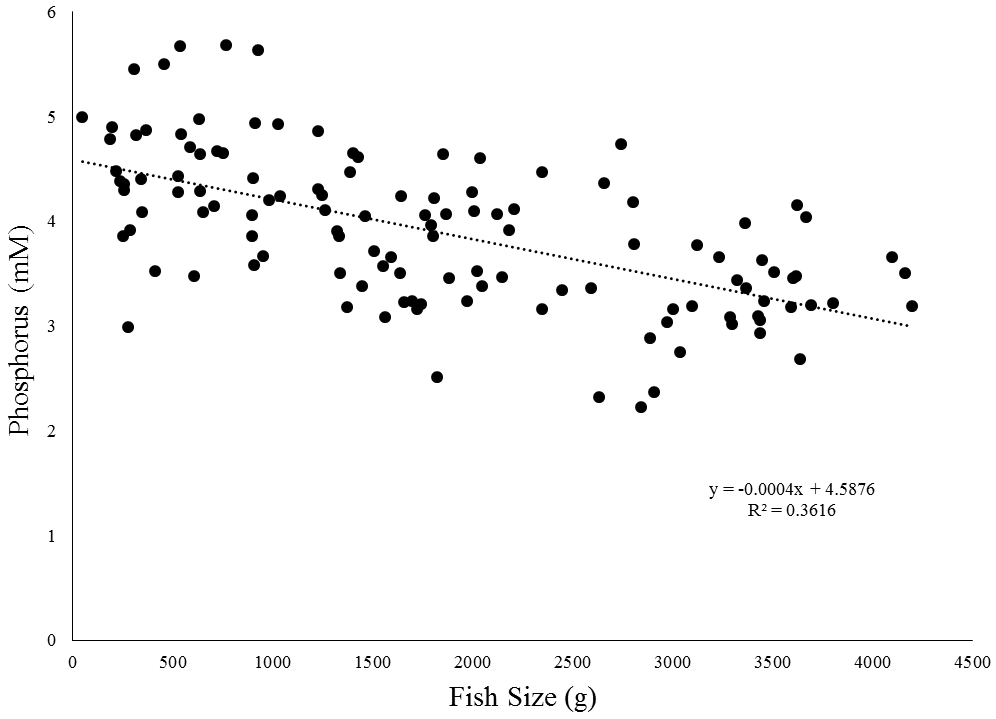

Figure 74: Correlation between fish size and plasma phosophorus in seemlingly healthy fish across the whole data set

At the end of the first month of Block 1 there was no effect of diet on the level of GPx in the plasma of fish (P = 0.18), which ranged from 58.5 to 130.5 $U.g^{-1}$ of Hb. However, at the end of the second month, those fish fed Diet A had the highest level of GPx (69 $U.g^{-1}$ Hb), significantly higher than those fed Diet C (38 $U.g^{-1}$ Hb), but not different to those fed Diet B (58 $U.g^{-1}$ Hb). At month 3, fish fed Diet C were found to have significantly lower plasma GPx (50 $U.g^{-1}$ Hb) than those fed Diet A (70 $U.g^{-1}$ Hb) and Diet B (76 $U.g^{-1}$ Hb), which did not differ from each other. No effects of diet on plasma GPx were found in Block 2 and GPx was not measured in Block 4. In the first month of Block 3 GPx in the fish fed Diet A (63) was significantly lower than those being fed Diet B (79) and Diet D (80), with no other differences in subsequent months. There was no correlation between fish size and plasma GPx across the whole data set.

As described above, the small pellet sizes of Diet A (3, 4 and 6 mm used in Block 1) had been enhanced with additional organic selenium. Le and Fotedar (2014) found that GPx increased with increasing dietary selenium content. All of the diets used in this trial had significantly different selenium contents, however significant differences were not always found in plasma GPx. A plot of plasma GPx versus dietary selenium does however reveal a positive correlation between these two factors (Figure 75). But as previously described, the correlation between dietary selenium and fillet selenium was poor.

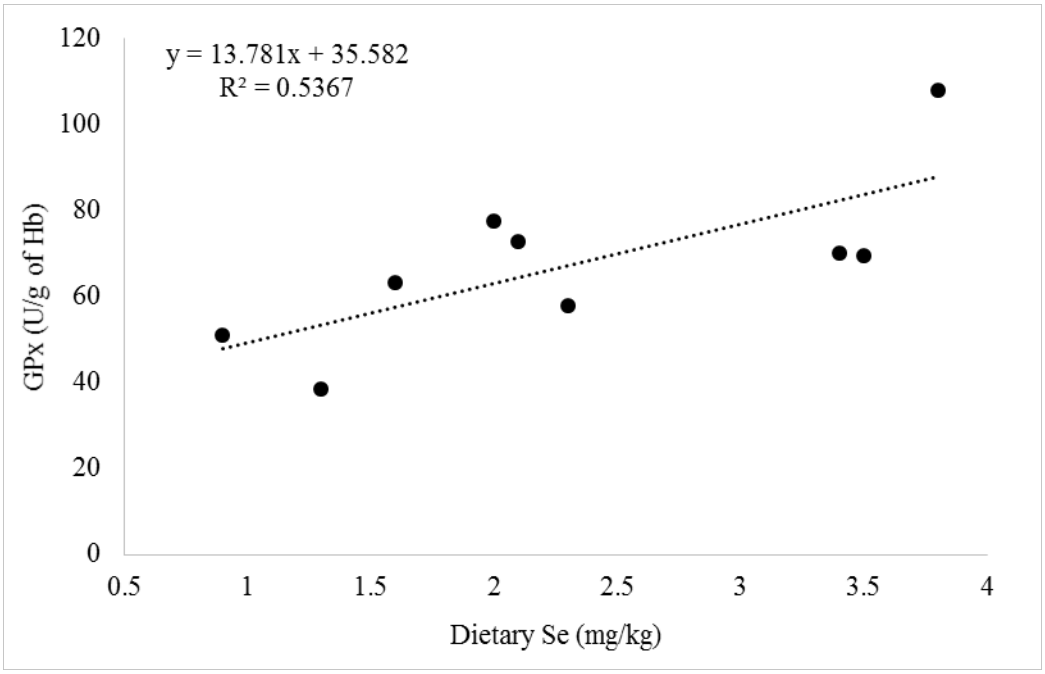

Figure 75: Relationship between dietary selenium and plasma GPx in fish from Block 1.

At the end of the first month of Block 1, haemoglobin measured in the red cell pellet was affected by both strain (P = 0.03) and diet (P 0.001). Fish from Strain 1 had significantly higher haemoglobin in red cells (219 $g.L^{-1}$) than those from Strain 2 (203 $g.L^{-1}$). Fish fed Diet C had the lowest haemoglobin (185 $g.L^{-1}$), significantly lower than both Diet A (210 $g.L^{-1}$) and Diet B (239 $g.L^{-1}$), which were also different to each other. At the end of the second month, fish fed Diet B again had the highest level of haemoglobin (293 $g.L^{-1}$). The haemoglobin of those fish fed Diet C had increased from the previous month's value of 185 $g.L^{-1}$ to 251 $g.L^{-1}$, but this was still significantly lower to both Diet A (286 $g.L^{-1}$) and Diet B (293 $g.L^{-1}$)(P = 0.04). Whilst Strain 1 fish at the end of month 1 were shown to have significantly higher haemoglobin than those from Strain 2, there was no effect of strain on haemoglobin at the end of month 2 (P = 0.79). At the end of month 3, fish fed Diet C had increased their haemoglobin content to 238 $g.L^{-1}$ and two way ANOVA revealed there to be no effect of diet or strain on haemoglobin. Whilst fish fed Diet C had increased their haemoglobin content, those fed Diets A (256 $g.L^{-1}$) and B (220 $g.L^{-1}$) had both decreased slightly from the previous month. The lower haemoglobin content of fish fed Diet C at the end of months 1 and 2 may have been attributed to the slightly lower iron content of this diet (see Table 9) or its significantly lower selenium content, as Le et al. (2013) showed that haemoglobin concentrations decreased with decreasing dietary selenium. This, however, cannot account for the recovery of the haemoglobin content in month 3, as the dietary iron content did not increase in the 6 mm Diet C pellets and the selenium content of these 6 mm pellets was lower than the 3 and 4 mm pellets. The reason for the improvement in haemoglobin over time is therefore not clear, but may be related to the relative reduction in growth rate over time. In Block 2, there was no effect of diet or time on the haemoglobin measured in the red cell pellet, with values ranging from 237 to 281 $U.L^{-1}$, similar to the values obtained in Block 1.

For Blocks 1 and 2 haemoglobin in whole blood was calculated by multiplying the red cell pellet value by (100-PCV). For Block 3, the values of haemoglobin in whole blood came directly from the haematology analyser. Block 1 values could not be statistically compared after month 1 as PCVs were not obtained for all fish. At month 2 and month 3, there was no effect of diet or strain on haemoglobin in whole blood, despite the differences described above for haemoglobin in red pellet. This was due to the higher degree of variation introduced by the conversion. In Block 2 there was no effect of diet on haemoglobin in whole blood and average

values pooled across diets ranged from 107 to 156 $g.L^{-1}$. In Block 3 haemoglobin in whole blood was only measured in month 2 and 3. No effect of diet was found and values ranged from 100 – 115 $g.L^{-1}$. The values we obtained in all blocks (95 to 156 $g.L^{-1}$) were much higher than those reported by Le at al. (2013) in 60 to 70 g YTK, which ranged from 69 to 97 $g.L^{-1}$. They are also much higher than those reported by Mansell et al (2005) in YTK of ~200 gram of 5 to 40 $g.L^{-1}$. These authors acknowledged that these values were very low and attributed these low values to the fact that fish had previously been infested with gill fluke and were therefore anaemic. Our values, however, are similar to those reported by D'Antignana et al (2013) for farmed and wild YTK of 101 ± 15 and 118 ± 24 $g.L^{-1}$, respectively and slightly lower than those reported by Sakai et al (1998) for Japanese yellowtail of 157 $g.L^{-1}$. Combined, our PCV and haemoglobin data demonstrate that none of the fish sampled were anaemic.

4.11. Haematology

At the completion of Block 2, samples of whole blood were analysed for haematology by VetPath services. A range of calibrations and modification to the standard mammalian methods were required. The data from these analyses are presented in Table 22:

Table 22: Haematology parameters measured in YTK at the end of Block 2.

		Diet B +ACT		**Diet B + AG**		**Diet B**		**Diet B_exC**	
Parameter	Unit	Mean	SE	Mean	SE	Mean	SE	Mean	SE
RBC	$x10^{12}/L$	3.5	0.1	3.6	0.1	3.5	0.2	3.9	0.1
HGB	g/L	121.7	6.4	114.3	7.1	114.3	2.5	115.3	6.0
MCHC	g/L	197.2	5.4	189.5	10.2	193.8	6.3	190.3	16.4
PCV	L/L								
MCV	fL	172.0	2.9	161.8	2.9	170.8	2.4	161.0	6.1
MCH	pg	33.6	1.4	30.8	1.9	33.0	8.4	30.0	8.4
Platelets	$x10^{9}/L$	32.8	8.1	15.3	1.9	40.3	8.4	27.7	8.4
WBC	$x10^{9}/L$	10.9	1.7	11.8	1.8	12.5	1.1	10.6	2.0
Neutrophils	% of WBC	6.2	2.1	7.5	1.4	4.0	0.6	23.3	8.1
Lymphocytes	% of WBC	81.7	2.3	84.5	2.3	80.4	3.8	57.8	13.1
Monocytes	% of WBC	12.3	2.5	8.0	0.9	15.6	3.3	19.0	5.9

ANOVA revealed no effect of diet on any of the measured parameters with the exception of the percentage of WBC types, which were significantly different in the DietB_exC treatment, which had significantly more of the WBCs as neutrophils compared with lymphocytes. The reasons for this are not clear.

Measurement of these parameters in the early part of this block, particularly during the mortality event, would have been useful however during the early part of the trial these procedures were still being refined.

Haematology data in Block 3 were only obtained at the end of Months 2 and 3 (Table 23). Two way analysis of variance revealed no effect of diet on any of the measured parameters with the exception of MCHC but a significant effect of time for a number of parameters shown in Table 23. For MCHC, the effect of both diet and time were significant, in addition to the interaction term. This significant interaction term was brought about by the fact that Diet A had equal MCHC in Months 2 and 3, but both Diet B and Diet D had significantly lower MCHC contents in Month 3 than Month 2. A reduction in MCHC can indicate that fish are struggling to make haemoglobin and can be an early sign of anaemia and indeed fish fed Diet B did experience a decline in haemoglobin in the third month. Whilst two-way ANOVA found no effect of diet or the interaction of diet and month, the leverage plot clearly demonstrates that Diet B fish had a much lower level of Hb in Month 3 than those from Diet A and D. Likewise, one-way ANOVA comparing Hb contents between diets only in Month 3 suggests a highly significant difference in Hb between diets ($P = 0.01$), with fish fed Diet B having significantly lower Hb than both other diets. The lower Hb concentration of fish fed Diet B in Month 3 is consistent with the very low iron content of the 9 mm Diet B offered in this month of the trial.

Table 23: Haematology parameters measured in YTK during Block 3.

Parameter	Unit	*P*	Month 2	Month 3
RBC	$x10^{12}$/L	0.18	4.1 ± 0.1	3.9 ± 0.1
HGB	g/L	0.02	114.9 ± 4.5[a]	100.3 ± 4.1[b]
MCHC	g/L	0.00004	186.0 ± 4.5[a]	160.0 ± 6.9[b]
PCV	%	0.78	62 ± 2	63 ± 1
MCV	fL	0.04	169.1 ± 1.7[a]	164.6 ± 5.6[b]
MCH	pg	0.005	27.9 ± 0.6[a]	25.2 ± 1.0[b]
Platelets	$x10^{9}$/L	0.72	28.9 ± 4.6	25.5 ± 6.9
WBC	$x10^{9}$/L	0.08	4.7 ± 0.7	9.2 ± 2.1
Neutrophils	% of WBC	0.06	12.1 ± 2.0	5.4 ± 2.0
Lymphocytes	% of WBC	0.07	75.3 ± 2.3	83.6 ± 3.9
Monocytes	% of WBC	0.56	12.3 ± 1.7	10.6 ± 2.9

4.12. Gut Microbiome

These data from Block 1 gut samples have been provided for this report by Dr Sarah Catalano and Dr Andrew Oxley of SARDI.

Out of the nine gut samples that were submitted to SARDI (three replicates per dietary treatment), only five positively amplified with the primers and were sent for sequencing. These samples were two of the Diet A replicates and all three of the Diet C replicates. Due to the low number of replicates, it was difficult to perform any statistical analyses however there did appear to be a difference in the microbiome between the two diets

In general, the Diet C samples clustered separately to the Diet A samples, however one Diet C sample was more similar to the Diet A grouping. However, as noted above results are somewhat difficult to interpret due to low sample sizes within the two diets groups (n=2 and n=3).

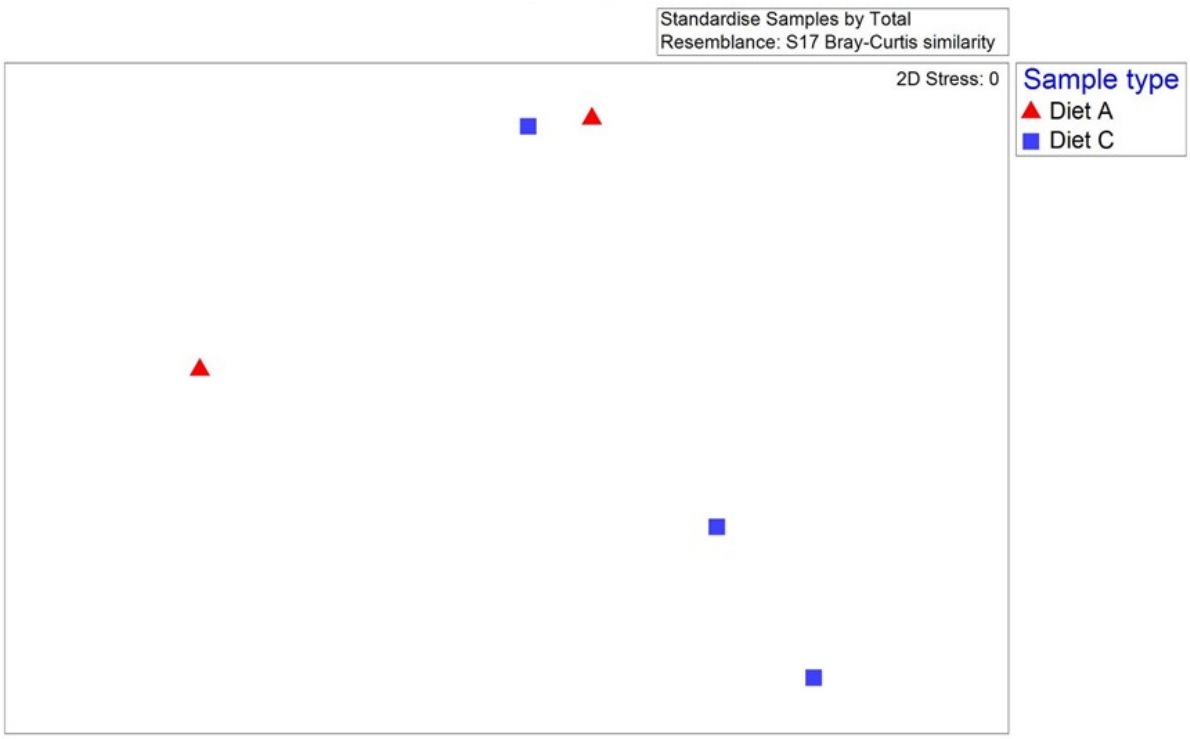

Figure 76: Difference between global bacterial community structure of 5 diet samples from Block 1 (combined mid- and hindgut scraping) as analysed by nMDS

The bacterial phyla associated with both diet types was similar, with particular dominance by representatives of the Proteobacteria, Bacteroidetes and Fusobacteria. There was also significant representation by bacterial taxa from the phyla Actinobacteria for one of the Diet A samples. These findings are similar to those recorded in the literature for other fish species, which highlight the dominance of microbes from the Proteobacteria (Sullam et al., 2012). Other studies also report phyla Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Fusobacteria as residents of the fish gut community (Gajardo et al., 2016). Whilst all nine phyla listed on the figure legend are present in at least one of the five samples, their contribution to the overall taxa are very low and therefore indistinguishable on the graph.

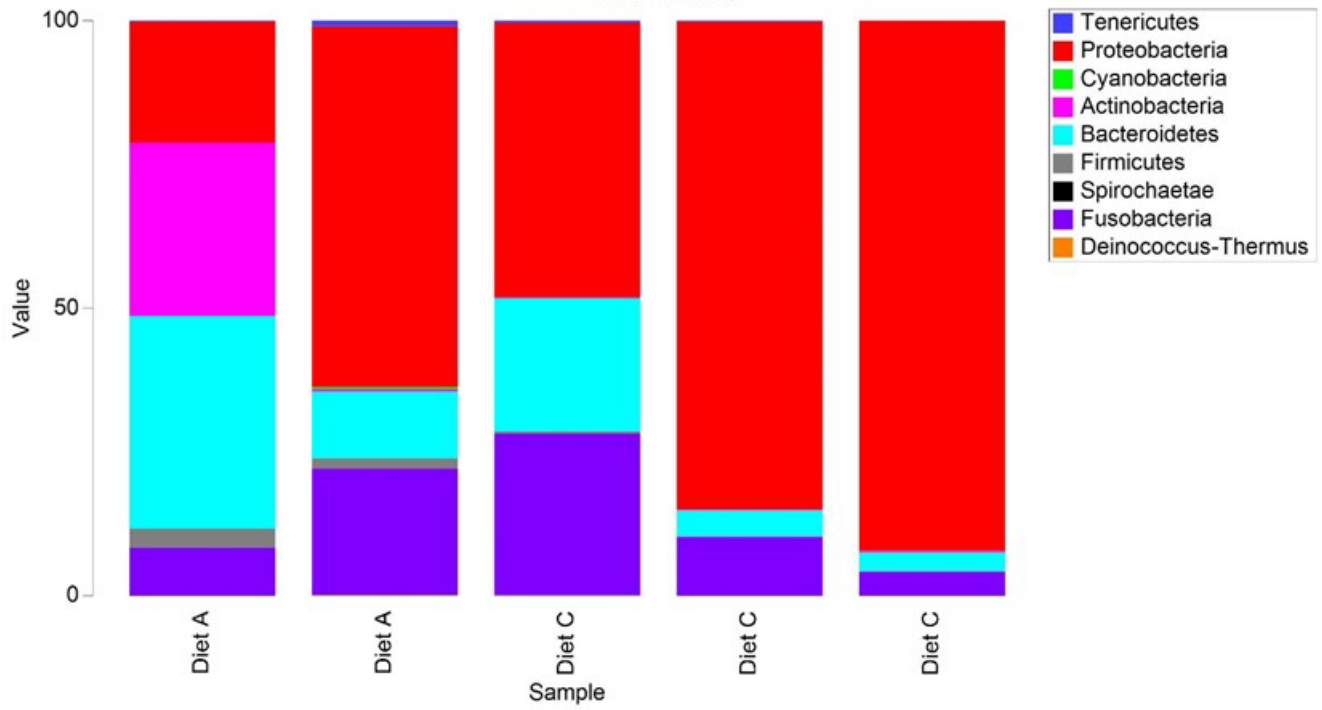

Figure 77: Relative percent abundance of bacterial phyla associated with the gut scrapings of fish from Block 1 across two diet types.

In general for this feed analysis component, we have noted that cultivation is a driving factor that influences the gut microbiome, with significant differences seen in previous samples analysed under different aspects of this K4P project between tank versus sea-cage samples, and that certain feeds present a more consistent microbiome with greater evenness and diversity, thus possibly providing the capacity to contribute to a greater array of functions which may support growth and/or performance. Whilst one sample of the marine fish Diet A appeared to have more evenness

It is clear from this analysis that more replicate samples would be required to elucidate significant differences between treatments and that we need to allow for samples that fail to amplify

4.13. Moribund Fish

The majority of mortalities during Block 1 occurred overnight and therefore were not sampled for fish health assessment. A 282 gram moribund fish with skin lesions was sampled on 4th January 2016. This fish had nothing of significance histologically. A bacterial swab of the skin lesion revealed *Vibrio harveyi, V. alginolyticus*, a *V. tubiashii*-like bacterium and

Tenacibaculum discolour. No bacteriology on organs was conducted. Many of the measured blood parameters of this fish fell outside of the range of normal values determined in healthy Block 1 fish and these differences are shown in bold in Table 24 below. The higher than normal PCV in the moribund fish and imbalance in electrolytes is likely the result of dehydration. The low levels of cholesterol, urea, protein, albumin and globulin are noteworthy and may suggest that the fish had not been feeding. Data from more moribund fish is required to increase our understanding of the pathogenesis of different conditions and types of sick fish and determine how these differences are reflected in blood chemistry.

Table 24: Blood parameters from a single moribund fish compared with the range of normal values found in fish at month 1 (from Table 15)

	Healthy	Moribund
PCV	**43 -56**	**60**
CK	**527 - 1698**	**1708**
ALT	10 - 29	21
GGT	<1	<1
GLDH	24.5 - 46.0	41.6
BT	<2	<2
UREA	**5.7 - 10.8**	**2.4**
CREAT	**15.5 - 21.0**	**11.0**
BOHB	< 0.1	< 0.1
CHOL	**2.6 - 6.3**	**1.1**
Na	**193 - 196**	**226**
K	**4.4 - 6.4**	**9.1**
Cl	**164 - 169**	**220**
PROT	**34 - 41**	**12**
ALB	**9.5 - 10.5**	**3**
GLOB	**24 - 30**	**9**
Ca	**3.58 - 3.78**	**3.31**
P	**4.11 - 5.16**	**5.19**
Mg	**1.19 - 1.99**	**2.55**
GPx U/g Hb	63 - 131	102
Hb g/L	178 - 251	211

As previously noted, sick (n = 4) and healthy (n = 8) fish were sampled during the Block 2 mortality event (29th March 2016). Routine biochemistry (Table 25) revealed that the only difference between sick and healthy fish on this occasion was haemoglobin in the red cell pellet (P = 0.02), with sick fish having significantly lower Hb (239 ± 11 g.L^{-1}) than healthy fish (276 ± 8 g.L^{-1}); suggesting the sick fish were slightly anaemic. This difference wasn't reflected in the PCV (P = 0.63), which is probably a less sensitive indicator of anaemia.

Table 25: Blood biochemical parameters in sick and healthy fish from the mortality event in Block 2 (pooled across diets).

		P	Sick	Healthy
PCV	%	0.63	46.5 ± 2.3	48.5 ± 3.3
CK	U/L	0.39	512 ± 431	979 ± 305
ALT	U/L	0.21	10.2 ± 3.4	15.8 ± 2.4
GLDH	U/L	0.43	31.5 ± 5.6	25.9 ± 3.9
UREA	mmol/L	0.72	5.4 ± 0.8	5.7 ± 0.5
CREAT	μmol/L	0.59	31.7 ± 10.6	38.8 ± 7.5
CHOL	mmol/L	0.38	5.1 ± 0.4	5.6 ± 0.3
Na	mmol/L	0.35	196 ± 4	191 ± 3
K	mmol/L	0.92	4.7 ± 0.7	4.8 ± 0.5
Cl	mmol/L	0.4	166 ± 4	162 ± 3
PROT	g/L	0.2	42.2 ± 1.8	39.2 ± 1.3
ALB	g/L	0.4	12.0 ± 0.6	11.3 ± 0.4
GLOB	g/L	0.15	30.2 ± 1.3	27.8 ± 0.9
Ca	mmol/L	0.31	3.56 ± 0.08	3.44 ± 0.06
P	mmol/L	0.47	3.91 ± 0.39	4.27 ± 0.28
Mg	mmol/L	0.53	1.90 ± 0.26	1.68 ± 0.18
GPx	U/g of Hb	0.06	61.7 ± 5.4	75.7 ± 3.8
$Hb_{(red)}$	g/L	**0.02**	239 ± 11	276 ± 8
$Hb_{(w)}$	g/L	0.05	122 ± 9	148 ± 6

Gel electrophoresis was also conducted on serum from these sick and healthy fish to differentiate globulin types. These data are presented in Table 26 and demonstrate that sick fish had significantly higher total protein (P = 0.03), total globulins (P = 0.05) as well as higher β2 globulins (P = 0.03) and γ globulins (P = 0.04) than healthy fish. This is the expected response, as the largest fraction of the γ globulins are immunoglobulins which are used to fight infection. Interestingly, however, when analysed via routine biochemical methods, the differences in total protein and total globulins were not significant (P = 0.20 and 0.15, respectively Table 25); the reason being that electrophoresis is more accurate (but also significantly more expensive) than the biochemical methods. This therefore demonstrates that electrophoresis should be the preferred method when looking for differences in immune response. Another interesting finding that arose from the comparison between gel electrophoresis and routine biochemistry was the significantly higher level of albumin measured via the former method (Figure 78). The reason for this difference is that the routine

biochemical method measures albumin by colorimetric means, following binding of the albumin to bromocresol green. This dye does not bind as predictably with albumin from non-mammalian species (birds, reptiles and fish) and for this reason some laboratories have stopped reporting albumin in non-mammalian species.

Table 26: Comparison of protein, albumin and various globulin fractions in sick and healthy fish from Block 2.

		P	Sick	Healthy
Protein	g/L	**0.03**	46.0 ± 0.0	38.3 ± 1.6
Albumin	g/L	0.23	4.95 ± 0.55	4.33 ± 0.06
Total globulins	g/L	**0.05**	41.05 ± 0.65	34.00 ± 1.70
α1 globulins	g/L	0.21	11.23 ± 0.35	9.43 ± 0.86
α2 globulins	g/L	0.9	12.15 ± 1.65	11.90 ± 1.07
β1 globulins	g/L	0.24	8.10 ± 0.70	5.83 ± 1.13
β2 globulins	g/L	**0.03**	7.05 ± 0.05	5.13 ± 0.38
γ globulins	g/L	**0.03**	2.50 ± 0.10	1.70 ± 0.15

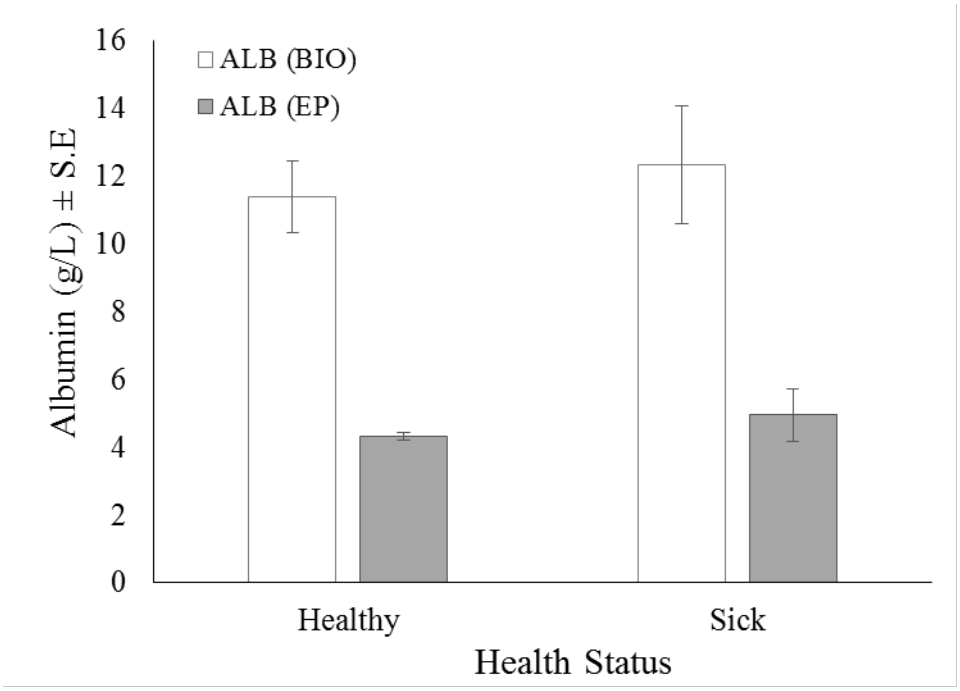

Figure 78: Differences in plasma albumin values between biochemical and electrophoretic methods.

4.14. Challenge Trials, Immunology and Flow Cytometry

No fish died, or showed signs of bacterial infection, during either of the Block 1 live challenge trials despite the very high concentrations of bacteria used or the various administration routes tested. This could suggest that either the culture process of the bacteria resulted in the loss of virulence, or that the fish were effectively able to fight off this secondary pathogen. Unlike *Photobacterium damselae* subsp *piscicida* which is a primary pathogen with known virulence factors, those of *P. damselae* subsp *damselae* are presently unknown. Natural antibody production in these large fish may have increased to such a level that enabled the fish to combat the challenge, particularly as they had been exposed to the bacteria previously in the experimental tanks. PCV values of the surviving fish ranged from 46% to 51%, demonstrating that they were not anaemic despite being exposed to haemolytic bacteria. At the end of the challenge trials, *P. damselae* subsp *damselae* was isolated from the gut of four of the six fish sampled, with a *Vibrio* species similar to *Vibrio ponticus* isolated from the gut of the other two fish. In addition the gut of all of these fish also contained other unidentifiable bacteria, and in three cases, *Aliivibrio fisheri*. This is in contrast to the fish sampled from the nutrition trial which typically contained a monoculture of bacteria in their gut. No *P. damselae* subsp *damselae* was isolated from the kidney or liver of any of the six fish sampled, however an unidentifiable bacteria was isolated in both organs from all fish. One of these fish did not contain this unidentifiable bacteria in these organs but both organs did contain two *Vibrio* species and another unidentifiable bacteria that was different to that found in the other five fish. This same fish also had a lesion on its skin and a swab of this lesion revealed the same two species of bacteria that were isolated in its kidney and liver.

The purpose of these initial challenge trials was to determine a dose of bacteria that would affect 50% of the population (ED_{50}) of healthy fish prior to conducting a more detailed study on the effects of the three diets on fish immunity. As an ED_{50} could not be determined, the final study was not undertaken. Had we included fish from the Diet C treatment in these preliminary challenge trials they may have experienced some effect as they were taurine deficient and hypocholesterolaemic and their diet contained a low level of selenium. However, these fish did not exhibit higher mortality than those fed Diets A and B in the tank trial despite having been demonstrated to be naturally infected with this bacterium.

The response of fish exposed to 7 x 10^8 CFU of heat-killed *Streptococcus iniae* in terms of lysozyme and complement activity are shown in Figure 79 and Figure 80, respectively. Two-way analysis of variance revealed significant effects of diet (0.001) and time (<0.0001) on lysozyme activity, but not the interaction term (P = 0.74). Those fish fed Diet C had significantly lower lysozyme activity (111 $U.mL^{-1}$) than those fed Diet A (204 $U.mL^{-1}$) and Diet B (230 $U.mL^{-1}$), which did not differ. In terms of time, there was no difference in lysozyme activity between treatments at t = 0 (181 $U.mL^{-1}$) and t = 4 (174 $U.mL^{-1}$), but lysozyme at 24 hours post challenge (282 $U.mL^{-1}$) was significantly higher than both earlier times. These data demonstrate that fish fed Diet C began with a lower lysozyme activity and as a result their response to the bacterial challenge was not as strong as those in Diet A and Diet B.

In all treatments, complement activity dropped 4 hours post challenge and then recovered to pre-challenge values, however there were no significant effects found for diet (P = 0.13) or time post challenge (P = 0.10) on complement activity.

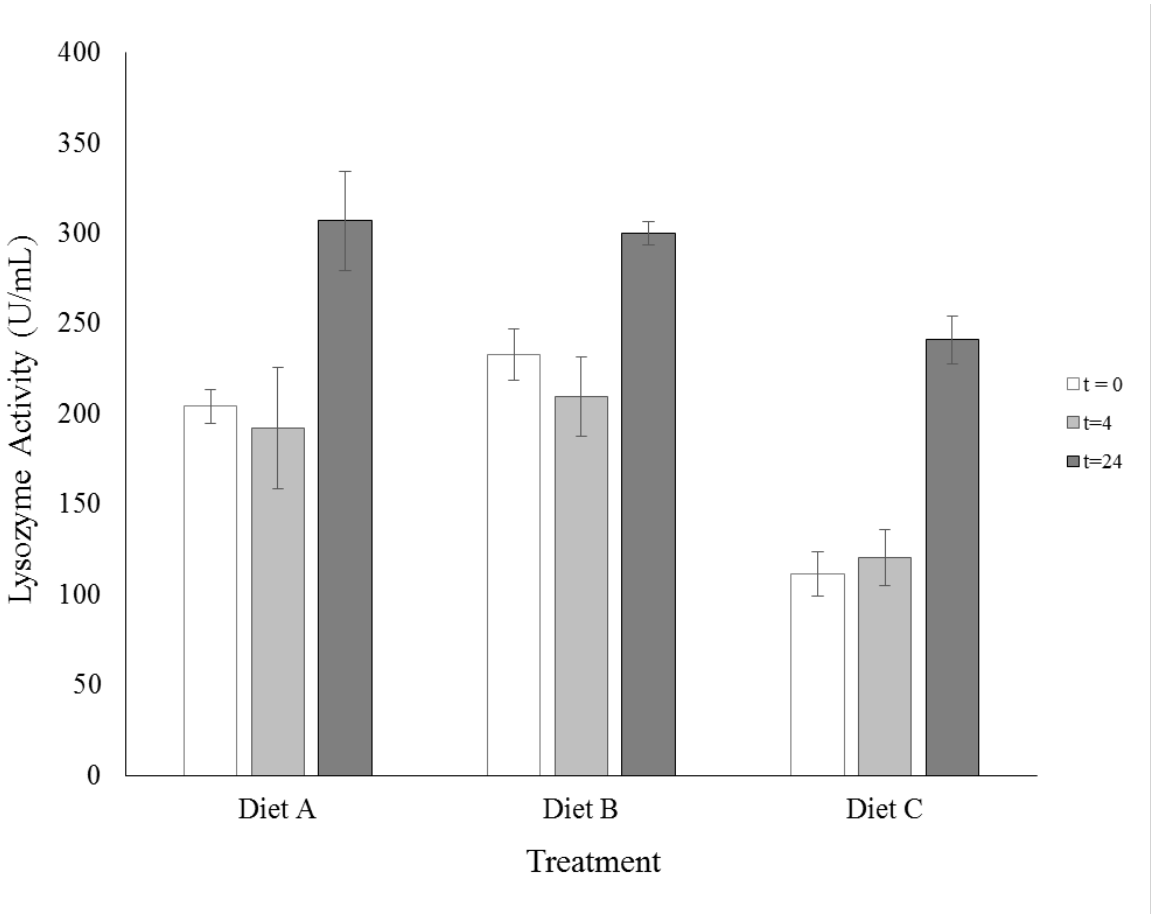

Figure 79: Lysozyme activity in serum of fish fed Block 1 diets at three different times following challenge with heat-killed *Streptococcus iniae.*

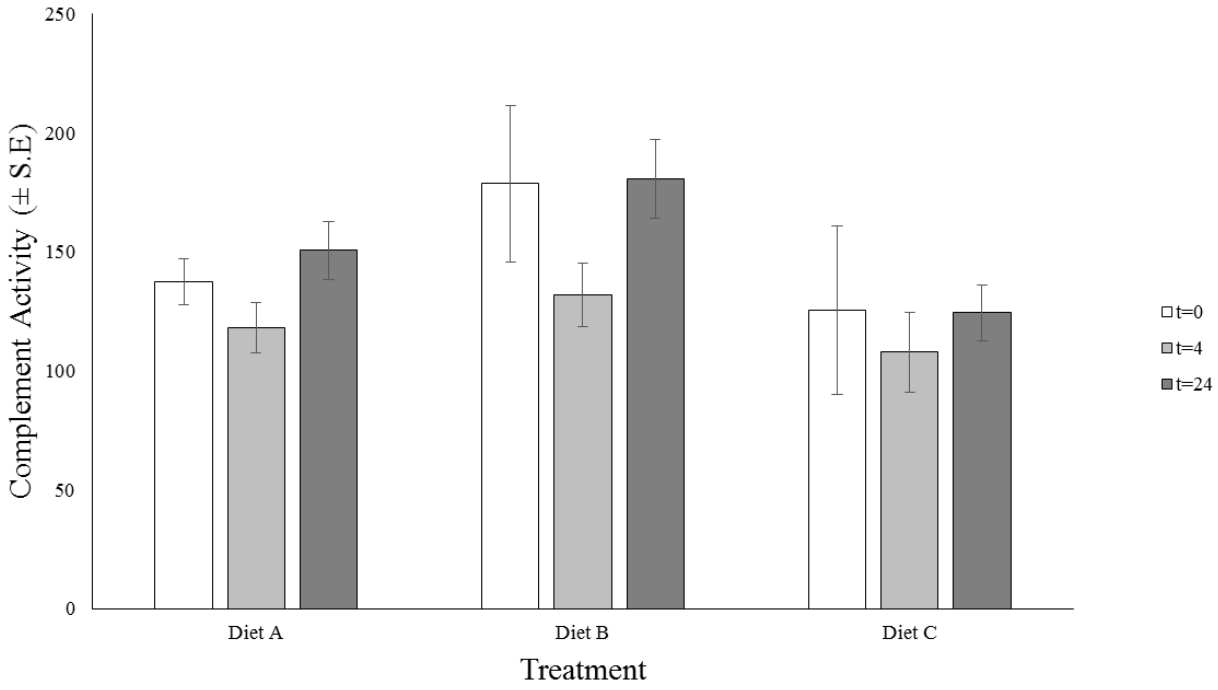

Figure 80: Complement activity in serum of fish fed Block 1 diets at three different times following challenge with heat-killed *Streptococcus iniae*.

At the conclusion of Block 2, lysozyme and complement analyses were performed to determine the effect of immunostimulant addition to Diet B and these data are presented in Figure 81 and Figure 82. ANOVA revealed there to be no effect of diet on lysozyme activity ($P = 0.07$) or serum complement activity ($P = 0.46$). The patterns in the data, however, were consistent with the more in depth flow cytometery methods, which did reveal significant differences. These differences are discussed in more detail below.

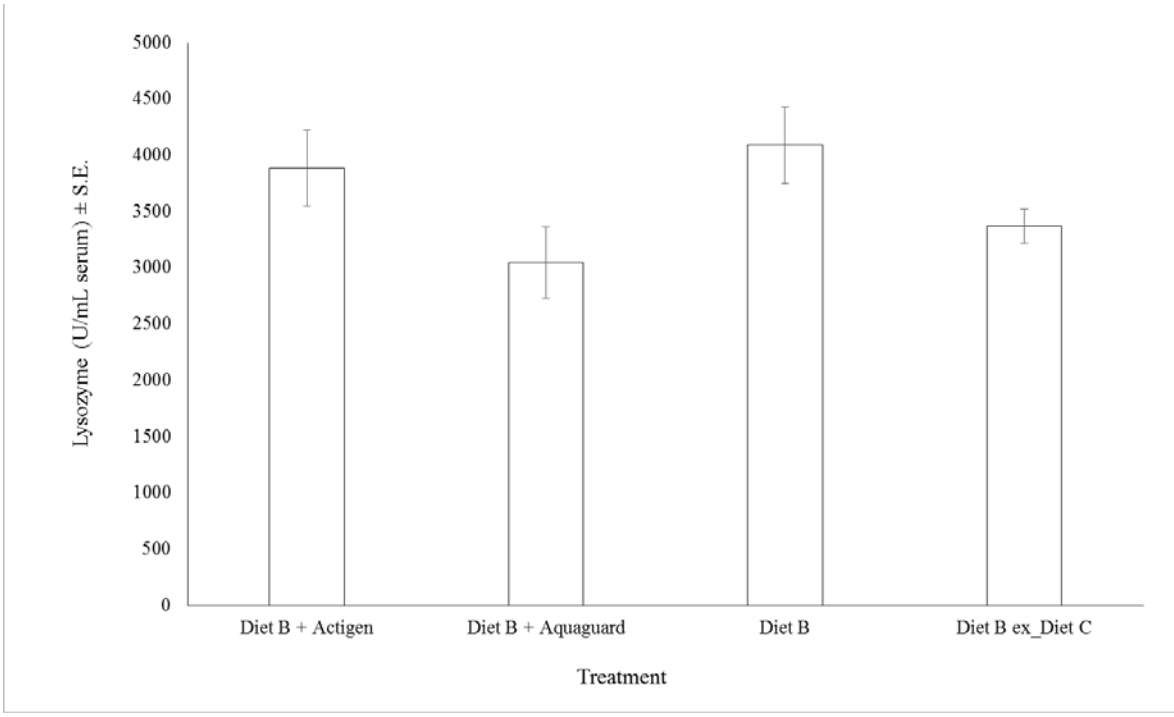

Figure 81: Lysozyme activity in the serum of fish fed various diets during Block 2.

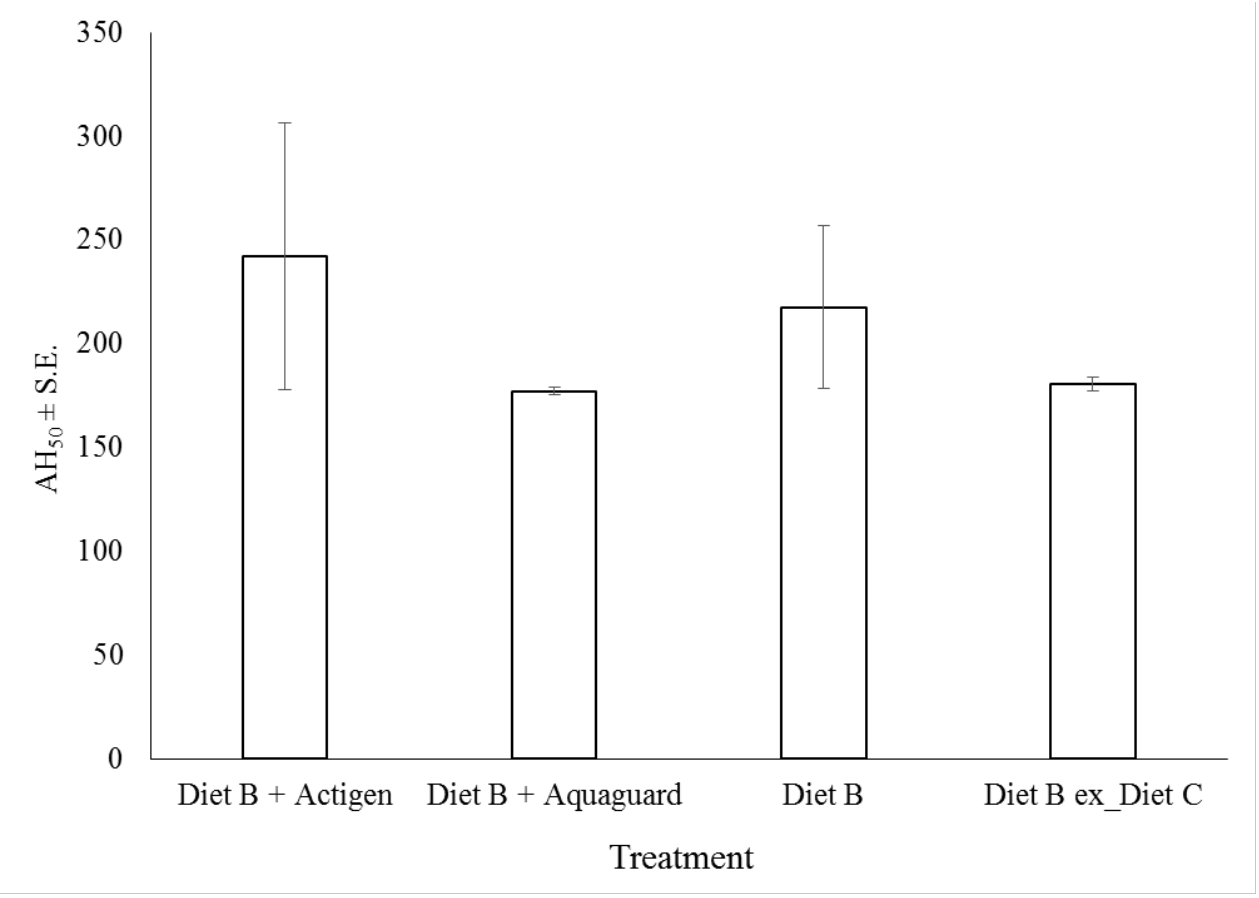

Figure 82: Complement activity in the serum of fish fed various diets during Block 2.

Lysozyme was measured at the end of month 1 and month 3 in Block 3 and on both occasions there was no difference in activity between treatments, with values ranging from 272 to 328 $U.mL^{-1}$. Similarly, at the end of Block 4 there was no difference ($P = 0.58$) in lysozyme activity between fish fed Diet B (212 ± 32 $U.mL^{-1}$) and Diet D (244 ± 48 $U.mL^{-1}$).

Complement activity at the end of the first month of Block 3 was equal between treatments (range 92 to 125 $U.mL^{-1}$)($P = 0.53$). Similarly at the end of Block 4 there was no difference ($P = 0.79$) in complement activity between Diet B (112 ± 14 $U.mL^{-1}$) and Diet D (122 ± 27 $U.mL^{-1}$).

Flow cytometry identified two main populations of leucocytes in the cell cultures isolated from blood taken at the end of Block 2, based on their size and cell complexity (Figure 83). The majority of cells were in the P2 population (87.2%), with 12.6% in the P1 population. D'Antignana et al (2013) reported that $73 \pm 6\%$ of leucocytes were lymphocytes in farmed yellowtail kingfish and $21 \pm 10\%$ were granulocytes (a combination of neutrophils or heterophils). Monocytes comprised ca. 5% of the leucocytes and basophils were not found. We aim to more accurately identify the types of leucocytes within these two populations as our work with flow cytometry continues, however the complexity of the P1 cells suggests these leucocytes to comprise the more mature cells which contain granules such as mature macrophages and neutrophils, which is consistent with the data from D'Antignana et al (2013). Further cross-referencing of the flow-cytometry data with manual white blood cell counts performed on blood smears suggests that the P2 cell population is a mixture of leucocytes (neutrophils and heterophils) and thrombocytes (platlets).

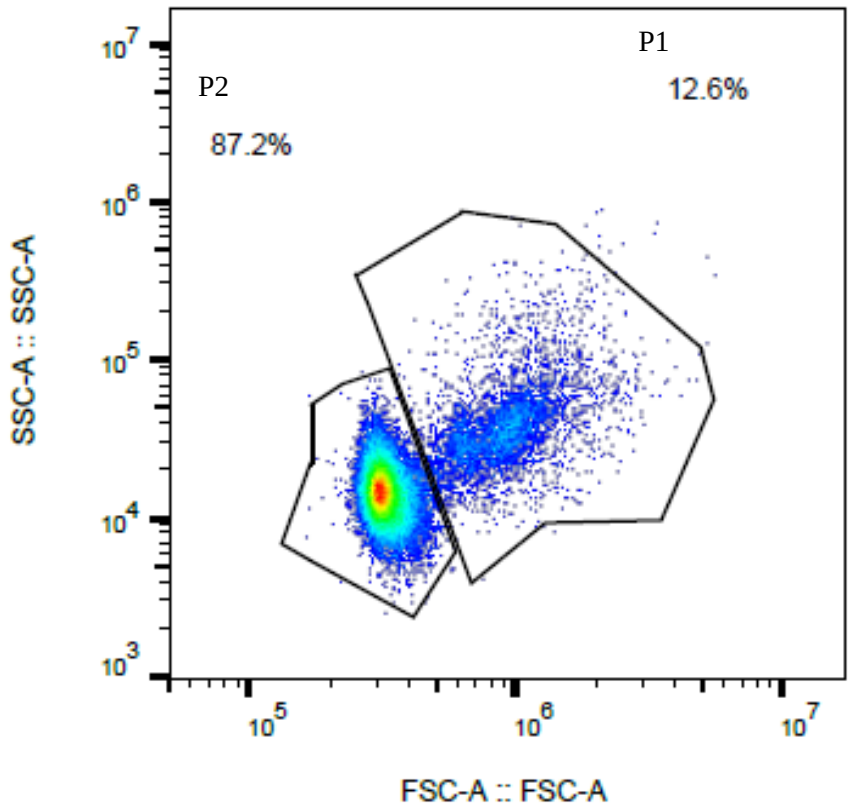

Figure 83: Cytogram showing two populations of leucocyctes in yellowtail kingfish.

Due to a delay in the availability of the *Streptococcus* bacteria for challenging the fish leucocytes, the samples were firstly analysed fresh (after being incubated in culture media for 18 hours) without challenging them with bacteria and then again 8 days later with the dead and live bacteria. The percentage of dead cells in each of these three tests is shown in Figure 84. The number of dead cells in the fresh sample was much lower (range 6 to 18%) than those stored in cell culture media for 8 days (range 40 to 67%). We have therefore not directly compared the results obtained in fresh samples against those challenged later.

There was a significant effect of diet on the percentage of dead cells in the fresh cell sample and also in the cells challenged with live bacteria after 8 days in culture media. In the fresh samples there were significantly more dead cells in those fish fed Diet B supplemented with Aquaguard than all other treatments. Those fish fed Diet B with no supplementation had the lowest number of dead cells, significantly lower than those fed on Diet B + Aquaguard and

those in the exDiet C treatment. These data suggest that the continuous supplementation with Aquaguard used in this trial had a negative impact on the cells, decreasing their viability.

To understand the intracellular oxidative activity and the lysosomal presence data in the present study, the process of how the host fights against an infection and the process of phagocytosis is briefly explained here. Once bacteria pass the host barrier, they face the first line of host defence which is phagocytosis by neutrophils and macrophages. In macrophages, the fusion of the vesicle containing the internalised microorganism with endosomes decreases the pH, resulting in a harsh and lytic environment that kills and degrades the bacteria (Baumgartner et al., 2014). Ultimately, maturing phagosomes fuse with lysosomes becoming phagolysosomes (Van Der Vaart et al., 2012). Lysosomes are membrane-delimited organelles with an acidic internal pH (≤5). These organelles contain active proteases and lipases, hydrolytic enzymes, and bactericidal peptides and have the ability to generate toxic oxidative compounds that help microbial degradation (Tapper, 1996) (Van Der Vaart et al., 2012). These compounds help degrade phagocytosed microbes (Roos and Winterbourn, 2002). Consequently, an increase in the lysosomal presence (given by the dye lysotracker) as well as an increase of the intracellular oxidative activity (given by the dye DCFH-DA) indicates that the host is immunocompetent and has built an immune response against a pathogen.

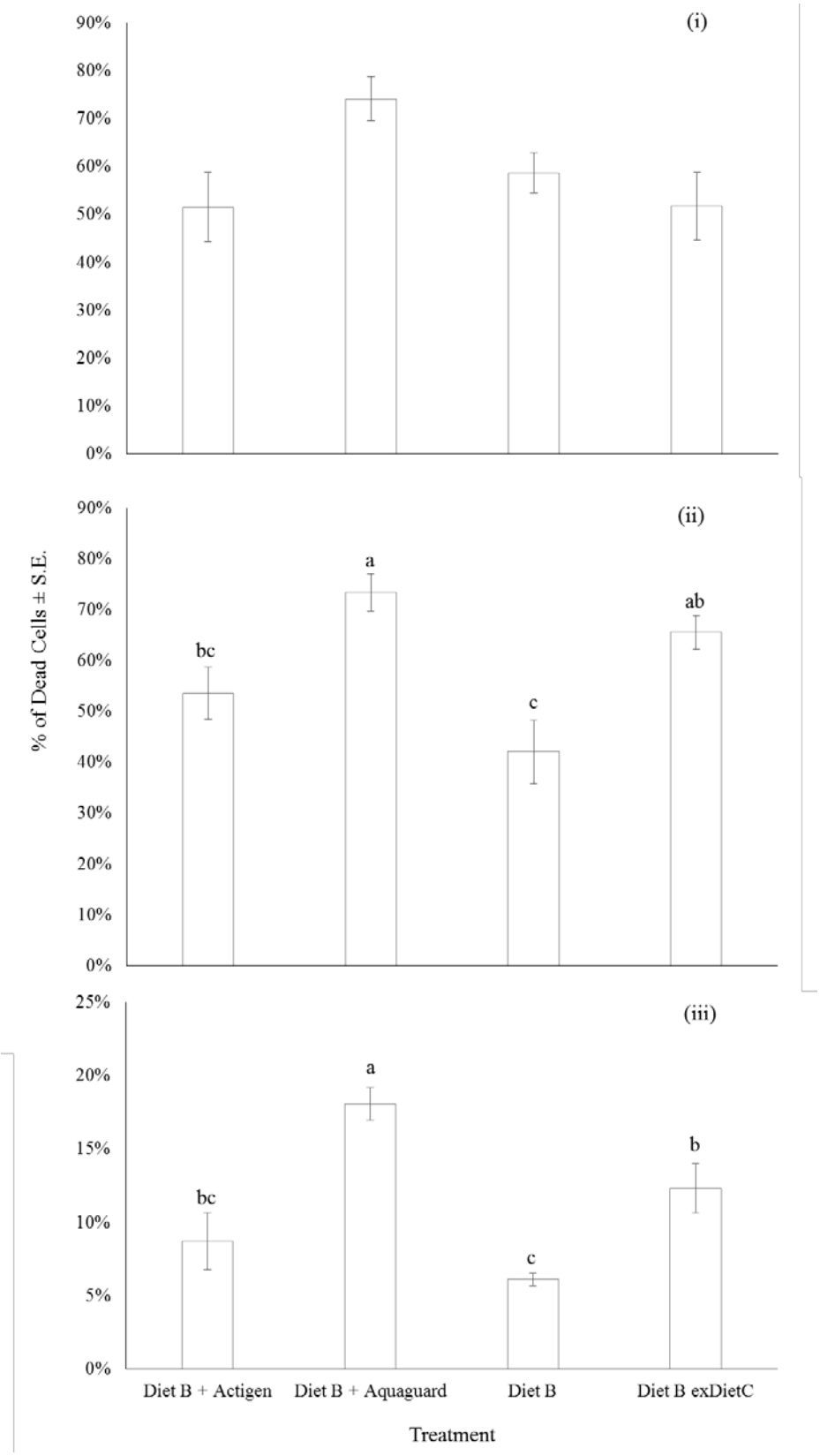

Figure 84: % of dead cells in lysed blood samples. Leucocytes challenged with (i) dead or (ii) live *S. iniae* cells after being held in cell culture media for 8 days. (iii) leucocytes not challenged with bacteria after being held in cell culture media for 18 hours.

No data was obtained for intracellular oxidative activity in leucocytes for the fresh cell cultures for the Diet B supplemented with Aquaguard, due to an issue with the concentration of fluorochrome used in these samples. Data on the intracellular oxidative activity within the other samples and treatments are shown in Figure 85 and Figure 86, respectively. In the P1 population of leucocytes there was no effect of diet on intracellular oxidative activity in fresh cells or in those challenged with dead *S. iniae*, however those cells from fish fed Diet B supplemented with Aquaguard and challenged with live *S.iniae* showed significantly lower oxidative activity than those fed Diet B without any supplementation. This supports our hypothesis above that continually feeding this immunostimulant for four months had a negative effect on the immune system of these fish, impairing their capacity to defend against pathogens. Intracellular oxidative activity in the P2 leucocytes challenged with live *S. iniae* was highly variable between treatments and therefore there was no effect of diet, however fish fed Diet B with Aquaguard again had the lowest intracellular oxidative activity in these cells when challenged with live *S. iniae*. Those cells from fish fed Diet B alone had significantly lower oxidative activity in P2 leucocytes than those in the exDiet C treatment in fresh cells.

The effect of diet on the lysosome activity in P1 and P2 leucocytes are shown in Figure 87 and Figure 88, respectively. There were significant and consistent dietary effects on these parameters in both fresh cells and stored cells challenged with live *S. iniae*, however no effects on stored cells when challenged with dead bacteria. Fish fed Diet B supplemented with Aquaguard had consistently lower lysosome activities in both cell populations than other diets, results that are consistent with the intracellular oxidative activity. These data again suggest that our long-term continual feeding regime with this immunostimulant had a negative impact on the immune system of these fish. These data also suggest that in this case, challenging the leucocytes with bacteria was not necessary to see a difference in lysosome activity, as long as the cells were fresh. Indeed fresh cells had a lower variation and therefore better resolution of treatment differences than those stored cells challenged with live bacteria. Similarly, challenging cells that have been kept in cell culture media for 8 days with dead bacteria is ineffective at eliciting a response from these cells. Also of note is the fact that the pattern of lysozyme with treatment shown above in Figure 81 and mirrors exactly that of lysosome activity in both leucocyte populations. That is, fish fed Diet B supplemented with Aquaguard also had the lowest level of serum lysozyme, whilst those fed Diet B or Diet B supplemented with Actigen had higher and equal levels of lysozyme. Lysosomes are the organelles

responsible for the production of lysozyme and it therefore follows that these two parameters should be consistent. These data also suggest that the measurement of lysosome activity via flow cytometry is more sensitive than the kit used to measure lysozyme in serum, where no significant differences between treatments were found despite showing the same pattern.

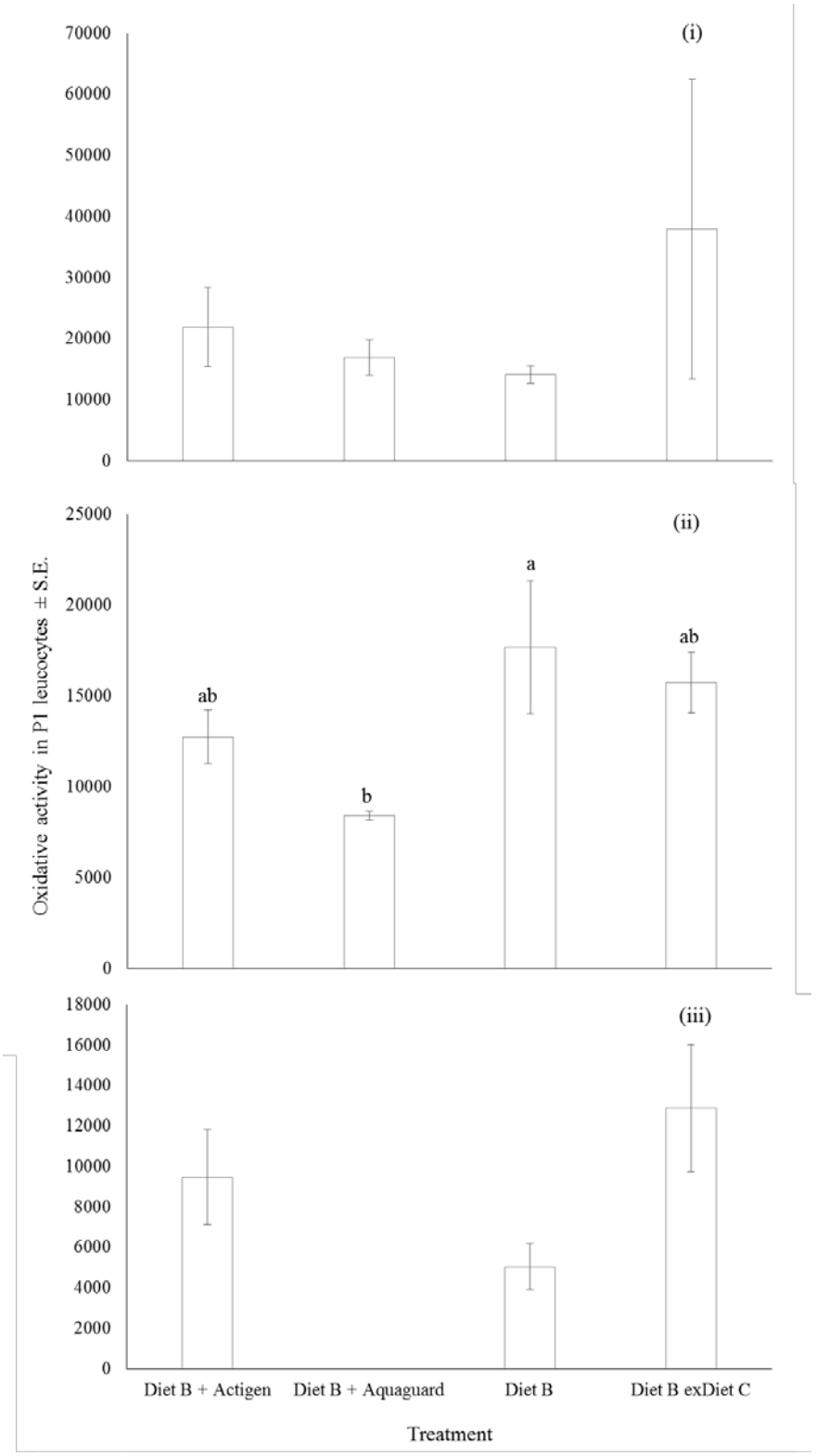

Figure 85: Intracellular oxidative activity in P1 leucocytes challenged with (i) dead or (ii) live *S. iniae* after being held in cell culture media for 8 days. (iii) cells not challenged after being held in cell culture media for 18 hours.

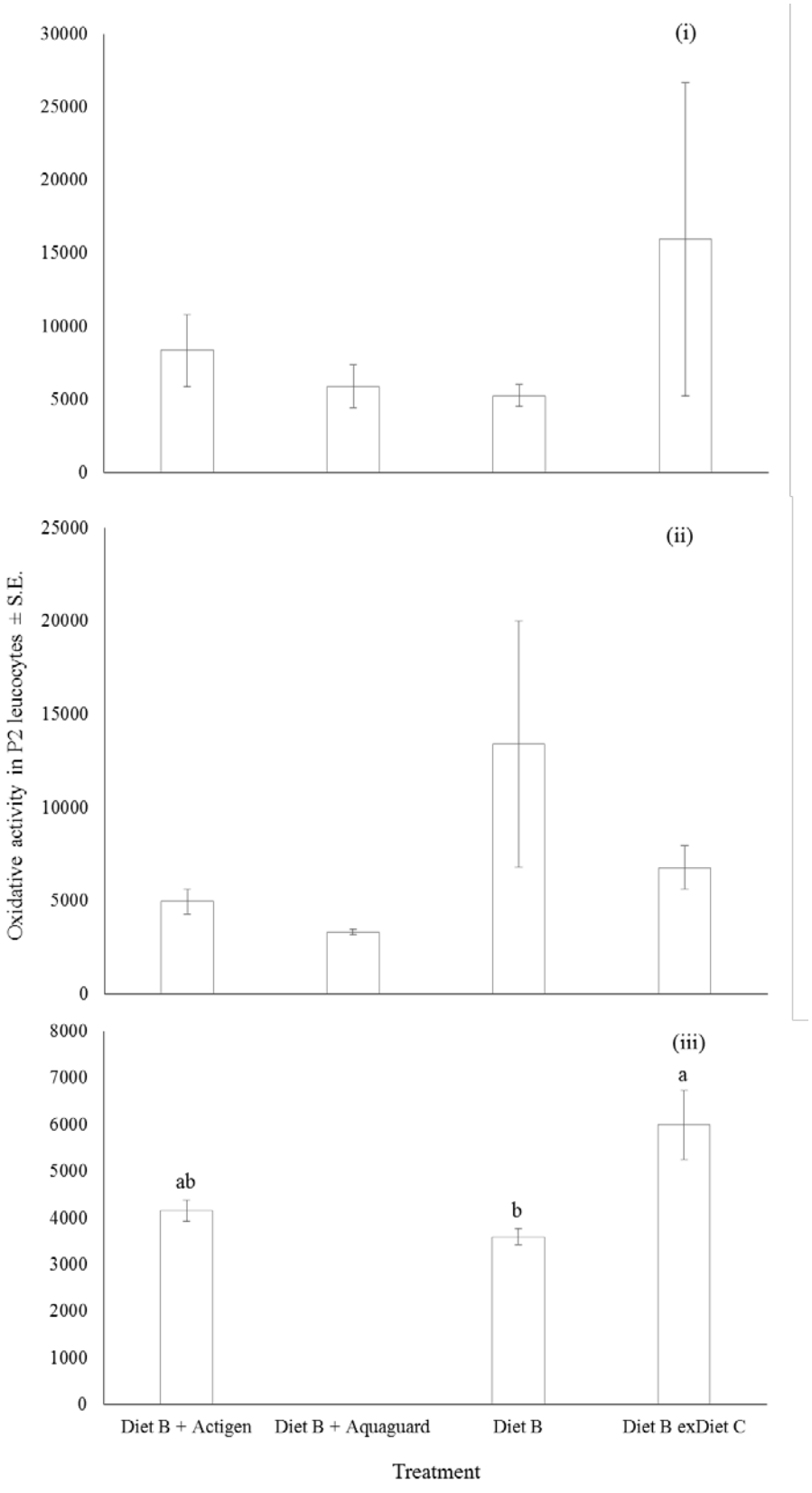

Figure 86: Intracellular oxidative activity in P2 leucocytes challenged with (i) dead or (ii) live *S. iniae* after being held in cell culture media for 8 days. (iii) cells not challenged after being held in cell culture media for 18 hours.

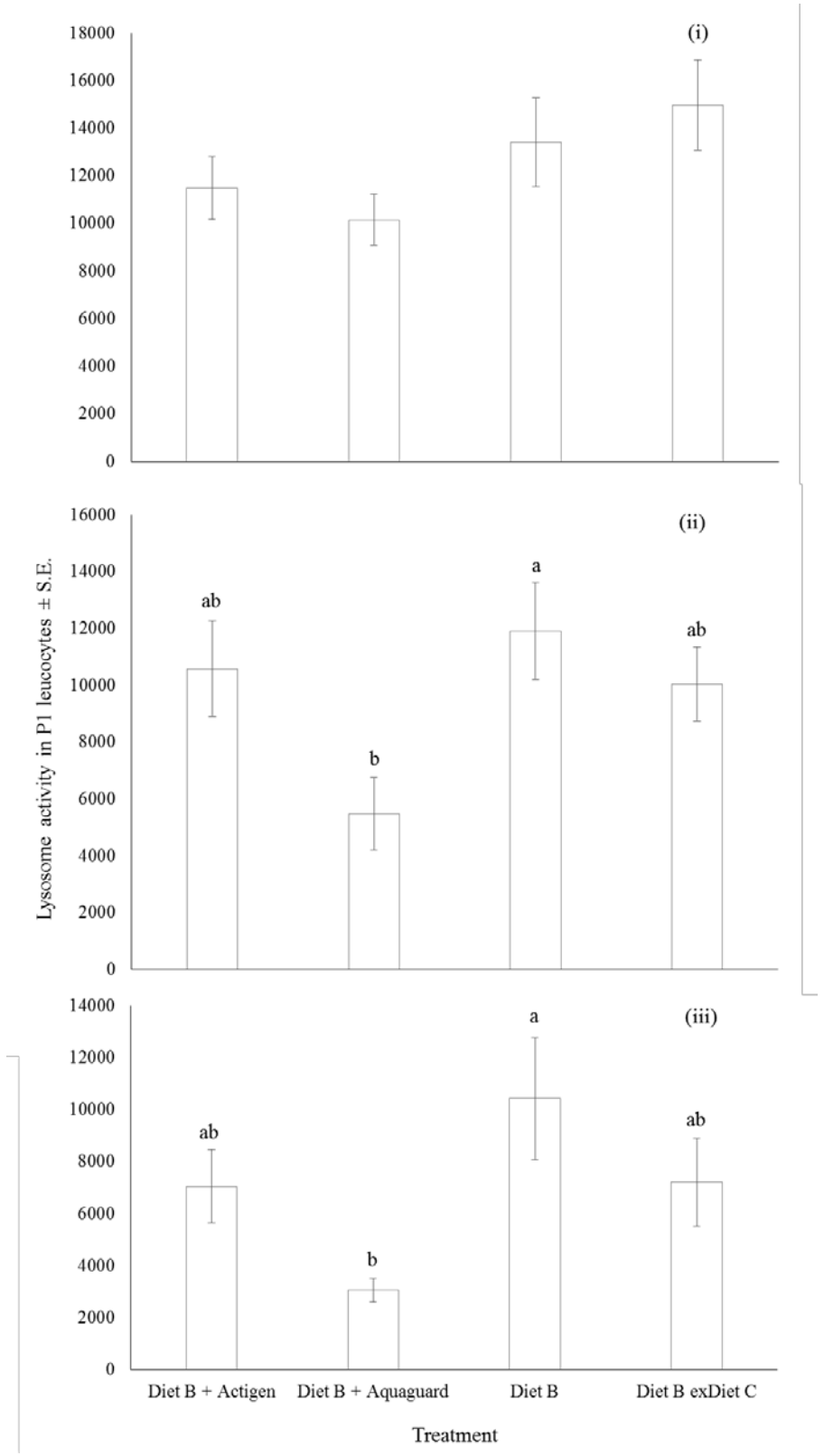

Figure 87: Lysosome activity in P1 leucocytes from lysed blood samples challenged with (i) dead or (ii) live *S. iniae* after being held in cell culture media for 8 days or (iii) cells not challenged after being held in cell culture media for 18 hours.

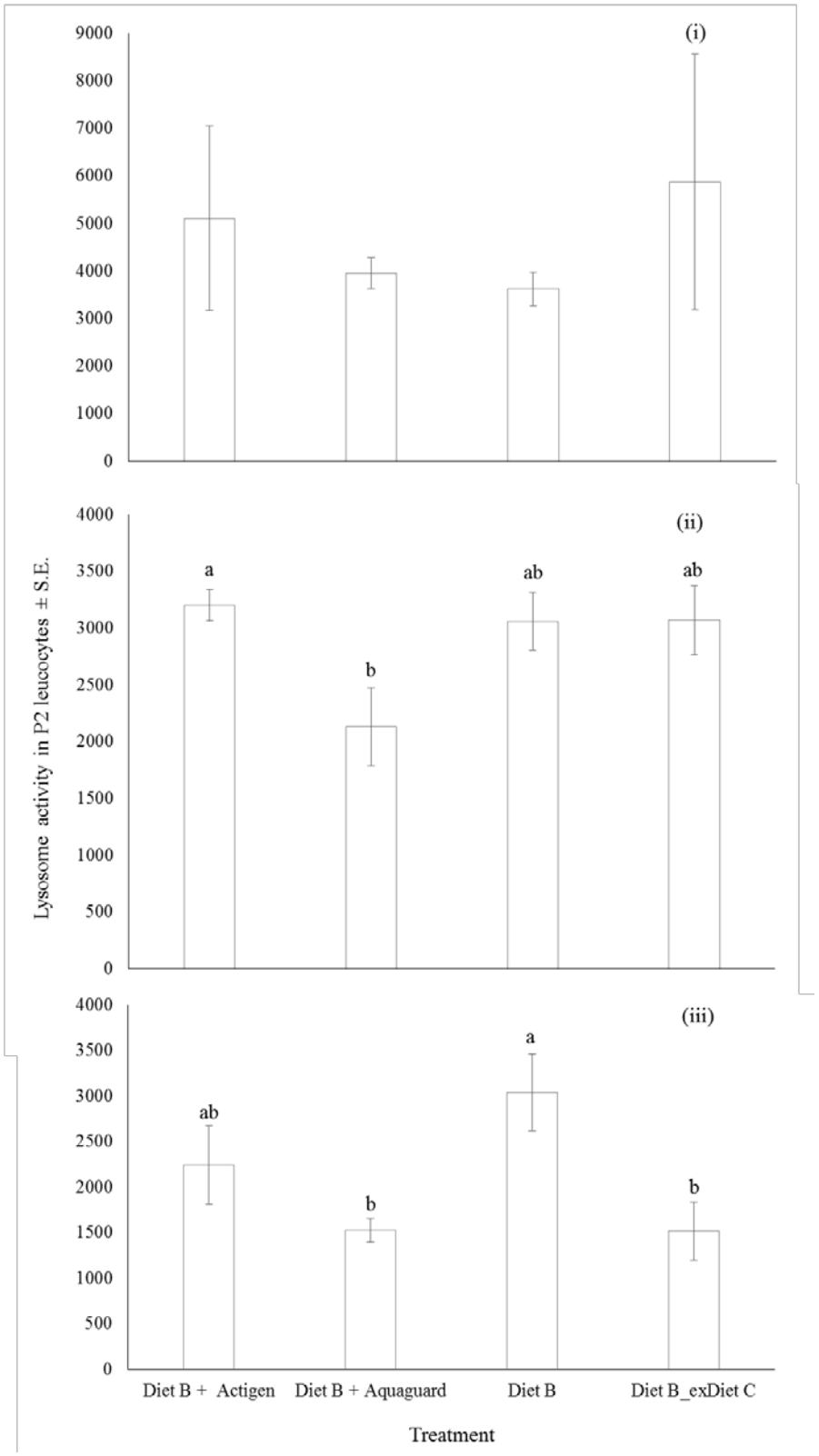

Figure 88: Lysosome activity in leucocytes from lysed blood samples. Cells challenged with (i) dead or (ii) live *S. iniae* after being held in cell culture media for 8 days. (iii) cells not challenged after being held in cell culture media for 18 hours.

As noted above, survival of fish fed Diet B supplemented with Aquaguard, resulted in significantly higher survival following a natural infection of *Photobacterium damselae sub damselae* and *Vibrio harveyi*, suggesting that Aquaguard had a positive impact on the immune response of these fish. It is noteworthy, however that this mortality event occurred in the 2-3 weeks after the trial started. There is varying and somewhat conflicting evidence on the optimum feeding regime for immunostimulants (i.e. continuous feeding versus stop-start feeding). Given that we observed higher survival in the Aquaguard fed fish after three weeks of feeding but evidence from the end of the trial (12 weeks of feeding) that Aquaguard is having a negative impact on the immune response suggests that short-term feeding is beneficial but longer term feeding is potentially detrimental.

Using those same studies described above, in which no growth benefit was found when feeding various yeast-based immunostimulants to fish – we review here the results of those same studies in terms of their ability to boost the fish's immune system. In the study by Ai et al(2007) yellow croaker fed 0.09% beta-glucans for 8 weeks had improved serum lysozyme, phagocytosis, respiratory burst and survival in challenge, however the higher dose of 0.18% did not improve any of these factors, suggesting that overstimulation of the immune system may have occurred at the higher dose. In the study by Shelby et al (2009) which investigated commercial beta glucans and mannan oligsaccardies in Nile tilapia, there was no benefit to any of the immune parameters measured (immunoglobulins, lysozyme and complement activities) or survival in challenge after feeding these products for two to four weeks. Cook et al (2001) supplemented snapper diets with beta-glucans for 84 days and measured various immune parameters at routine intervals during this time. As in the current trial, supplementation failed to increase complement activity at any of the measured time points, however macrophage superoxide anion production was significantly elevated in fish fed supplemented diets at lower water temperature. In warm water, this activity was slightly but not significantly elevated. These data (together with the aforementioned growth data) suggest there to be no benefits of feeding beta glucans to snapper in warm water, but benefits at sub-optimum temperatures. Li and Gaitlin (2004) measured significantly greatly superoxide anion production of head kidney macrophages and significantly higher survival post-challenge in hybrid striped bass fed yeast products for four to seven weeks at 1% to 2%, however similar to this trial there was no difference in serum lysozyme between treatments. Misra et al (2006)

found an elevation in total serum protein, globulins, superoxide anion production, phagocytic index, serum lysozyme activity, complement activity and serum bactericidal activity in rohu fed beta glucans until 42 days but then all of these values declined at 56 days, suggesting that chronic overstimulation may be occurring when fed for this period, which was considerably shorter than the period used in the current study (112 days). The doses used by Misra et al. (2006) were also lower (up to 0.5 g/kg) than those used in the current study, which may suggest that overstimulation may be enhanced at higher doses. When Atlantic cod larvae were fed rotifers enriched with beta glucans extracted from the microalgae *Chaetoceros muelleri*, survival was significantly improved, but not in those larvae fed rotifers enriched with yeast beta glucans (Skjermo et al., 2006). Whilst Sealey et al (2008) could find no differences in respiratory bust response, lysozyme or TNF-α mRNA expression in rainbow trout fed on different beta-glucans, there were significant differences in survival following a viral challenge, suggesting that improvements in immune function may not always be captured depending on the measurements taken and that challenge trials are therefore the best method of categorically determining whether any such benefits exist. Similarly, Welker et al (2007) measured no differences in any of the measured haematology parameters (haematocrit, haemoglobin, red cell count, white cell count) or serum lyosyzme, complement or bactericidal activity, despite achieving up to a 17.5% improvement in survival following challenge in fish fed beta-glucan supplemented diet. In the study in which Whittington et al (2005) fed beta-glucan containing diets to Nile tilapia for 14 weeks, there was no effect on survival post challenge with *Streptococcus iniae* and those fish fed the highest level of beta-glucan had significantly lower serum lysozyme 14 days post challenge. Grisdale-Helland et al. (2008) conducted a study of the same duration as this study in which Atlantic salmon were fed MOS. At the end of this trial, fish fed the MOS diet had significantly lower serum lysozyme than those fed the control diet, which is consistent with this study. The results of these studies combined demonstrate that yeast derived beta glucans and MOS are not a panacea to improving immune function and that overstimulation is likely to occur with chronic use. Further research is required to determine the optimum dose and duration of application of these products in yellowtail kingfish and whether these products may be particularly useful in cooler water. Some of these issues issue will be addressed in FRDC Project 2017-030, which will determine the impact of feeding beta-glucan for shorter periods.

5. Conclusion

This project demonstrated significant differences in the performance of YTK fed different diets across a commercially relevant time frame. It has generated growth and FCR data that are superior to those reported previously and that will be used to generate more industry-relevant growth models for optimising feeding and food conversion ratios in warm-water environments. The project has been highly successfully in generating large volumes of baseline data on health and has generated new techniques and skill sets within Western Australia that will be of key importance to the developing YTK industry in this state. The project has identified several areas for future work that have potential in improving YTK health and subsequently the profitability and sustainability of the industry

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7. Implications

Feed costs for finfish aquaculture can account for up to 40% of the cost of production. Feeds that do not suit the requirements of the species not only lead to poor performance in the fish and increased costs, but can leave the fish susceptible to disease through a lowered immunity. Developing feeds that suit the fast growing yellowtail kingfish are therefore vital to the ongoing development and viability of the industry.

This project both identified suitable existing feeds for yellowtail kingfish and increased the knowledge of the implications of some of the ingredients and formulations that may be used. The research led to significantly improved growth and performance over previous growout trials for yellowtail kingfish in WA.

This project, run as it was in parallel to a pilot seacage trial for yellowtail kingfish off the coast of Geraldton, Western Australia, led to substantial improvement in the at-sea fish performance and helped to explain some of the observations of ill-thrift in the fish on a real time basis. It identified a suitable feed for yellowtail kingfish growout, and one that could be used as a base for further trials of the inclusion of additives to improve the immunity of the fish to disease challenges.

The research also further developed the yellowtail kingfish growth model for warm water culture and provided insights into the implications of ingredients and combination of ingredients into feed formulations.

Perhaps the most important implication of this research was the further development of the excellent collaboration that exists with Western Australia between the marine fish farming industry, the Fish Health Group at WA Fisheries and with the team at the Australian Centre for Applied Aquaculture Research. The commitment, skills and experience this team has developed has given the industry proponent, Indian Ocean Fresh Australia, the confidence to commence commercial farming of yellowtail kingfish off the Mid West coast.

This research also has implications for disease management in the expanding yellowtail kingfish industry. Bacterial disease has the potential to seriously impact on profitability and this research has generated a substantial amount of information that will be used in future projects to advance the science of fish health and reduce mortality.

8. Recommendations

8.1. Further development

A proposal to build on the fish health knowledge gained during this project has been put forward to the FRDC. The project will concentrate on understanding and controlling yellowtail kingfish encounters with identified bacteria species and attempt to boost the immune system of the yellowtail kingfish to withstand such encounters. The research will also build on the diet development knowledge of the team of collaborators across three states of Australia to further improve fish growth and boost the fish immune systems to increase the disease resistance to infectious agents.

9. Extension and Adoption

This research has been conducted with the direct involvement of the industry partner Indian Ocean Fresh Australia (IOFA). This company not only contributed substantial cash to the project, but was directly involved in developing the research direction that were taken throughout the project. This not only ensured that the research was directed to deliver the outcomes required by industry, but also ensured the results were taken up by industry.

9.1. Project coverage

There was no media associated with this ACAAR FRDC project.

10. Project materials developed

No project materials have been developed at the time of publication of this report. .

11. Appendices

Appendix 1: Summary of histological findings.

	Block 1 Month 1		
	Diet A	**Diet B**	**Diet C**
Liver Fatty	2	1	2, with large fat vacuoles
	2, large numbers of necrotic hematocytes	3	3, with large fat vacuoles
	2	2	3
	2	2, occasional necrotic hepatocyte	2
Gill	-	-	-
	-	-	-
	-	-	-
	-	-	-
Spleen	-	Congestion, formalin pigment	-
	Large amount of formalin pigment	-	-
	Congestion	Congestion, formalin pigment	Congestion, formalin pigment
	Very congested	Very congested	-
Foregut	-	-	-
	-	-	-
	-	-	-
	-	-	-
Midgut	-	-	-
	-	-	-
	-	-	-
	-	-	-
Hindgut	-	-	-
	-	-	-
	-	-	-
	-	-	-
Pyloric cacae	-	-	3, villi well developed
	-	-	Shorter villi, dilated lumens
	-	-	-
	-	-	-
Gonad	-	-	-
	-	-	-
	-	-	-
	-	-	-
Kidney	-	-	-
	Few eosinophilic droplets	Eosinophilic globules, dilation of tubules	Few eosinophilic droplets
	-	-	Moderate eosinophilic droplets
	-	-	Moderate eosinophilic droplets
Skeletal Muscle	-	1FD	-
	1FD	2FD	3FD
	-	-	3FD
	3FD	3FD	2FD
Skin thickness (Microns)	-	-	-
	-	-	-
	-	-	-
	-	-	-
Mean skin mucous cells/ 100 microns of	-	-	-
	-	-	-
	-	-	-
	-	-	-
Heart	-	-	-
	-	-	Severe multifocal aggregations of lymphocytes in the ventricle
	-	-	-
	-	-	-
Brain	-	-	-
	-	-	-
	-	-	-
	-	-	-

	Block 1 Month 2		
	Diet A	**Diet B**	**Diet C**
Liver Fatty	2	1	1
	2	1	1
	2	2	1
	2	2	1
Gill	-	-	-
	-	-	-
	-	-	-
	-	-	-
Spleen	Mild congestion and depletion	Mild depletion	Congestion, formalin pigment
	Low number of small melanomacrophages	-	-
	-	Mild depletion, many melanomacrophages	Very congested, few melanomacrophages
	-	Many melanomacrophages	Moderate melanomacrophages
Foregut	-	-	-
	-	Villi with few mucous cells	-
	Very long villi	Less than nomal number of mucous cells	3, long thin villi
	3, moderate length villi	Less than nomal number of mucous cells	3, very long villi
Midgut	-	-	-
	-	-	-
	Moderate lenth villi	-	3, long thin villi
	3, moderate length villi	Long, thin villi	3, very long villi
Hindgut	-	-	-
	-	-	-
	3, moderate length villi	-	3, long thin villi
	Short villi	Thickened lamina propria	3, very long villi
Pyloric cacae	-	Thin villi, prominent lumen	-
	Long thin villi	-	Thin villi, prominent lumen
	Long thin villi	-	Long, thin villi
	-	-	3, very long villi
Gonad	-	-	-
	-	-	-
	-	-	-
	-	-	-
Kidney	Low eosinophilic droplets	-	-
	-	-	Few eosinophilic droplets
	Moderate eosinophilic droplets	-	-
	Few eosinophilic droplets	-	Large numbers of eosinophilic droplets
Skeletal Muscle	1FD	2FD	-
	-	3FD	-
	2FD	-	2FD
	-	-	2FD
Skin thickness (Microns)	-	-	-
	-	-	-
	-	-	-
	-	-	-
Mean skin mucous cells/ 100 microns of	-	-	-
	-	-	-
	-	-	-
	-	-	-
Heart	-	Small area of chronic epicarditis	-
	-	-	-
	-	-	-
	-	-	-
Brain	-	-	-
	-	-	-
	-	-	-
	-	-	-

	Block 1 Month 3		
	Diet A	**Diet B**	**Diet C**
Liver Fatty	2	1, atrophic hepatocytes	3
	2	1, atrophic hepatocytes	2
	1	2	2
	2	1, atrophic hepatocytes	3
Gill	-	-	-
	-	-	-
	-	-	Sever hyperplasia
	-	Mild hypertrophy, fusion of lamellae	-
Spleen	Occasional melanomacrophages	Moderate congestion, mild depletion	-
	Slight depletion	Few melanomacrophages	Occasional melanomacrophages
	Very congested, few melanomacrophaes	-	-
	Low melanomarcophages	Few melanomacrophages	Few melanomacrophaged
Foregut	-	-	*P. damselae* present
	-	Long villi, fewer mucous cells	-
	P. damselae present (light growth)	*P. damselae* present (haemolytic)	*P. damselae* present (heavy growth)
	3	-	-
Midgut	-	-	-
	-	-	-
	-	Mild congestion	Mild necrosis
	3	-	3, long villi
Hindgut	Friable mucosa	-	-
	-	-	-
	-	Increased eosinophilic cells	-
	3	-	Increased eosinophilic cells
Pyloric cacae	-	-	-
	-	-	Short, fat villi and large lumens
	-	-	Congestion
	3	-	-
Gonad	-	-	-
	-	-	-
	-	-	-
	-	-	-
Kidney	-	-	Dilated ducts with mineral material present
	Moderate eosinophilic droplets	-	Moderate eosinophilic droplets
	-	-	Dilated ducts with mineral material present
	Few eosinophilic cells	-	-
Skeletal Muscle	2FD	-	-
	1FD	-	-
	-	-	-
	2FD	2FD	2FD
Skin thickness (Microns)	-	-	-
	-	-	-
	-	-	-
	-	-	-
Mean skin mucous cells/ 100 microns of	-	-	-
	-	-	-
	-	-	-
	-	-	-
Heart	-	-	-
	-	-	-
	-	-	-
	-	-	-
Brain	-	-	-
	-	-	-
	-	-	-
	-	-	-

	Block 2 Month 1			
	Diet B + Actigen	**Diet B + Aquaguard**	**Diet B**	**Diet B_ex-Diet C**
Liver	2 to 3	1	2	2
	2	3	2	2
	2	-	1	1
Gill	Lamellar fusion at some filament bases	-	-	Lamellar fusion and moderate hyperplasia
	-	Hypertophy at lamellae tips	Mild fusion of lamellae at filament bases	Nodular hyerplasia and hypertrophy
	One small area of lymphocytes	-	Moderate to severe hyperplasia	Some fusion of the lamellae tips
Spleen	Congestion, few melanomacrophages, some depletion	Congestion, moderate depletion	Formalin pigment, melanomacrophages	Depletion
	Occasional melanomacrophages	Formalin pigment	Formalin pigment	-
	Occasional melanomacrophages	-	-	-
Foregut	3, short villi with thick lamina propria	-	-	3, long villi
	Long, thin villi	3, Moderate congestion. Short villi, thick lamina propria	Congestion, thick lamina propria	Long thin villi with congestion
	-	-	Thick villi	Mild congestion, villi short and unbranched
Midgut	Sparse, thin villi	-	Congested, long thin villi	3, congestion long thin villi
	-	3, Moderate congestion	Long thin villi	-
	-	-	Short, thin villi	3, short villi, mild congestion
Hindgut	-	-	Congestion	-
	Long villi	3, Moderate congestion	-	-
	-	-	Short, thick villi with mild congestion	Long villi, good structure
Pyloric cacae	-	-	-	Mild congestion, and sloughing
	-	-	-	-
	-	-	-	-
Gonad	-	-	-	-
	-	-	-	-
	-	-	-	-
Kidney	-	-	-	-
	Large number of eosinophilic cells	-	Moderate eosinophilic cells	Moderate eosinophilic cells
	Enlarged ducts with necrotic material	-	-	-
Skeletal Muscle	1FD	3FD	-	-
	-	-	2FD	1FD
	1FD	-	-	-
Skin thickness (µm)	-	-	-	-
	-	-	-	-
	-	-	-	-
Mean skin mucous cells/ 100 µm of skin length	-	-	-	-
	-	-	-	-
	-	-	-	-
Heart	-	-	-	Bulbus arteriosus has mild necrosis
	-	-	-	-
	-	-	-	-
Brain	-	-	-	-
	-	-	-	-
	-	-	-	-

	Block 2 Month 2			
	Diet B + Actigen	**Diet B + Aquaguard**	**Diet B**	**Diet B_ex-Diet C**
Liver	3 2 4	2 3 3	4 1 3	2 3, some chronic inflammation 3
Gill	Mild telangiectasis Sever fusion and clubbing of lamellae -	Fusion, congestion and clubbing of lamellae - -	- Mild fusion and clubbing of lamellae Mild telangiectasis	- - -
Spleen	Depletion Moderate amount of formalin pigment Formalin pigment, reduced haematopoietic tissue	Formalin pigment, reduced haematopoietic tissue Formalin pigment, reduced haematopoietic tissue Formalin pigment, reduced haematopoietic tissue	Formalin pigment, reduced haematopoietic tissue Depleted. Congestion Formalin pigment, reduced haematopoietic tissue	Formalin pigment Formalin pigment Heavy formalin pigment
Foregut	- Moderate congestion -	- - -	- - -	- - -
Midgut	- Moderate congestion -	- - -	- - -	- - -
Hindgut	- Moderate congestion -	- - -	- Moderate congestion -	Slight increase in lymphocytes in lamina propria Slight increase in lymphocytes in lamina propria -
Pyloric cacae	- - -	- - -	- Short villi and reduced mucous cells -	- - -
Gonad	- - -	- - -	- - -	- - -
Kidney	- - -	- - -	- - -	- Large area of mineralisation in ducts Some eosinophilic droplets and mineralisation in ducts
Skeletal Muscle	- 2FD Occasional degenerative fibre	- 2FD -	2FD - Occasional degenerative fibre	2FD Occasional degenerative fibre Occasional degenerative fibre
Skin thickness (µm)	- - -	- - -	- - -	- - -
Mean skin mucous cells/ 100 µm of skin length	- - -	- - -	- - -	- - -
Heart	- - -	- - -	- - -	- - -
Brain	- - -	- - -	- - -	- - -

	Block 2 Month 3			
	Diet B + Actigen	**Diet B + Aquaguard**	**Diet B**	**Diet B_ex-Diet C**
Liver	3	3	3	3, mild chronic inflammation
	3	2 to 3	3	2
	2 to 3	3	2 to 3	1
Gill	-	Moderate fusion and clubbing of lamellae	Occasional hyerplasia	Mild hyperpalsia of epithelium and clubbing of lamellae
	-	Mild telangiectasis and hyperplasia of epithelium	-	Diffuse, mild hyperpalsia of epithelium and clubbing of lamellae
	-	-	Severe fusion and clubbing of lamellae	-
Spleen	-	-	Moderate formalin pigment	-
		Moderately depleted haematopoietic tissue	Heavy formalin pigment	Heavy formalin pigment, reduced haematopoietic tissue
	Heavy formalin pigment	Mild depletion of haematopoietic tissue	-	-
Foregut	-	-	Short villi that are moderately sparse	-
	-	Short, thick sparse villi	-	-
	Long thin villi	-	-	-
Midgut	-	-	-	-
	-	Short, thick sparse villi. Moderate congestion	-	-
	Long thin villi	-	3	-
Hindgut	-	-	-	-
	-	Short, thick sparse villi	Increased lymphocytes in lamina propria and mucosa	-
	Long thin villi	-	-	-
Pyloric cacae	-	-	-	-
	-	-	-	-
	Long thin villi	-	-	Congestion
Gonad	Ovary, immature	Testis, sperm	Testis, sperm	Testis, sperm
	Ovary, immature	Ovary, immature	-	Ovary, immature
	Ovary, immature	-	Ovary, immature	Ovary, immature
Kidney	-	Moderate eosinophilic droplets	Mineral in a duct lumen	Moderate eosinophilic droplets
	Moderate eosinophilic droplets	-	Moderate eosinophilic droplets	-
	-	-	-	-
Skeletal Muscle	1FD	-	1FD	-
	2FD	1FD	-	Occasional necrotic fibre
	2FD, variable sized fibres	1FD	-	-
Skin thickness (µm)	-	-	-	-
	-	-	-	-
	-	-	-	-
Mean skin mucous cells/ 100 µm of skin length	-	-	-	-
	-	-	-	-
	-	-	-	-
Heart	-	Mild chronic endocarditis	-	-
	-	-	-	-
	-	Mild chronic endocarditis	-	-
Brain	-	-	-	-
	Vaculoar change in white matter	-	-	Vaculoar change in white matter
	-	-	-	-

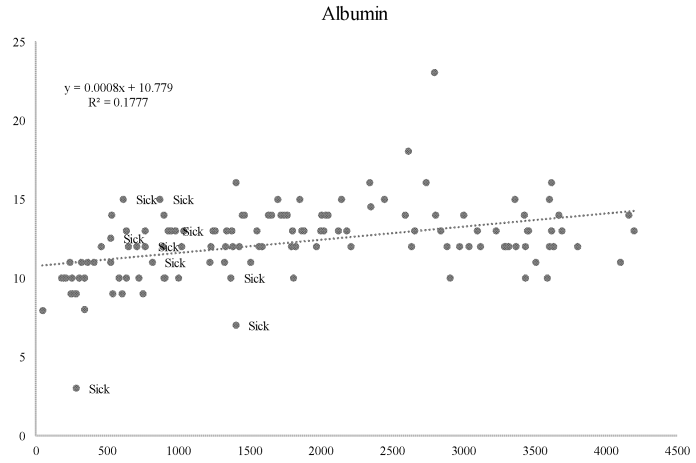
	Block 2 Month 4			
	Diet B + Actigen	**Diet B + Aquaguard**	**Diet B**	**Diet B_ex-Diet C**
Liver	3	2	3	1
	1 to 2	2	2	2
	2	1	2 to 3	2
Gill	-	-	Diffuse, mild hyperpalsia of epithelium and clubbing of lamellae	Moderate fusion and clubbing of lamellae
	Mild fusion of lamellae tips	-	Occasional fusion of lameallar tips and mild telangiectasis	Mild clubbing of lamellae tips
	Moderate fusion and clubbing of lamellae	Moderate fusion and clubbing of lamellae	Severe fusion and clubbing of lamellae	-
Spleen	Mildly depleted haematopoietic tissue	Mildly depleted haematopoietic tissue, congestion	Congestion, formalin pigment	-
	-	Serevely depleted haematopoietic tissue,	Mildly depleted haematopoietic tissue	Depleted haematopoietic tissue
	Mildly depleted haematopoietic tissue	Mildly depleted haematopoietic tissue, congestion	Very depleted haematopoietic tissue	Mildly depleted haematopoietic tissue
Foregut	-	-	-	-
		-	Short villi with small epithelial cells	Short villi with atrophic enterocyes
	-	-	-	-
Midgut	-	-	-	-
	-	-	Short villi with small epithelial cells	-
	-	-	-	-
Hindgut	-	-	-	-
	-	-	Increased lymphocytes in lamina propria	-
	-	-	-	-
Pyloric cacae	-	-	-	-
	-	-	-	-
	-	-	Lymphocyte aggregation in lamina propria	-
Gonad	Ovary, immature	-	Ovary, immature	Ovary, immature
	-	Ovary, immature	-	-
	-	-	-	-
Kidney	-	-	Mineral present within ducts	-
	-	-	Enlarged ducts containing mineral	-
	-	-	-	-
Skeletal Muscle	3FD	1FD	2FD	1FD
	-	2FD	1FD	1FD
	2FD	2FD	2FD	-
Skin thickness (µm)	85 to 132, no eosinophilic outer layer	121 to 147	63	172
	140	140	65 to 110	71 to 145, thin friable eosinophilic layer
	160 to 224	170	107 to 130	121, no eosinophilic outer layer
Mean skin mucous cells/ 100 µm of skin length	-	-	-	-
	-	-	-	-
	-	-	-	-
Heart	Mild chronic endocaditis	-	-	-
	-	Mild chronic inflammation and fibrosis of the conus arteriosus	-	-
	-	-	Mild, chronic epicarditis with haemorrhage and fibrosis	-
Brain	-	-	-	-
	-	-	-	-
	-	-	-	-

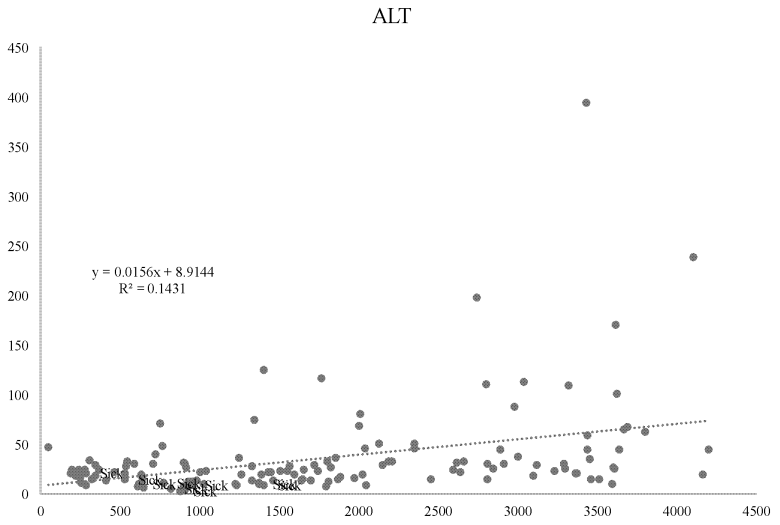
	Block 3 Month 1		
	Diet D	**Diet B**	**Diet A**
Liver Fatty	2	2	3
	2	2	2
	2	2	2
Gill	-	-	-
	some fused lamella	-	-
	-	Multifocal fusion of lamellae	-
Spleen	mild depletion	melanomacrophages	melanomacrophages
	mild depletion	moderate depletion	-
	mild depletion	very congested	-
Foregut	3	3	3
	3	2	3, short villi
	2	-	3
	3	3, thickening of lamina propria	3, thin villi
Midgut	3	short, sparse villi	3
	3	3	Sparse, thin villi
	3	3	3
Hindgut	3	Long thick villi	3
	2	-	1
	-	-	-
Pyloric cacae	-	-	-
	-	-	short, sparse villi
Gonad	Ovary, immature	testis sperm	testis sperm
	testis sperm	Ovary, immature	testis sperm
	testis sperm	testis sperm	testis sperm
Kidney	-	-	-
	low eosinophilic cells	-	-
	-	-	high eosinophilic droplets
Skeletal Muscle	-	2FD	occasional FD
	1 FD	large amount of fat	3FD
	1FD	1FD	2FD
Skin thickness (Microns)	-	-	-
	-	-	-
	-	-	-
Mean skin mucous cells/ 100 microns of	-	-	-
	-	-	-
	-	-	-
Heart	-	-	-
	-	-	-
	-	-	-
	-	-	-
Brain	-	-	-
	-	-	-
	-	-	-

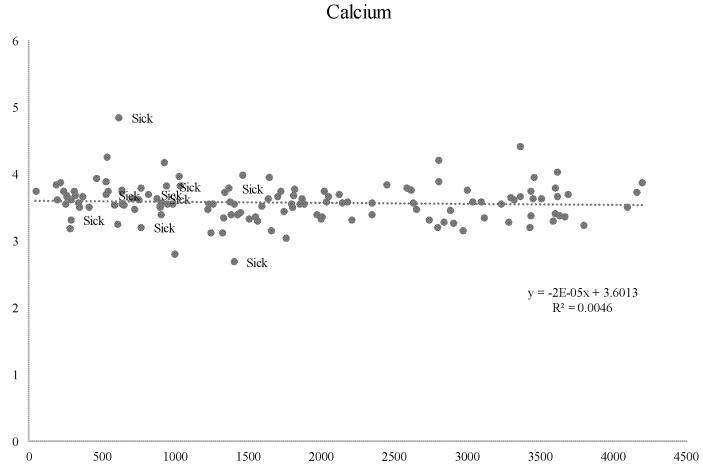
	Block 3 Month 2		
	Diet D	**Diet B**	**Diet A**
Liver Fatty	2	2	2
	2	2	2
	1	2	3
Gill	-	1 hyperplastic nodule	macrophages at filament tips
	some granule cells and macrophages	1 hyperplastic nodule	hyperplasia of lamellae
	-	Multifocal fusion of lamellae	1 hyperplastic nodule
Spleen	Mild depletion melanomacrophages & necrotic erythrocytes	Mild depletion	-
		Mild depletion	few melanomacrophages
	congested	-	few melanomacrophages
Foregut	2	-	-
	Mildly thickened lamina propria	-	short, sparse villi
	3	-	-
	2	short villi	3
Midgut	Mildly thickened lamina propria	some sloughed villi tips	-
	-	3	-
	-	Lymphocytes in lamina propria	-
Hindgut	Mildly thickened lamina propria	Thickened lamina propria, with lympocytes present	-
			-
	-	-	
	-	-	Some short villi
Pyloric cacae	low eosinophilic cells	Mild slouging of mucosa of villi tips	-
	-	-	-
Gonad	testis sperm	Ovary, immature	testis sperm
	testis sperm	Ovary, immature	testis sperm
	Ovary, immature	testis sperm	Ovary, immature
Kidney	-	-	-
	-	-	-
	Mild sloughing of epithelial cells	-	-
Skeletal Muscle	-	1 FD	1 FD
	-	2 FD	3 FD
	-	-	-
Skin thickness (Microns)	96	175	180
	165	111	170
	-	102	138
Mean skin mucous cells/ 100 microns of	2.57	3.88	2.53
	2.83	2	4.86
	-	2.26	2.6
Heart	-	-	2 small lymphocyte aggregations in endo- and epicardium
	Multifocal aggregation of lymphocytes in endo- and epicardium	-	-
	-	-	-
Brain	-	-	-
	-	-	-
	-	-	-

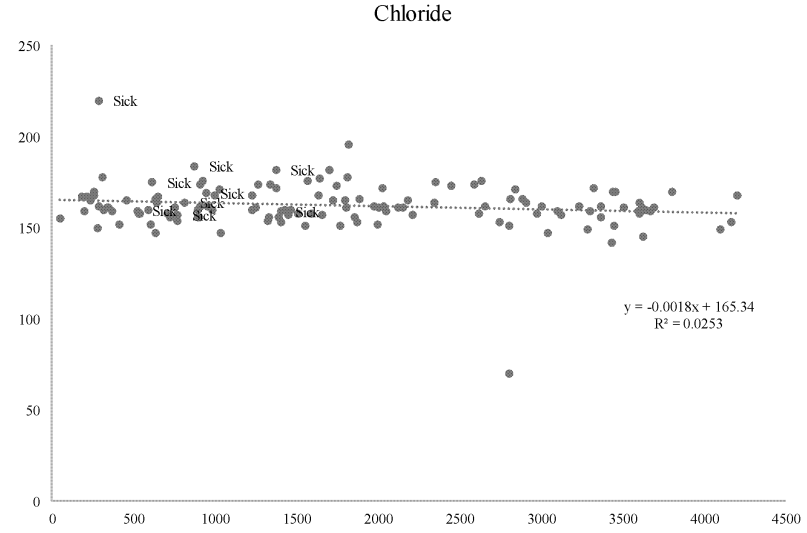
	Block 3 Month 3		
	Diet D	**Diet B**	**Diet A**
Liver Fatty	2	2	1
	2	2	1
	3	4	3
Gill	-	2 clubbed lamellae	Mild hypertrophy & clubbing
		mild clubbing and fusion and moderate hyerplasia	Moderate fusion of lamellae
	1 lymphocyte nodule	-	2 hyperplastic nodules
	Multifocal fusion of lamellae		
Spleen	-	Congestion, mild depletion	Congestion, slight depletion
	Mild depletion	Congestion	Moderate congestion
	moderate melanomacrophages	Congestion	-
Foregut	-	-	-
	-	-	-
	Mild increase in cellularity of lamina propria	Increased inflammatory cells in lamina propria	-
	-	-	-
Midgut	Increased number of lymphocytes	Increased in cells & thickness of lamina propria	-
	Mild increase in cellularity of lamina propria	Increased in cells & thickness of lamina propria	-
	-	Minor Increas in cells & thickness of lamina propria	-
Hindgut	Increased number of lymphocytes	Increased in cells & thickness of lamina propria (Low grade enteritis)	-
	Mild increase in cellularity of lamina propria (Low grade bacterial infection)	Chronic inflammation of the lamina propria (Low grade enteritis)	-
	-	Sloughing of epithelium	Large lumens, short villi
Pyloric cacae	-	Sloughing of epithelium, lumen dilated and short villi	-
	-	-	-
Gonad	testis sperm	Ovary, immature	testis sperm
	testis sperm	Ovary, immature	testis sperm
	Ovary, immature	testis sperm	Ovary, immature
Kidney	-	-	-
	Granulomas in one area	Mild cellular debris in tubules	Sloughed cells in lumens, mild
	Moderate eosinophilic cells	-	-
Skeletal Muscle	-	1 FD	1 FD
	-	-	-
	-	-	-
Skin thickness (Microns)			
Mean skin mucous cells/ 100 microns of			
Heart	Occasional small lymphocyte nodule	-	Several small inflammatory cells in endo- and epicardium
	-	-	-
	-	-	One small inflammatory cell in atrium
Brain	-	-	-
	-	-	-
	-	-	-

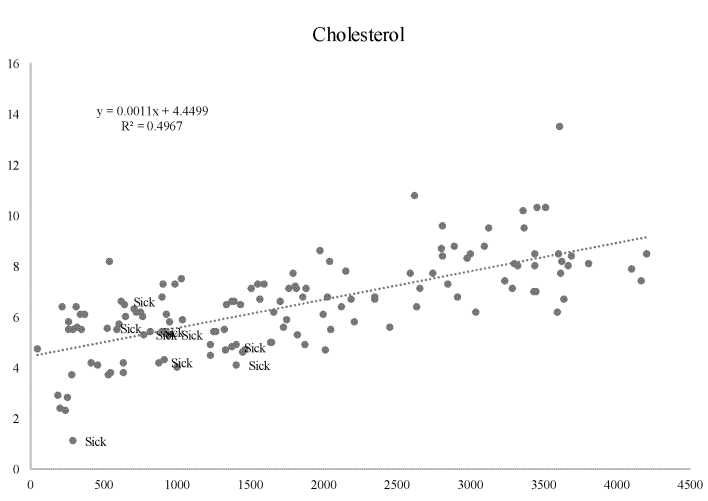
Appendix 2: Detailed plots of blood parameters versus fish size

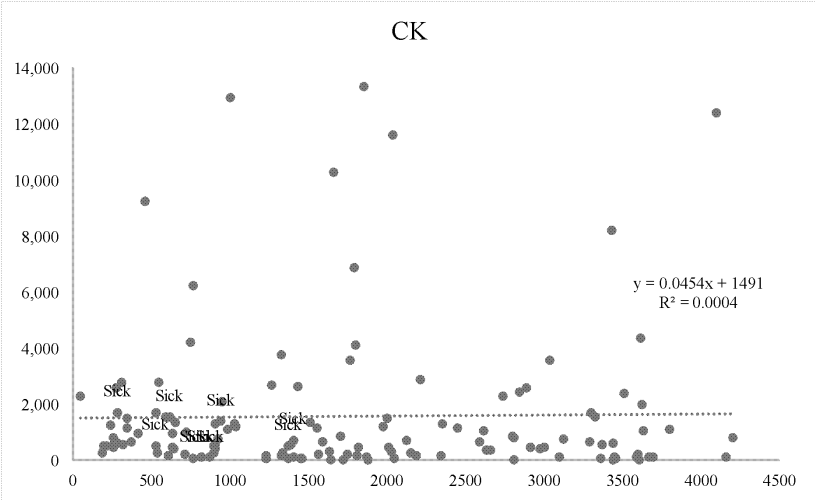


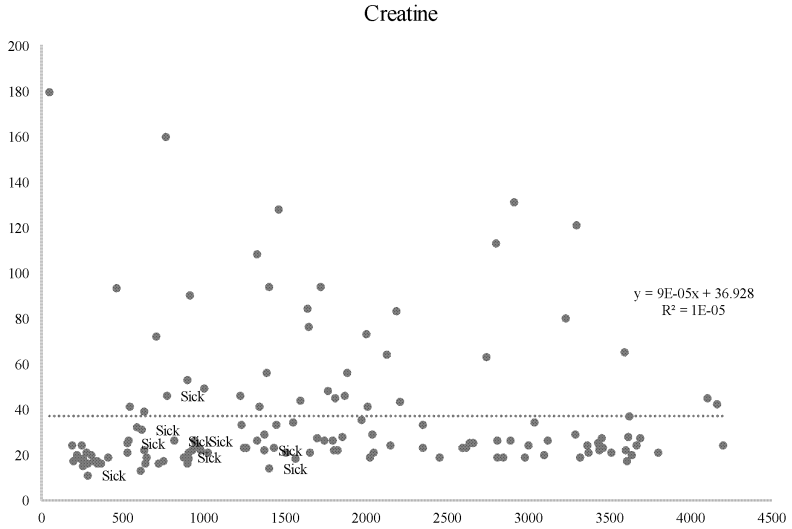


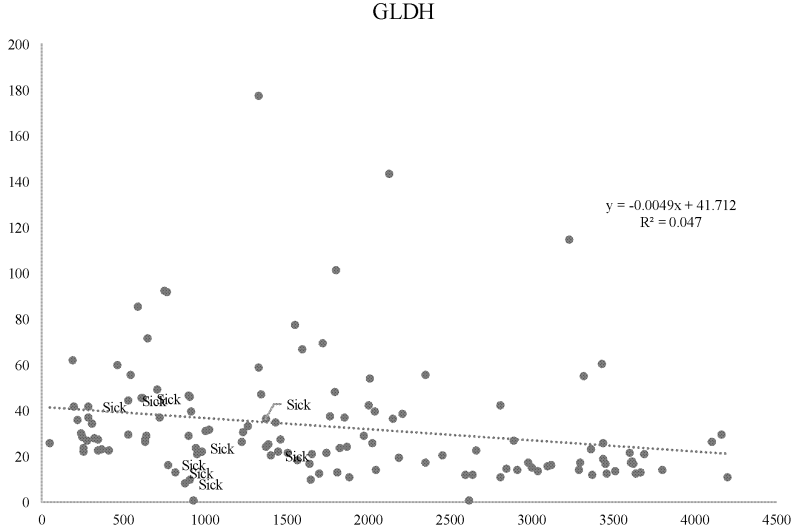


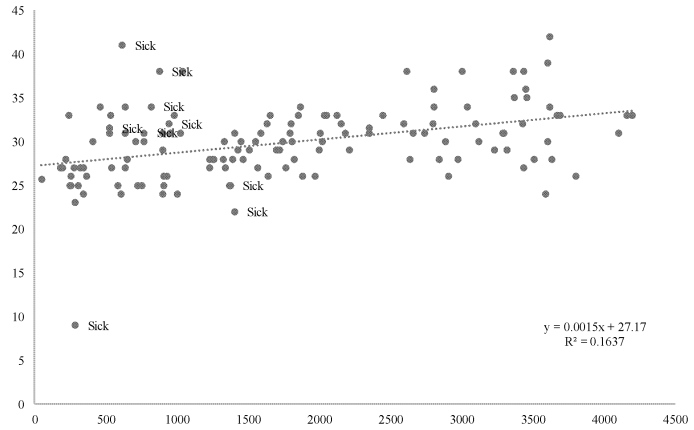


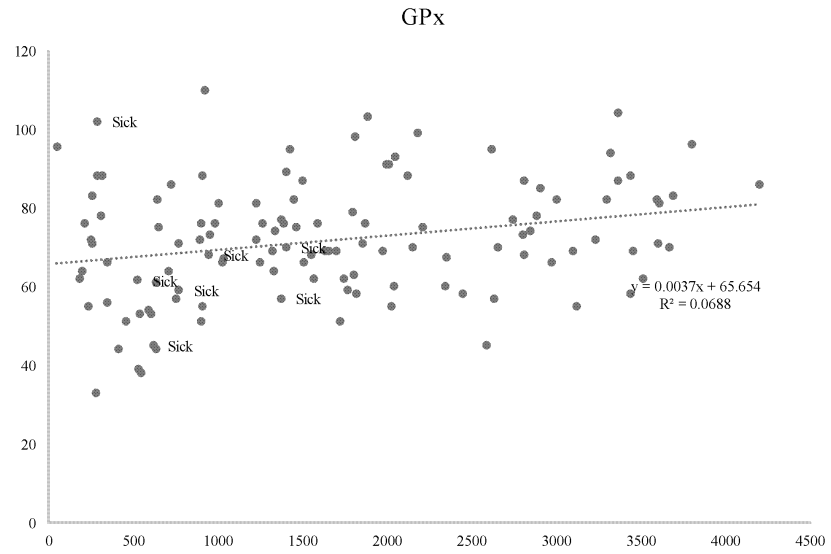

Cholesterol
y = 0.0011x + 4.4499
R² = 0.4967
Sick

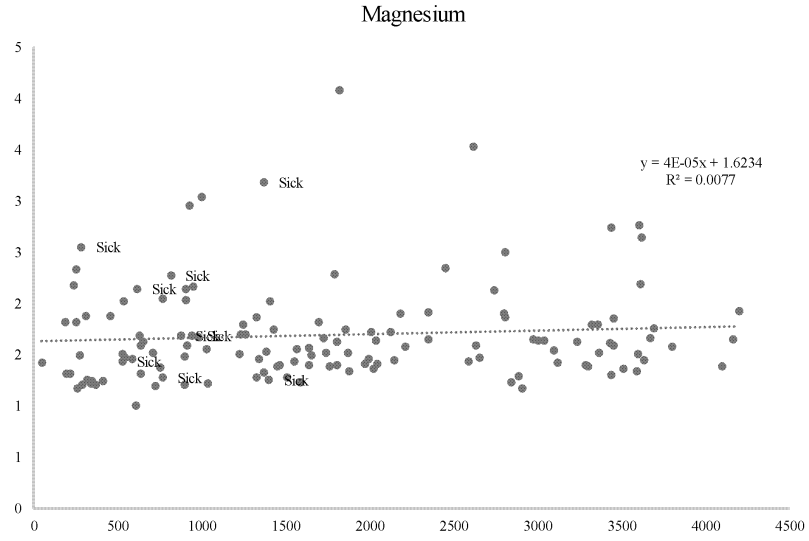

CK
y = 0.0454x + 1491
R² = 0.0004
Sick

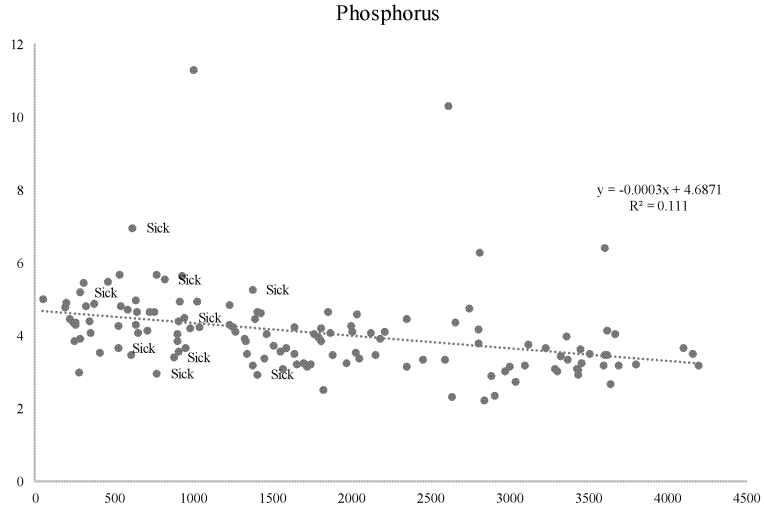

Creatine
y = 9E-05x + 36.928
R² = 1E-05
Sick

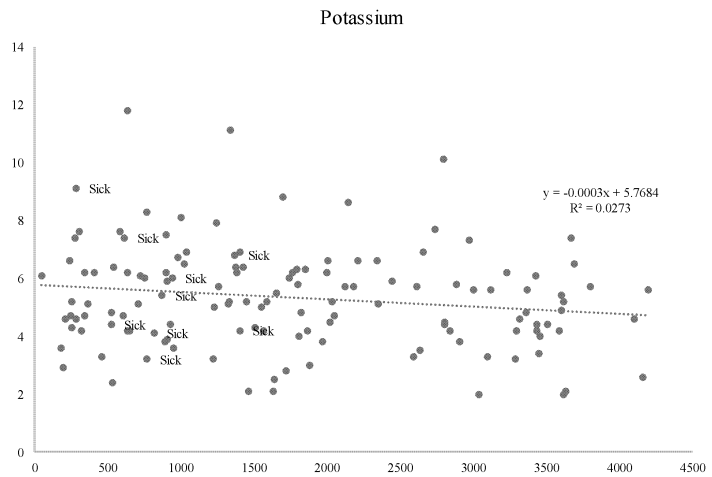

GLDH
y = -0.0049x + 41.712
R² = 0.047
Sick

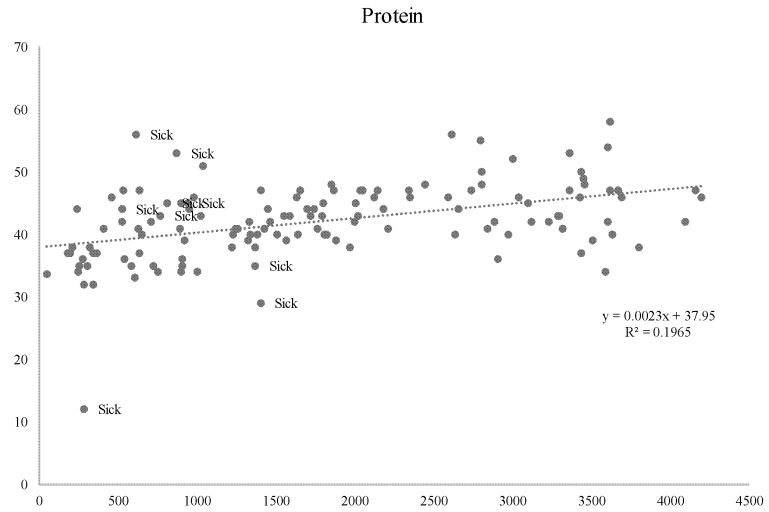

Globulin
y = 0.0015x + 27.17
R² = 0.1637
Sick

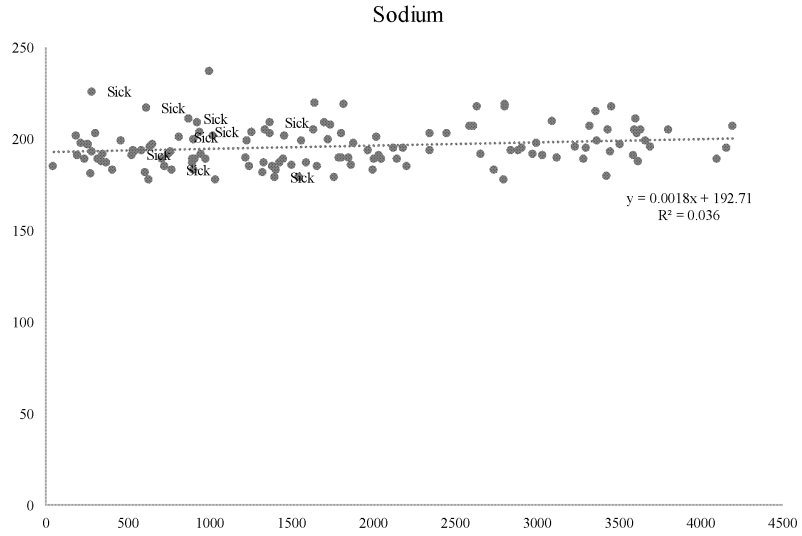

GPx
y = 0.0037x + 65.654
R² = 0.0688
Sick

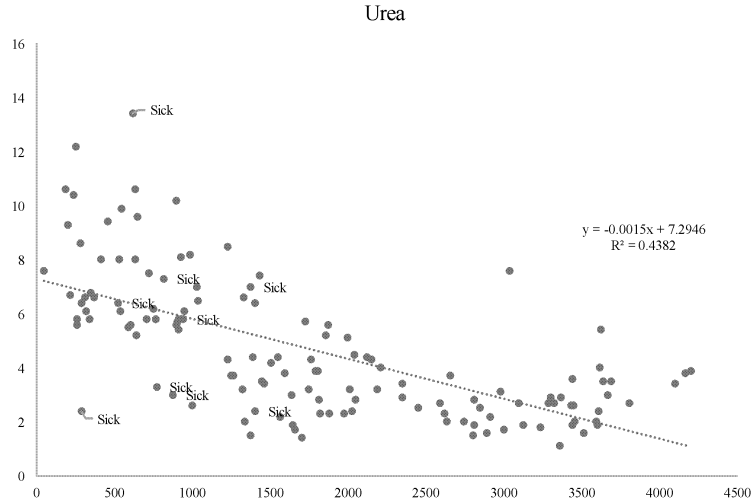

Magnesium
y = 4E-05x + 1.6234
R² = 0.0077
Sick


Phosphorus
y = -0.0003x + 4.6871
R² = 0.111
Sick


Potassium
y = -0.0003x + 5.7684
R² = 0.0273
Sick


Protein
y = 0.0023x + 37.95
R² = 0.1965
Sick


Sodium
y = 0.0018x + 192.71
R² = 0.036
Sick


Urea
y = -0.0015x + 7.2946
R² = 0.4382
Sick