Effects of graded dietary docosahexaenoic acid in combination with other long-chain polyunsaturated fatty acids in post-smolt Atlantic salmon (*Salmo salar*) : Performance characterisation, health and behavioural effects

Brett D. Glencross[1], Douglas R. Tocher[2], Chessor Matthew[3], J. Gordon Bell[2]

1. CSIRO Food Futures Flagship - CSIRO Marine and Atmospheric Research, GPO Box 2583, Brisbane, QLD 4001, Australia
2. Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland
3. Marine Environment Research Laboratory, Machrihanish PA28 6PZ, Scotland

(p) 61-7-3833-5926
(e) Brett.Glencross@csiro.au

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Abstract

A dietary dose-response study with varying docosahexaenoic acid (DHA; 22:6n-3) inclusion levels (1 g/kg, 5 g/kg, 10 g/kg, 15 g/kg and 20 g/kg) was conducted with post-smolt (111 ± 2.6g; mean ± S.D.) Atlantic salmon (*Salmo salar*) over a nine week feeding period. Further diets included DHA at 10 g/kg in combination with either eicosapentaenoic acid (EPA; 20:5n-3) or arachidonic acid (ARA; 20:4n-6), both included at 10 g/kg, and a diet where both EPA and DHA were included at 5 g/kg (total of 10 g/kg of long-chain polyunsaturated fatty acids, LC-PUFA). Fish were fed using a pair-feeding feeding regime to eliminate feed and energy intake variability. Fish were weighed every three weeks, and carcass, blood and tissue samples collected after nine weeks. Behavioural parameters were assessed weekly. A minor improvement in growth was seen with increasing inclusion of DHA. However, the addition of EPA provided a further improved growth response while addition of ARA had no effect on growth. An improvement in feeding behaviour was seen with increasing DHA up to 10 g/kg, and addition of EPA or ARA had only minor effects on behavioural responses. Temporal differences were observed in the survival of fish, with the addition of ARA resulting in a progressive decline in survival relative to fish fed the other diets. In contrast, the survival of fish on the low DHA diet (1 g/kg) was initially high but declined from 6 weeks onwards. As expected, the fatty acid composition of whole body lipid largely reflected the diets. Deposition efficiency of dietary fatty acids was generally unresponsive to the different dietary treatments with the notable exception of DHA. At very low inclusion levels DHA deposition efficiency was substantially higher (~300 %) than that for all other inclusion levels (31 % to 58 %). The inclusion of EPA in the diet also had a positive effect on the deposition efficiency of DHA but EPA deposition efficiency was variable. Deposition efficiency of ARA was unaffected by DHA inclusion, but addition of either EPA or ARA resulted in a substantial reduction in the deposition efficiency of ARA. In the present study, the results suggested that inclusion of DHA of at least 10 g/kg diet is optimal. Addition of either EPA or ARA had a nominal influence on the effects of DHA, although inclusion of EPA appeared to improve performance. When the total n-3 LC-PUFA content of the diet was the same but consisted of either DHA alone or as a combination of EPA plus DHA the performance effects were similar, but behavioural effects in this study were more related to DHA content than total n-3 LC-PUFA content.

1. Introduction

The relatively static global supply of fish oil resources has increased the level of alternative (vegetable and terrestrial animal) oil resources being used in fish feeds (Tacon and Metian, 2008). Many alternatives have been evaluated in a wide range of fish species over the last few years and, in most cases, it has been demonstrated that high-level replacement of fish oils is possible (Sales and Glencross, 2010). However, most of the alternatives now commonly being used (e.g. rapeseed, soybean and poultry oils) are notable in their lack of long-chain polyunsaturated fatty acids (LC-PUFA) and, with increasing use of these alternative lipid sources and the concomitant use of alternative protein resources rather than fish meals, some diets are beginning to encroach on documented dietary levels of essential fatty acids (EFA) for some species (Glencross, 2009).

Most aquatic species have some form of requirement for EFA as dietary nutrients (reviewed by Glencross, 2009). However, which specific fatty acid (n-3 or n-6, long-chain or C18) satisfies EFA requirements, their concentration in the diet, and how the value of an EFA is influenced by the presence of other dietary fatty acids appears to vary markedly among species (Glencross and Smith, 2001). It is generally considered that marine species have a higher, or more defined requirement, for the LC-PUFA docosahexaenoic acid (DHA; 22:6n-3) or eicosapentaenoic acid (EPA; 20:5n-3), while diadromous species have a lower requirement and some freshwater species appear to have no requirement at all for LC-PUFA (Bell et al., 1986; Castell et al., 1994; Tocher, 2003; Glencross, 2009).

Unlike most marine species salmonids possess the ability to elongate and desaturate α-linolenic acid (LNA; 18:3n-3) to produce EPA and DHA (Castell et al., 1972a; b; Thomassen et al., 2012). However, in the absence of dietary LNA or other n-3 LC-PUFA rainbow trout have been shown to produce elevated levels of 20:3n-9 (Castell et al. 1972c). It has been suggested that the ratio of 20:3n-9 to DHA in liver phospholipids of rainbow trout serves as an indicator of EFA deficiency (Castell et al. 1972b). In terms of the dietary requirement of salmonids for n-3 LC-PUFA, this has been reported to range from 10 g/kg to 25 g/kg of the diet depending on species and experimental conditions (reviewed by Glencross, 2009). The early studies of Castell et al. (1972a; 1972b) with rainbow trout (*Oncorhynchus mykiss*) focussed on the requirement for linoleic acid (LOA; 18:2n-6) or LNA and found that growth was significantly better with LNA over LOA or an EFA deficient diet. However, the value of LNA as an EFA for trout exists only because it has significant ability to desaturate and elongate LNA to the biologically active LC-PUFA, EPA and DHA (Castell et al., 1972a,b; Thomassen et al., 2012). These studies also demonstrated that there was no requirement for n-6 PUFA by rainbow trout (Castell et al., 1972a,b), though this notion was later challenged by the assertion that trout may require small amounts of n-6, specifically arachidonic acid (ARA; 20:4n-6), for prostaglandin and leukotriene synthesis (Henderson et al., 1985; Villalta et al., 2008).

Although the early work of Castell et al. (1972a, b) defined a requirement for n-3 PUFA, it was a series of studies on Atlantic salmon fry undertaken by Ruyter et al. (2000a) that went on to

examine the quantitative and qualitative EFA requirements for LOA, LNA and, EPA and DHA in combination. It was shown that inclusion of LNA, and EPA and DHA in combination both provided significant nutritional benefits to the fish. Of all treatments examined, the 50:50 combination of EPA and DHA at 10 g/kg provided the greatest benefit. Interestingly, Ruyter et al. (2000a) also showed poorer growth of fish fed 20 g/kg of either LNA or EPA and DHA. This was consistent with the earlier reports of Yu and Sinnhuber (1979), who also reported that excess levels of LC-PUFA had a negative impact on growth. Therefore, there is accumulating evidence that providing an excess amount of n-3 LC-PUFA in the diet can have adverse effects on growth and food utilization. However, in the work by Ruyter et al. (2000a) the roles of the LC-PUFA of DHA and EPA were only examined in combination. Recent studies with Asian seabass (*Lates calcarifer*; aka barramundi) have reported discrete effects of DHA alone or in the presence of EPA, and verified the synergistic importance of both (Glencross and Rutherford, 2011). Additionally, the influence of the n-6 LC-PUFA, ARA, has also been assessed and showed some contrasting effects to that observed with the inclusion of EPA, reflective of the competing roles that these fatty acids have in the pathways for eicosanoid synthesis (Terano et al., 1986; Garg et al., 1990; Garg and Li, 1994; Bell et al., 1995). A more recent study by Codabaccus et al., (2012) also examined the response of post-smolt Atlantic salmon to DHA. However, this study showed no growth effects to the manipulation of the dietary fatty acid profile, but we believe this result was constrained by the design of the experiment.

Based on the earlier work by Ruyter et al (2000a), it was hypothesized that post-smolt Atlantic salmon will have a quantitative dietary DHA requirement of around 10 g/kg. Based on studies with Asian sea bass it was also hypothesized that the response to this dietary level of DHA may be affected by the presence of other LC-PUFA in the diet (Glencross and Rutherford, 2011). The present study therefore examined the inclusion of incremental levels of dietary DHA on performance attributes of post-smolt Atlantic salmon. The study was not primarily designed to determine optimal feed specifications but rather aimed to specifically investigate the requirement for dietary DHA and its interaction with other dietary LC-PUFA. Accordingly, a paired-feeding strategy was adopted to eliminate the confounding effects of variation of intake of other dietary nutrients and energy. The study included a further three treatments to examine the effect of dietary EPA and ARA on the response of this species to dietary DHA. Whilst the primary parameter evaluated in this study was growth performance it was hypothesized that many of the dietary effects would be sub-clinical and therefore a range of biochemical, compositional, health and behavioural studies were also included to elucidate the specific mechanisms of DHA and possible interactions with other key LC-PUFA in the diet.

2. Materials and Methods

2.1 Diet manufacture and management

A single basal diet was formulated to provide protein and lipid at 460 g/kg and 200 g/kg diet at a gross energy level of 22.0 MJ kg^{-1} (estimated digestible protein and energy of 440 g/kg and 19.5 MJ kg^{-1}, respectively). Of the dietary lipid, 185 g/kg was vacuum-infused post-extrusion and it was at this point that the different treatments were made by infusing different oil blends. The fishmeal was defatted by hexane extraction followed by drying at 60 ºC for 24 h to remove all residual solvent. The nutrient compositions of the main dietary ingredients are presented in Table 1. To produce the basal diet pellets, the dry ingredients were blended in 30 kg batches to make a pellet mash using a 60 L upright Hobart mixer (HL600, Hobart, Pinkenba, QLD, Australia). The mash was pelletized using a laboratory-scale twin-screw extruder (MPF24:25, Baker Perkins, Peterborough, United Kingdom). Extruder configurations were as defined in Glencross et al. (2012). A single 130 kg batch of basal diet was extruded using the same operational parameters for consistency.

A series of five DHA inclusion levels (D1, D5, D10, D15 and D20) were created by blending oils including an algal-derived (*Crypthecodinium* sp.) DHA oil source (DHASCO) and a blend of butterfat and olive oil to provide the lipid base (Table 2). Three additional treatments were included to examine the accessory inclusion of ARA or EPA. The ARA was added as the fungal-derived concentrate (ARASCO) to provide inclusion levels of 10 g/kg each of ARA and DHA (D10A). As there was no equivalent EPA concentrate equivalent to DHASCO or ARASCO, anchovy oil containing both EPA and DHA in equal amounts was used to formulate the DHA/EPA diets, with addition to provide inclusion levels of either 10 g/kg (D10E) or 5 g/kg (D5E) each of EPA and DHA. Full diet compositions are given in Table 3. The oil blends were prepared prior to vacuum coating and were thoroughly mixed before being applied. The butterfat was melted at 60 ºC for 2 h and any remaining solids decanted to waste prior to addition to the oil blends. Dry basal pellets were placed in a mixer (Hobart, Sydney, Australia) and the prescribed allocation of oil blend applied whilst mixing, after which the bowl was sealed with a Perspex lid and a vacuum applied. Once all visible signs of air escaping from the pellets had ceased, the vacuum chamber was re-equilibrated to atmospheric pressure and the oil infused into the pellet.

2.2 Fish management

Pre-smolt Atlantic salmon were sourced from Howietoun fish hatchery (Bannockburn, Scotland) and transferred to the Marine Environmental Research Laboratory (MERL, Machrihanish, Argyll, Scotland). At MERL the fish were allocated to two 10,000 L seawater tanks and on-grown to 110.9 ± 2.61 g (mean ± S.D) prior to the experiment. All fish were anesthetised using benzocaine prior to handling. The fish were weighed on an electronic top-loading balance to 0.5 g accuracy and 20 fish allocated to each of 24 x 500 L tanks. Fish were re-weighed at day 21, day 42 and finally on

day 62 of the experiment. The experiment was conducted in a flow-through, ambient water temperature, 500L x 24-tank array. Water temperature was 14.0 ± 0.82 ºC (mean ± S.D.) and dissolved oxygen was at 7.8 ± 0.60 mg L^{-1} (mean ± S.D.) for the duration of the 62-day experiment. Three replicates (tanks of 20 fish) were used for each treatment.

During the experiment, feeds were provided on a restricted pair-wise feeding regime with uneaten feed collected to accurately determine feed intake per tank (Helland et al., 1996). A correction factor was applied to recovered uneaten pellets to account for soluble losses incurred between feeding and collection to improve accuracy of feed intake assessment. The initial restrictive rations were estimated based on an 80 % demand as estimated for a 19.5 MJ kg^{-1} diet at 12 ºC using a bioenergetic model for salmon (B. Glencross, unpublished). Subject to all rations being consumed by each tank, the ration allocations were incrementally increased uniformly by 0.25 g $fish^{-1}$ each week from a base allocation of 1.0 g $fish^{-1}$. Total feed fed per fish is presented in Table 4.

2.3 Sample preparation and analysis

At the beginning of the study, six fish representative of the initial population were euthanized, dried of residual surface moisture and frozen for subsequent compositional analysis in three lots of two fish. At the end of the experiment (day 62), after final weighing, five fish from each tank were similarly sampled and frozen for compositional analysis. The fish from each tank were minced together after defrosting and a sample taken for dry matter analysis and another frozen prior to being freeze-dried. The freeze-dried whole fish samples were milled to a fine powder before being analysed for dry matter, nitrogen (protein), ash, total lipid content, and fatty acid compositions. Blood samples (~10 mL total) were collected from the caudal vein of an additional two fish per tank and pooled within a single Falcon™ tube, containing 100 IU of lithium heparin. About half the blood was transferred to three Eppendorf™ tubes before being centrifuged at 1000 x g for ~2 min to sediment the erythrocytes and the plasma transferred to a Cryotube™ prior to being frozen in liquid nitrogen. The remaining blood was kept on ice and haematological analysis performed within 24 h of collection (Scottish Agricultural College Veterinary Services, Penicuik, Scotland). Clinical biochemical analysis was performed on frozen plasma using an automated chemistry analyser (AU400, Olympus Optical Co. Ltd) using standard assay kits developed for the auto-analyser (Scottish Agricultural College Veterinary Services, Penicuik, Scotland).

Moisture and ash contents of diets and fish were determined according to standard procedures (AOAC, 2000). Dry matter was assessed gravimetrically following oven drying at 105 ºC for 24 h, and gross ash content was determined gravimetrically after combustion in a muffle furnace at 550 °C for 12 h. Energy contents of the diets were measured by bomb calorimetry using a Parr 6200 calorimeter according to standard procedures. Crude protein levels were calculated from the nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyser, Foss, Warrington, UK). Lipid contents were determined gravimetrically after extraction according to Folch

et al. (1957). Fatty acid compositions were determined by gas chromatography of methyl esters essentially according to Christie (2003). Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980; Tocher and Harvie, 1988), and quantified using Chromcard for Windows (version 1.19).

2.4 Behaviour analysis

Two methods were used to assess the behavioural response of tanks of fish. Method one scored feeding activity after one round of feed had already been fed as described previously for Asian sea bass (Glencross and Rutherford, 2011). A score of 2 was given when fish actively came to the surface and pursued feed, a score of 1 for fish feeding actively but not breaking the surface to pursue feed, and a score of 0 for fish that were slow to feed and appeared lethargic in their behaviour. This semi-subjective assessment was carried out by the same person once a week for each of the nine weeks of the study. Scores were averaged across time to give a repeated-measures response to each tank, and each tank was used as a replicate within each treatment. This is similar to that which was reported in Glencross and Rutherford (2011). However, the behaviour of the salmon in this experiment was substantially different to that of the Asian sea bass in the work by Glencross and Rutherford (2011), with the fish always remaining timid and never being able to relate the presence of a person to feed allocation. As such the behaviour scoring was more difficult in the case of salmon.

A second, more objective, indicator of feeding behaviour, the number of pellets remaining in the feed container after two rounds of feeding relative to the total number of pellets allocated, was determined. The fish were fed to a point of notable decline in active feeding before cessation of feeding. This assessment was also carried out by the same person once a week for each of the nine weeks of the study except weeks 5 and 7. Scores were again averaged across time to give a repeated-measures response to each tank and each tank used as a replicate within each treatment.

2.5 Physical health assessment

At the end of the study, a series of physical condition features were noted and recorded for all fish within each tank. The incidence of conditions such as pectoral fin erosion, caudal fin reddening or erosion, skin lesions/scale loss, pin-heading and eye malformations were assessed to give a percentage value for each parameter for each tank. A systemic outbreak of amoebic gill disease (AGD) was also present in the facility during the experimental period at low levels and was controlled by giving all fish a 2 h freshwater bath treatment once a month. Accordingly, at week 9 an AGD gill score was given for each fish based on that reported by Taylor et al. (2009). The scoring for health parameters was carried out by the same person for all fish and at each time point.

232 *2.6 Statistical analyses*

233 All figures are mean ± SEM unless otherwise specified. Effects between D10, D20, D10A,
234 D10E and D5E were examined by ANOVA using the software package Statistica (Statsoft®, Tulsa,
235 OA, USA). Levels of significance were determined using an LSD planned comparisons test, with
236 critical limits being set at $P < 0.05$. Effects of DHA inclusion level (D1, D5, D10, D15 and D20) were
237 analysed by regression analysis.

238 Relative deposition efficiency (%) of specific fatty acids (DHA, EPA, ARA, LNA and LOA)
239 was calculated using the mean intake per fish in each tank and the mean gain in mass of specific fatty
240 acids by fish in that tank, over the duration of the study, to give tank-specific values that were then
241 used to derive a treatment mean. The formula used was based on that reported by Glencross et al.,
242 (2003), where *FAf* is the absolute amount of a specific fatty acid in the fish at the end of the study and
243 *FAi* is the absolute amount of that specific fatty acid in the fish at the beginning of the study. *FAc* is
244 the amount of that specific fatty acid that the fish consumed over the study period, such that:
245

$$Fatty\ Acid\ Deposition\ Efficiency\ (\%) = \left(\frac{FAf - FAi}{FAc}\right) \times 100$$

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3. Results

3.1 Fish growth, feed utilisation and survival

Fish in this study generally grew at an equivalent rate to those reported in Codabaccus et al. (2012), and at a better rate than those reported by Miller et al. (2007), despite being pair-fed in the present study and to apparent satiety in the other studies. A significant effect of dietary fatty acid composition on growth (as final weight, weight gain and gain rate) after 9-weeks was observed (Table 4a and 4b). No significant differences were seen among the different DHA inclusion levels when analysed using regression (Table 4a), but fish fed diet D10E had significantly better growth than fish fed the diets with the two lower and the two higher DHA inclusion levels (Table 4b). Interestingly, during this period of growth the fish fed the D10A treatment was best.

As intended, there were no significant differences in feed intake among any of the treatments (Table 4a,b). Some significant differences among the treatments for FCR were observed. Both of the EPA treatments showed significantly lower FCR than the lower DHA inclusion levels (D1 and D5). No significant improvements in FCR were noted with increasing DHA inclusion based on regression analysis (Table 4a). No other significant differences in FCR were present.

No significant effects of treatment on survival were noted with increasing DHA content when based on regression, albeit $P = 0.060$). Fish fed the D10A treatment had the poorest survival of all treatments but, despite a difference of 11 % between this treatment and the treatment with highest survival (D5E), variability among replicates meant this was not significantly different (Table 4). However, notable were both the consistent decline in survival of fish in the D10A treatment and the sudden decline in survival from day 40 onwards, prior to the day 42 weighing event, in fish fed D1.

3.2 Tissue composition and fatty acid retention

The fatty acid composition of fish largely reflected that of the dietary treatments (Table 5). As expected there was an increasing percentage of DHA in the tissues of fish fed increasing dietary DHA content. However, the concentration of DHA was typically always marginally higher than that of the diet. Somatic EPA content declined substantially from the initial fish in all treatments and at the end of the experiment represented around 3 % of total fatty acids (Table 5). Even with the treatment containing a higher level of added EPA it still only represented 4 % of the total fatty acids. Somatic ARA was low in fish fed all treatments (< 1 %) except for fish fed diet D10A where it increased to around 3 % of the total fatty acids in the fish (Table 5). Total SFA content of fish at the end of the experiment was generally very consistent at around 30 % of total fatty acids (range 29 – 32 % of total fatty acids). MUFA content of fish from each of the treatments ranged from 35 % in the initial population sample to 51 % in fish fed the D5 treatment. Among most treatments there was more variation in MUFA content than the SFA content (range 44 % to 51 %). The proportions of total LC-PUFA in the whole body tissues of the treatment diet fed fish varied from 11 % to 17 %. It was

highest in the treatment fed the D20 diet and lowest in the fish fed the D5 diet. However, the initial fish had the highest LC-PUFA content of all at 23% of total fatty acids. There was only a minor treatment effect in whole body content of LC-PUFA with increasing DHA inclusion in the diet (range 11% to 17%). Even the lowest inclusion level of LC-PUFA (the D1 diet) was relatively conserved at a total LC-PUFA level of 12% of total fatty acids.

There was considerable difference in the fatty acid deposition efficiencies of the different PUFA and LC-PUFA. DHA deposition efficiency was the most dramatically affected by DHA inclusion level, but not by the inclusion of either EPA or ARA in the diet. At the lowest inclusion level of DHA the deposition efficiency exceeded 300 %, but as DHA inclusion increased there was a curvilinear decline in deposition efficiency such that at the next inclusion level of DHA (D5) retention had declined to around 58 % and by the highest DHA inclusion level (D20) the deposition efficiency had declined to just over 30 % (Fig. 1a).

The deposition efficiency of EPA was clearly affected by DHA inclusion level, and also by the inclusion of either EPA or ARA in the diet. At the lowest inclusion level of DHA, negative deposition efficiencies of EPA were observed. Deposition efficiency of EPA increased in a curvilinear fashion with increasing DHA in the diet before declining again to negative deposition efficiency at the highest DHA inclusion (Fig. 1b). However, among the D10, D10A and D10E diets there were marked differences. Addition of ARA reduced EPA deposition efficiency to -18 %, while inclusion of EPA or DHA increased it to 18 % or 28 %, respectively.

In contrast, the deposition efficiencies of ARA, LNA and LOA were largely unaffected by DHA inclusion level with, in most cases, a consistent level of retention of each of the respective fatty acids. However, the level of retention of each fatty acid varied substantially among each other. Thus, ARA retention was generally high at around 180 %, and addition of EPA or ARA to the diet at the 10 g/kg inclusion level reduced ARA retention to around 40% (Fig. 1c). The lower inclusion level of EPA had little effect on the efficiency of ARA retention. Deposition efficiency of LNA was also largely unaffected by DHA inclusion level (Fig. 2a). Although there was some variation in the LNA deposition efficiency there were no consistent patterns in response to DHA dose, or EPA or ARA inclusion. Similarly, deposition efficiency of LOA was also unaffected by DHA inclusion level, or the inclusion of ARA or EPA in the diet (Fig. 2b).

3.3 Fish behaviour

There were some significant differences among the treatments in the assessed behaviour indices (Fig. 3a,b). There was a relatively clear and significant dose response effect between both behavioural indices and the DHA content of the diet. The effect was most obvious with the more subjective assessment of a behaviour score, but these results were a direct inverse reflection of the completely objective assessment based on the proportion of pellets remaining after a defined feeding period. Both the EPA treatments (D10E and D5E) and the ARA treatment (D10A) showed improved

behaviours relative to the low LC-PUFA diet (D1), although these effects could not be fully discriminated from dietary DHA content.

3.4 Plasma chemistry

There were several significant differences among the plasma chemistry parameters relative to dietary treatment (Table 6). Only those parameters that showed significant effects that could be clearly attributed to dietary treatment are discussed below.

There was a significant increase in plasma activities of the liver marker enzymes, alanine aminotransferase and asparagine aminotransferase, with the inclusion of ARA in the diet, but varying the levels of DHA or inclusion of EPA had little or no effect on their activities. Although there were significant differences in activities of glutamate dehydrogenase among the treatments a consistent pattern was not clearly observed. Creatinine levels were not significantly influenced by the level of DHA in the diet, but were significantly affected by the inclusion of ARA or EPA. Plasma calcium levels were significantly different among the treatments with a pattern suggesting a trend towards lower concentrations with increasing dietary DHA. The addition of EPA to the diet increased plasma calcium to significantly greater levels than those in fish fed the equivalent diet with DHA, though addition of ARA did not have the same effect. Plasma cholesterol levels were higher in fish fed the diet D10E. Red blood cell counts showed a significant suppression in the ARA treatment relative to the EPA treatment and also some of the DHA treatments but there did not appear to be a dose response to dietary DHA.

3.5 Physical health assessment

A range of physical pathology signs were assessed at week 9 (Table 7). It was notable that fish fed the low LC-PUFA diet (D1) showed few signs of physical pathologies. Increasing dietary DHA initially increased signs of physical pathology (D5 and D10) but these declined at higher levels (D15 and D20. The only notable effects attributable to EPA or ARA inclusion were increased incidence of pectoral fin erosion (D10E) or scale-damage (D10A).

4. Discussion

4.1 What is so essential about essential fatty acids?

The results from the present study provide empirical evidence of the roles of DHA and DHA in combination with either EPA or ARA in the diets of Atlantic salmon. Despite much evidence of the essentiality of dietary n-3 LC-PUFA, the present results suggest that the essentiality is relatively minor in salmon. However, the findings support that, while DHA alone can satisfy most requirements, the addition of EPA stimulates performance on a range of levels beyond that achieved with DHA alone. This suggests that EPA plays an equally important role in lipid metabolism in this species. Notably, dietary EPA along with DHA promotes better growth and health than that achieved by the same level of DHA alone suggesting potential of distinct roles for each LC-PUFA. It was also shown that the inclusion of ARA in addition to DHA had little effect on growth, but had effects on animal health. The outcomes of the present study in salmon were generally consistent to those reported in a similar study with Asian seabass (barramundi), although the current study also showed distinct differences, particularly in regards to the clinical observations (Glencross and Rutherford, 2011). These findings raise the question of why such differences between Atlantic salmon and Asian sea bass were observed and whether they relate to the environmental preferences/lifecycle of each species. To address this question it is pertinent to first consider the effects in relation to previous research in this area to examine why some findings were different and others similar.

It had been reported that the dietary requirement of salmonids for n-3 LC-PUFA was in the range from 10 g/kg to 25 g/kg of the diet depending on species and experimental conditions (see Glencross, 2009). The first comprehensive series of studies on EFA requirements of a salmonid were those of Castell et al. (1972a, b) with rainbow trout (*Oncorhynchus mykiss*). In those early studies, fish were fed one of four diets in which treatments included a fat-free diet, a diet in which the 50 g/kg lipid in the diet was provided as 18:1n-9, a diet with 40 g/kg 18:1n-9 and 10 g/kg LOA, and a diet with 40 g/kg 18:1n-9 and 10 g/kg LNA. The results of this work demonstrated that salmonids had a defined n-3 requirement, albeit as LNA in this case. However, the value of LNA as an EFA for trout was proposed to exist only because it had significant ability to desaturate and elongate LNA to the biologically active LC-PUFA, EPA and DHA (Tocher, 2003). In the present study it was interesting to note that the fish fed the D1 diet (albeit not completely devoid of LC-PUFA) showed relatively good survival and growth. Indeed classic signs of EFA deficiency were not observed even after 62 days of the experiment. However, the most obvious feature of fish fed that diet was the sudden decline in performance after about 40 days. If the initial decline in survival around day 7 is discounted (probably due to handling losses) then the effects observed on survival in this experiment are even more telling. The results provide good support that the fish were relying on endogenous stores of LC-PUFA during this initial period before levels reached critical limits. Interestingly, if the growth response to DHA dose is examined solely from week 6 onwards a much clearer response to DHA can

be observed, which further affirms the notion of a requirement for this fatty acid and also that the optimal requirement level is around 10 g/kg. In retrospect it would have been of value to have continued this study for longer to allow these effects to consolidate.

Over the past decades, since the foundation work of Castell (1972a, b), there have been further studies to ascertain what the critical requirement (quantitative or qualitative) might be for the n-3 LC-PUFA, EPA and DHA, in salmonid species (Brodtkorb et al., 1997; Ruyter et al., 2000a,b). An important methodological point of difference in the present study, compared to previous studies, was the use of a pair-fed feeding regime to isolate other nutrient intake effects. In using this strategy we have demonstrated that the requirement for DHA by Atlantic salmon is relatively minor, and have been able to isolate the confounding effects of feed intake variability and thereby remove protein and energy intake effects from the interpretation.

It has also been previously reported that an excess amount of n-3 LC-PUFA in the diet can have an adverse effect on growth and food utilization by fish. An increase in EPA or DHA content to four-times the proposed requirement level reportedly resulted in poorer growth and feed efficiency, and the fish showed signs of EFA deficiency (Takeuchi and Watanabe, 1976). However, almost every modern salmonid diet tends to have lipid levels almost double those used in the 1970's and 1980's and, until recently, dilution of fish oil with alternatives (largely vegetable oils), these diets also had EPA + DHA inclusion levels considerably above the reported threshold without the apparent negative effects of inclusion of high amounts of n-3 LC-PUFA reported previously (Takeuchi and Watanabe, 1976; Yu and Sinnhuber, 1976; 1979). However, some recent studies have demonstrated that high inclusion levels of LC-PUFA can have a significant negative effect on fish performance and health (Ruyter et al., 2000a; Ostbye et al., 2011; Betancor et al., 2011; Glencross and Rutherford, 2011). In the present study, such negative effects with the highest inclusion level of DHA, or a similar LC-PUFA inclusion level of EPA and DHA, did not result in increased mortalities, levels of physical pathologies, or aberrations in plasma biochemistry or haematology.

Formative studies by Ruyter et al. (2000a,b) used a series of trials on Atlantic salmon fry to determine their quantitative (0, 1, 2, 5, 10 and 20 g/kg of the diet) and qualitative requirements for LOA, LNA, and EPA and DHA in combination. These diets had relatively low lipid levels (~80 g/kg) in which the inclusion of LNA, and EPA and DHA in combination both provided significant benefits to the fish. The combination of EPA and DHA at 12.5 % total fatty acids provided the greatest benefit of all the treatments studied. While the data generally support this, the effects observed in the present study with post-smolt Atlantic salmon where not as dramatic.

4.2 Fish growth and feed utilisation

The growth of the fish in the present study, using the pair-fed regime, demonstrated that the stimulatory effect of DHA is minor at best. Indeed, although addition of EPA to the diet produced a significant improvement in growth relative to that observed in fish fed the low LC-PUFA diet, the

effect was still not as pronounced as that reported in some other species (reviewed by Glencross, 2009). However, this observation showed that, while DHA may be important to development in this species, it cannot be provided in exclusion to EPA and indeed low levels of dietary LNA may be sufficient to offset the requirements for either LC-PUFA based on the fact that Atlantic salmon has demonstrated capacity for endogenous LC-PUFA biosynthesis (Tocher et al., 2003). However, despite evidence of the importance of dietary EPA in addition to DHA, further exploration of the relationship between the n-3 LC-PUFA and the importance of the ratios between these key PUFA is required (Furuita et al., 1998).

A potential negative aspect of using a pair-fed feeding regime though is that the fish will grow somewhat slower than that expected of fish fed to satiety. However, in the present experiment the growth rates of the fish were equal to or better than that of Atlantic salmon parr and post-smolt fed to satiety in other recently published works (Miller et al., 2007; Codabaccus et al., 2012). Ironically the fish in the present study doubled their weight in 62 days compared with those in the study of Codabaccus et al. (2012) which doubled their weight from 71 g to 148 g in 75 days. However, consistent with the present study, Codabaccus et al. (2012) also found limited influence of the fatty acid composition on the growth or feed utilisation by post-smolt Atlantic salmon. These authors also stated that varying the ratio of EPA to DHA had no significant effect on growth or feed utilisation in post-smolt Atlantic salmon. Other studies with Atlantic salmon have shown that fish grew best with an inclusion of 10 g/kg LC-PUFA in their diet, when this was provided as an equal ratio of EPA to DHA (Ruyter et al., 2000a). Rainbow trout also grew best with an EPA to DHA ratio of 1 : 1, but with a total LC-PUFA inclusion level of 3 g/kg (Watanabe and Takeuchi, 1976). However, these authors also reported that EPA could be omitted from the diet and the LC-PUFA supplied solely as DHA to achieve the same effect (Watanabe and Takeuchi, 1976). Arguably this might also be possible with Atlantic salmon based on the present results, although the marginal improvements in performance observed with both EPA and DHA in the diet suggested both these LC-PUFA are possibly required.

4.3 Tissue composition and fatty acid retention

The changes in tissue fatty acid composition of fish fed each diet were consistent with those reported in most other studies on fish fed different lipid sources in that the tissue fatty acids were largely reflective of the respective diets (Sargent et al., 1999). However, it is the subtleties around the examination of the mass-balance relationship between dietary and tissue fatty acid compositions through the analysis of the deposition efficiency that often show distinct differences in the utilisation of different dietary fatty acids, particularly in the case of DHA intake (Glencross et al., 2003; Glencross and Rutherford, 2011).

The observation that DHA deposition efficiency decreased with increasing dietary DHA were consistent with other reports on utilisation of this fatty acid (Glencross et al., 2003; Glencross and Rutherford, 2011). At the lowest inclusion level (1 g/kg) the very high deposition efficiency (>300%)

461 suggested that there was high conservation and/or possible endogenous synthesis of DHA (Glencross
462 et al., 2003; Turchini and Francis, 2009). Certainly it is well established that Atlantic salmon have this
463 capability (Tocher et al., 2003; Thomassen et al., 2012). The negative deposition efficiency values of
464 EPA at both low and high DHA levels supports the position that some chain elongation and
465 desaturation of precursor fatty acids occurred, but this pattern of EPA deposition efficiency contrasts
466 with that observed in a similar study on Asian sea bass where there was clear retroconversion of DHA
467 to produce EPA (Glencross and Rutherford, 2011). However, the curvilinear response in EPA
468 deposition efficiency, peaking at a DHA inclusion level of 10 g/kg, is perhaps also supportive of
469 defining the optimal dietary inclusion level of DHA. As expected, the addition of EPA to the diet
470 resulted in relatively low deposition efficiency of EPA, but the addition of ARA to the diet produced
471 an even more interesting response in that it also induced a negative deposition efficiency of EPA. This
472 may be attributable to an increase in flux of EPA in the eicosanoid pathways, the activity of which
473 may have been heightened by the additional dietary ARA (Bell et al., 1995; Ghioni et al., 2002).
474 Notably there is a distinct symmetry in EPA retention between EPA and ARA diets, and this
475 counteractive concentration/response effect has been noted in other species (Xu et al., 2010). The
476 deposition efficiency of ARA was substantially higher (~180 %) than that reported in the counterpart
477 study (~120 %) with Asian sea bass (Glencross and Rutherford, 2011). ARA retention in Atlantic
478 salmon was largely unaffected by DHA inclusion, although the addition of EPA or ARA to the diet at
479 the 10 g/kg inclusion level resulted in a significant reduction in ARA retention to around 40 %,
480 although there were few effects of varying DHA inclusion level. This observation on ARA retention
481 contrasts with the competing symmetry effect noted for EPA deposition efficiency with EPA and
482 ARA inclusion.
483 The absence of any clear dose response effects of DHA on LNA (or LOA) deposition
484 efficiency suggested that this C18 PUFA appeared to be playing a limited role in DHA supply through
485 possible elongation and desaturation processes. This may be being restricted by the relatively low
486 levels of LNA in the diet (<1% of total fatty acids).
487
488 *4.4 Fish behaviour*
489 Previous studies on DHA dose response effects in Asian sea bass reported some distinct
490 behavioural elements attributed to DHA (Glencross and Rutherford, 2011). Therefore, feeding
491 behaviour was examined in the present study to determine if there was also a dose-response effect of
492 DHA in Atlantic salmon. Although salmon were more skittish in their behaviour compared to Asian
493 sea bass, a relative change in behaviour was still noted among the different DHA treatments. In the
494 present study, the complications in the behavioural assessment compared to that done in Asian sea
495 bass (Glencross and Rutherford, 2011) required both a reassessment of the behaviour score
496 parameters and also an additional, more objective, feeding behaviour assessment and, combined, they
497 provide confidence in our interpretation. Both methods showed that positive feeding behaviour

498 responded in a dose-response manner to increasing inclusion of DHA. This effect could not be
499 attributed to either EPA or ARA as the inclusion of DHA in each of the diets clearly accounted for the
500 responses observed and the addition of the other LC-PUFA did not alter these responses. Whether this
501 effect was via changes to brain function or visual development is unclear, but further analysis of brain
502 and eye compositions of fish from such a dose-response design may help clarify this issue (Tocher
503 and Harvie, 1988; Crawford, 1992; Brodtkorb et al., 1997). Interestingly, earlier work had more
504 strongly implicated dietary EPA as having a greater influence on the composition of lipids in neural
505 tissues, though an effect of DHA was also noted (Mourente and Tocher, 1992; Brodtkorb et al., 1997).
506
507 *4.5 Fish health and plasma chemistry*
508 There have been many reports examining the effects of LC-PUFA (both n-3 and n-6) on the
509 health of fish (Thompson et al., 1996; Richard et al., 2007; Ostbye et al., 2009; Betancor et al., 2011).
510 In the present study, the focus was on both a clinical assessment of physical signs of EFA deficiency
511 (poor growth, lesions, fin erosion) and also measurement of biochemical markers in plasma. Most
512 notable was the response to dietary ARA as indicated by increased scale damage/skin lesions. The
513 reduction in the level of fin damage with increasing levels of n-3 LC-PUFA was consistent with other
514 studies, although the relatively low levels of fin damage in the low LC-PUFA diet (D1) were perhaps
515 surprising (Castell, 1972a, b; Ruyter et al., 2000a).
516 The plasma biochemical markers perhaps provided a more objective assessment of the roles
517 of DHA, EPA and ARA in fish health, and in particular liver function (Glencross and Rutherford,
518 2011). However, the number of treatments and tests involved meant that significant, but clinically
519 irrelevant, effects may be observed and, therefore, the focus was on effects related to dose response,
520 or relative response to corresponding inclusion of different LC-PUFA. Notably, the present study with
521 Atlantic salmon showed a contrasting result to osmotic balance issues observed previously in Asian
522 sea bass in the relative absence of EPA in the diet (Glencross and Rutherford, 2011). In Atlantic
523 salmon there was little evidence of perturbations in urea, potassium or chloride levels. This suggested
524 that EPA, which plays an important role in regulating plasma osmolarity through eicosanoid
525 metabolism, was not particularly restricted in this study (Henderson et al., 1985; Beckman and
526 Mustafa, 1992). This could be a sign that EPA requirements for Atlantic salmon are either low, or that
527 endogenous biosynthesis was sufficient to maintain homeostasis.
528 More notable in the present experiment though were the changes in liver enzyme markers
529 such alanine aminotransferase, which showed an acute response to the presence of ARA, but was less
530 responsive to the inclusion of DHA or EPA. Other liver marker enzymes such as glutamate
531 dehydrogenase, however, also showed a dose response to dietary DHA, whilst still being elevated by
532 ARA inclusion. Inclusion of EPA resulted in a lower level of this enzyme activity in the plasma.
533 These observations provide some support to negative effects of ARA and DHA at high inclusion
534 levels (in the absence of EPA) on the liver health of this species.

4.6 Implications and conclusions

The present study showed that Atlantic salmon were not highly sensitive to dietary LC-PUFA manipulation and could perform relatively well with only low dietary levels of these fatty acids. However, the data indicated that dietary inclusion of 10 g/kg or above of DHA generally improved growth, feed conversion and feeding behaviour compared to fish fed a diet with 1 g/kg of DHA, albeit not all parameters were consistently significant. It was notable that the addition of EPA to the diet resulted in further improvements to growth and feed conversion, but did not appear to have an impact on feeding behaviour. In contrast to the results observed in similar studies, the absence of EPA in the diet did not induce any major pathologies (Glencross and Rutherford, 2011). Therefore, it is recommended, based on these findings, that at least 10 g/kg of DHA are required for optimal performance, but that 20 g/kg of EPA + DHA is preferable. Further investigation of whether the ratio of EPA to DHA can be altered and still achieve similar performance is required.

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Figure legends

Figure 1. Deposition efficiency (%) of DHA (a), EPA (b) and ARA (c) in fish from each of the experimental treatments. Treatments are indicated by (●) for those diets with incremental inclusion levels of DHA. Those diets with equivalent amounts of EPA (○) and ARA (Δ) are also indicated. Error bars show the pooled SEM.

Figure 2. Deposition efficiency (%) of LNA (a) and LOA (b) in fish from each of the experimental treatments. Treatments are indicated by (●) for those diets with incremental inclusion levels of DHA. Those diets with equivalent amounts of EPA (○) and ARA (Δ) are also indicated. Error bars show the pooled SEM.

Figure 3. Assessment of the behavioural responses of groups of fish within each treatment (n=3 tanks, error bars show the SEM for each treatment) using either of two assessment methods. (a) After two rounds of feeding in each tank the number of pellets remaining to be fed as a percentage of the total amount being fed was recorded, and (b) Fish were assessed as being highly-active (2), moderately active (1) or slow and lethargic (0) in terms of their response to being fed. Treatments are indicated by (●) for those diets with incremental inclusion levels of DHA. Those diets with equivalent amounts of EPA (○) and ARA (Δ) are also indicated.

760 **Tables and Figures**
761
762 Table 1. Nutrient compositions of major dietary ingredients (all values are g/kg DM unless
763 otherwise indicated).
764

Ingredient	SPI	WF	WG	DF	FO	DHA	ARA	OO	BF
Dry matter (g/kg)	93.7	87.7	93.1	94.8	99.7	99.9	99.6	99.9	99.9
Protein	94.6	12.6	82.2	71.8	0.4	0.6	0.8	0.9	0.6
Fat	1.1	1.1	2.9	2.0	94.0	96.4	92.9	97.3	94.6
Carbohydrate	1.0	85.7	14.2	8.4	5.6	3.0	6.3	1.8	4.8
Ash	3.3	0.6	0.7	17.8	0.0	0.0	0.0	0.0	0.0
Gross Energy (kJ/g)	22.6	16.0	21.7	18.7	39.1	39.1	38.4	39.3	37.5
All fatty acids are %TFA									
14:0	0.6	0.0	0.0	6.7	8.3	9.5	0.9	0.0	11.8
16:0	17.3	19.8	19.6	22.7	18.7	25.5	11.7	11.3	27.0
18:0	4.7	1.9	1.5	5.3	3.4	0.9	7.7	3.2	12.1
Total SFA	24.6	21.7	21.1	38.2	34.5	37.8	33.5	15.2	63.3
16:1n-7	0.8	1.0	0.0	7.1	10.2	1.9	0.3	0.8	1.0
18:1n-9	24.4	14.0	13.5	14.3	12.5	2.0	7.1	0.0	0.0
18:1n-7	1.9	1.2	1.0	0.0	0.0	0.0	0.0	73.0	31.4
Total MUFA	28.0	16.1	15.5	21.3	24.2	4.2	8.1	76.1	33.6
18:2n-6	41.7	56.0	59.7	1.8	1.3	0.5	0.0	8.2	3.1
18:3n-3	4.6	3.7	2.9	0.0	0.7	0.1	0.1	0.5	0.0
Total C18 PUFA	46.2	59.6	62.6	3.6	5.3	1.5	3.1	8.6	3.1
20:4n-6	0.0	0.0	0.0	1.5	1.1	0.9	49.7	0.0	0.0
20:5n-3	0.0	1.3	0.8	13.2	16.9	1.9	0.2	0.0	0.0
22:5n-6	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.0	0.0
22:6n-3	0.0	1.3	0.0	21.2	14.5	50.8	0.0	0.0	0.0
Total LC-PUFA	1.3	2.5	0.8	35.9	33.7	56.3	55.3	0.0	0.0
Total n-3	4.6	6.2	3.7	36.2	35.1	51.8	0.7	0.5	0.0
Total n-6	41.7	56.0	59.7	3.3	3.9	6.1	57.6	8.2	3.1
n-3 : n-6	0.1	0.1	0.1	10.9	9.0	8.5	0.0	0.1	0.0

765 SPI : Soy protein isolate, WF : Wheat flour, WG : Wheat gluten, DF : Defatted fishmeal, FO : Fish
766 oil, DHA : DHASCO, ARA : ARASCO, OO : Olive oil, BF : Butterfat.
767

768 Table 2. Formulations of the experiment diets (all values are g/kg).
769

	D1	D5	D10	D15	D20	D10A	D10E	D5E
Defatted Fish meal [a]	300.0	300.0	300.0	300.0	300.0	300.0	300.0	300.0
Pregelled starch [b]	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0
Wheat gluten [b]	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0
Wheat flour [b]	155.0	155.0	155.0	155.0	155.0	155.0	155.0	155.0
Soy Protein Isolate [c]	221.0	221.0	221.0	221.0	221.0	221.0	221.0	221.0
Fish oil [a]	0.0	0.0	0.0	0.0	0.0	0.0	75.0	30.0
Olive oil [d]	92.5	88.3	82.0	77.8	71.5	68.3	55.0	77.5
DHASCO [e]	0.0	8.4	21.0	29.4	42.0	21.0	0.0	0.0
ARASCO [e]	0.0	0.0	0.0	0.0	0.0	27.5	0.0	0.0
Butter fat [f]	92.5	88.3	82.0	77.8	71.5	68.3	55.0	77.5
L-Histidine [g]	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
L-Lysine [g]	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
DL-Methionine [g]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
L-Threonine [g]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Yttrium oxide [h]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$CaPO_4$ [g]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin/minerals [i]*	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0

770 [a] Fish meal (prior to being defatted): Chilean anchovy meal and oil, Skretting Australia, Cambridge, TAS, Australia. [b] Wheat
771 gluten, wheat flour and pregelatinised starch: Manildra, Auburn, NSW, Australia. [c] Soy protein isolate: ADM, Decatur, IL,
772 USA. [d] Refined olive oil: Conga Foods, Coburg North, VIC, Australia. [e] DHASCO and ARASCO oils: HuaTai BioPharm
773 Inc, Deyang, Sichuan, China. [f] Butterfat: Woolworths Dairies, Bella Vista, NSW, Australia. [g] Amino acids and monocalcium
774 phosphate: BEC Feed Solutions, Carole Park, QLD, Australia.. [h] Yttrium oxide: Stanford Materials, Aliso Viejo, California,
775 United States. [i]* Vitamin and mineral premix includes (IU kg^{-1} or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25
776 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3;
777 Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3
778 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g.
779
780
781

782 Table 3. Nutrient composition of experimental diets

	D1	D5	D10	D15	D20	D10A	D10E	D5E
Dry matter (g/kg)	958	967	952	961	943	921	946	944
Protein (g/kg DM)	525	526	511	513	521	519	517	518
Fat (g/kg DM)	181	176	204	205	204	178	186	182
Carbohydrate (g/kg DM)	186	239	230	253	206	214	194	213
Ash (g/kg DM)	82	72	68	69	71	86	82	74
Gross Energy (kJ/g)	22.3	22.4	23.1	22.7	22.1	22.7	22.5	23.0
Protein:Energy (g/MJ)	23.5	23.5	22.1	22.6	23.6	22.9	23.0	22.5
All fatty acid data are %TFA								
14:0	6.2	6.0	6.7	6.8	7.4	5.8	7.3	6.8
16:0	21.5	20.6	21.5	22.9	23.7	20.7	21.9	22.5
18:0	8.4	7.7	7.3	7.7	7.2	7.4	6.9	7.8
Total SFA	36.7	34.4	35.9	37.3	38.7	34.9	36.6	37.4
16:1n-7	1.4	1.3	1.8	1.9	2.1	1.4	5.1	3.2
18:1n-9	49.7	48.2	44.3	42.5	39.2	38.8	35.5	43.5
18:1n-7	4.0	3.9	3.7	3.7	3.7	3.1	3.8	4.0
Total MUFA	56.0	54.1	51.1	49.1	46.0	44.1	45.8	51.7
18:2n-6	5.8	6.7	5.9	5.3	4.9	6.5	5.6	6.1
18:3n-3	0.5	0.8	0.6	0.5	0.5	0.6	0.7	0.6
Total C18 PUFA	6.4	8.0	7.1	5.8	5.6	7.4	7.6	7.1
20:4n-6	0.1	0.1	0.1	0.1	0.1	5.1	0.4	0.1
20:5n-3	0.4	0.6	0.5	0.4	0.4	0.6	4.8	2.0
22:5n-6	0.0	0.6	1.3	1.5	1.6	1.3	0.0	0.0
22:5n-3	0.0	0.1	0.2	0.0	0.0	1.9	0.6	0.0
22:6n-3	0.5	2.0	3.6	5.7	7.6	4.1	3.9	1.7
Total LC-PUFA	1.0	3.4	5.8	7.8	9.8	13.5	10.0	3.8
Total n-3	1.4	3.8	5.3	6.6	8.5	7.1	11.3	4.7
Total n-6	5.9	7.7	7.7	6.9	6.8	13.8	6.3	6.2
n-3 : n-6	0.24	0.49	0.69	0.96	1.25	0.52	1.78	0.76

783 %TFA = percentage of total fatty acids.

Tables 4. Growth, feed utilisation and survival over the 62-day experimental period.

a	D1	D5	D10	D15	D20	R^2	F-value	P-value
Initial weight (g/fish)	110.8	112.5	110.7	113.7	109.2	0.022	0.070	0.808
Final weight (g/fish)	226.8	226.7	233.1	232.1	231.4	0.564	3.880	0.143
Weight gain (g/fish)	116.0	114.2	122.5	118.5	122.1	0.401	2.010	0.251
Gain rate d0-d62 (g/d)	1.87	1.84	1.98	1.91	1.97	0.401	2.010	0.251
Gain rate d42-d62 (g/d)	2.30	2.66	2.80	2.70	2.90	0.715	7.532	0.071
Feed intake (g/fish)	106.3	105.9	108.5	105.3	107.3	0.025	0.078	0.797
Feed Conversion (feed : gain)	0.95	0.96	0.90	0.90	0.90	0.534	3.440	0.161
Survival (%)	83%	85%	90%	88%	90%	0.744	8.709	0.060

b	D1	D10	D20	D10A	D10E	D5E	Pooled SEM
Initial weight (g/fish)	110.8	110.7	109.2	111.8	111.0	108.0	0.53
Final weight (g/fish)	226.8[a]	233.1[ab]	231.4[ab]	231.9[ab]	238.9[b]	229.6[ab]	1.49
Weight gain (g/fish)	116.0[a]	122.5[ab]	122.1[ab]	120.1[ab]	127.9[b]	121.6[ab]	1.39
Gain rate d0-d62 (g/d)	1.87[a]	1.98[ab]	1.97[ab]	1.94[ab]	2.06[b]	1.96[ab]	0.02
Gain rate d42-d62 (g/d)	2.30[a]	2.80[b]	2.90[b]	2.99[b]	2.92[b]	2.75[ab]	0.04
Feed intake (g/fish)	106.3	108.5	107.3	105.0	107.4	106.1	0.43
Feed Conversion (feed : gain)	0.95[b]	0.90[ab]	0.90[ab]	0.91[ab]	0.86[a]	0.87[a]	0.01

Table 5. Whole body proximate (g/kg live basis) and fatty acid (% of total) com

	Initial	D1	D5	D10	D15	D20	D10A	D10E
Dry matter	268	279	277	279	277	272	271	277
Protein	176	184	185	185	189	186	190	185
Fat	48 ^{a}	67 ^{b}	68 ^{b}	63 ^{ab}	65 ^{ab}	53 ^{a}	61 ^{ab}	64 ^{ab}
Ash	19	28	21	26	24	24	24	25
14:0	6.8 ^{b}	5.3 ^{a}	5.4 ^{a}	5.2 ^{a}	5.6 ^{ab}	5.6 ^{ab}	5.5 ^{ab}	5.9 ^{ab}
16:0	19.6 ^{b}	18.3 ^{a}	18.6 ^{ab}	17.4 ^{a}	19.4 ^{b}	19.5 ^{b}	19.0 ^{ab}	19.3 ^{b}
18:0	4.6 ^{a}	5.8 ^{b}	5.8 ^{b}	5.3 ^{a}	5.6 ^{ab}	5.4 ^{a}	5.5 ^{ab}	5.5 ^{ab}
Total SFA	33.0 ^{b}	30.5 ^{a}	30.7 ^{a}	29.1 ^{a}	31.6 ^{ab}	31.4 ^{ab}	31.0 ^{ab}	31.8 ^{ab}
16:1n-7	9.0 ^{c}	4.6 ^{a}	4.5 ^{a}	4.4 ^{a}	4.7 ^{ab}	4.7 ^{ab}	4.5 ^{a}	6.1 ^{b}
18:1n-9	18.5 ^{a}	38.4 ^{c}	39.3 ^{c}	36.9 ^{bc}	35.1 ^{bc}	33.1 ^{bc}	34.3 ^{bc}	31.8 ^{b}
18:1n-7	4.1	3.7	3.8	3.7	3.8	3.7	3.5	4.2
Total MUFA	34.6 ^{a}	50.1 ^{c}	51.0 ^{c}	48.4 ^{bc}	46.7 ^{bc}	44.2 ^{b}	44.7 ^{b}	45.0 ^{bc}
18:2n-6	6.1	6.1	6.2	6.5	6.3	6.4	6.8	6.3
18:3n-3	1.3 ^{b}	0.7 ^{a}	0.7 ^{a}	0.8 ^{a}	0.8 ^{a}	0.8 ^{a}	0.8 ^{a}	0.9 ^{ab}
Total C18 PUFA	9.1 ^{b}	7.6 ^{a}	7.6 ^{a}	8.2 ^{ab}	7.6 ^{a}	7.9 ^{a}	8.4 ^{ab}	8.0 ^{a}
20:4n-6	0.8 ^{a}	0.6 ^{a}	0.5 ^{a}	0.6 ^{a}	0.5 ^{a}	0.7 ^{a}	3.1 ^{b}	0.5 ^{a}
20:5n-3	8.0 ^{c}	3.0 ^{ab}	2.5 ^{a}	2.9 ^{a}	3.1 ^{ab}	3.1 ^{ab}	2.9 ^{a}	4.0 ^{b}
22:5n-6	0.3 ^{a}	0.2 ^{a}	0.4 ^{ab}	0.9 ^{b}	1.0 ^{b}	1.6 ^{c}	1.0 ^{b}	0.2 ^{a}
22:5n-3	2.6 ^{b}	1.1 ^{a}	0.9 ^{a}	1.2 ^{a}	1.1 ^{a}	1.1 ^{a}	1.0 ^{a}	1.5 ^{ab}
22:6n-3	10.3 ^{b}	5.4 ^{a}	5.1 ^{a}	7.3 ^{ab}	7.2 ^{ab}	9.0 ^{b}	6.5 ^{a}	7.6 ^{ab}
Total LC-PUFA	23.3 ^{c}	11.8 ^{a}	10.7 ^{a}	14.3 ^{a}	14.2 ^{a}	16.5 ^{b}	15.9 ^{ab}	15.2 ^{ab}
Total n-3	24.5 ^{b}	11.1 ^{a}	10.0 ^{a}	13.3 ^{a}	12.9 ^{a}	15.1 ^{a}	12.1 ^{a}	15.2 ^{a}
Total n-6	7.9 ^{a}	8.3 ^{a}	8.3 ^{a}	9.3 ^{ab}	8.9 ^{a}	9.4 ^{ab}	12.2 ^{b}	8.1 ^{a}
n-3 : n-6	3.09 ^{c}	1.35 ^{a}	1.21 ^{a}	1.44 ^{ab}	1.44 ^{ab}	1.62 ^{b}	0.99 ^{a}	1.88 ^{b}

Different superscripts indicate significant differences between means among treatments ($P<0.05$). Lac
implies that there were no significant differences.

Table 6. Plasma biochemistry and blood haematology parameters

	Units	D1	D5	D10	D15	D20	D10A	D10E	D5E	Pooled SEM
Alanine Aminotransferase	IU L^{-1}	6.0 [a]	5.7 [a]	5.7 [a]	5.3 [a]	9.3 [a]	12.7 [b]	6.0 [a]	6.3 [a]	0.7
Glutamate Dehydrogenase	IU L^{-1}	28.7 [ab]	28.3 [ab]	23.7 [a]	33.0 [ab]	38.0 [b]	35.7 [ab]	27.0 [ab]	31.0 [ab]	1.9
Asparagine Aminotransferase	IU L^{-1}	234 [ab]	193 [ab]	188 [a]	202 [ba]	205 [ab]	246 [b]	194 [ab]	213 [ab]	7.6
Creatine Kinase	IU L^{-1}	25973	17717	23790	18287	21653	25817	22777	24880	1749
Creatinine	µmol L^{-1}	29.7 [ab]	27.3 [a]	28.0 [a]	26.3 [a]	25.7 [a]	31.3 [b]	33.0 [b]	29.3 [ab]	0.8
Total Protein	g L^{-1}	38.0 [a]	40.3 [a]	39.3 [a]	40.0 [a]	39.7 [a]	40.0 [a]	44.7 [b]	42.3 [ab]	0.6
Cholesterol	mmol L^{-1}	6.8 [a]	7.6 [ab]	7.7 [ab]	7.1 [ab]	6.9 [a]	6.2 [a]	7.5 [ab]	8.2 [b]	0.2
Calcium	mmol L^{-1}	2.93 [ab]	2.93 [ab]	2.90 [ab]	2.90 [ab]	2.83 [a]	2.93 [ab]	3.00 [b]	2.97 [b]	0.02
Potassium	mmol L^{-1}	4.3	3.9	3.8	4.4	4.5	3.6	3.8	4.5	0.2
Sodium	mmol L^{-1}	168 [b]	168 [b]	164 [ab]	160 [a]	166 [ab]	164 [ab]	166 [ab]	164 [ab]	1.1
Chloride	mmol L^{-1}	133	133	131	129	133	130	130	131	0.8
Red Blood Cell Count	$x10^{12}$ L^{-1}	1.10 [ab]	1.20 [b]	1.10 [ab]	1.15 [b]	1.13 [b]	1.00 [a]	1.20 [b]	1.15 [b]	0.02
White Blood Cell Count	$x10^{9}$ L^{-1}	32.1	33.4	19.3	28.1	31.3	28.3	23.6	18.2	1.9
Haemoglobin	g L^{-1}	126.0 [ab]	132.0 [b]	123.0 [ab]	136.5 [b]	126.7 [ab]	130.0 [ab]	126.7 [ab]	121.0 [a]	1.3
Packed Cell Volume	mL mL^{-1}	0.54	0.57	0.53	0.60	0.54	0.57	0.54	0.53	0.01

Different superscripts indicate significant differences between means among treatments ($P<0.05$). Lack of a superscript implies that there were no significant differences.

Table 7. Clinical physical health assessment at the end of the 62-day experimental period. Other than gill score, data are percent (%) of total population presenting each symptom.

	D1	D5	D10	D15	D20	D10A	D10E	D5E	Pooled SEM
Pin head	0 [a]	2 [ab]	2 [ab]	4 [b]	0 [a]	4 [b]	5 [b]	0 [a]	0.8
Pectoral fin erosion	2 [a]	12 [b]	6 [ab]	2 [a]	0 [a]	0 [a]	13 [b]	4 [a]	1.5
Caudal fin erosion	7 [b]	2 [a]	7 [b]	0 [a]	2 [a]	0 [a]	2 [a]	7 [b]	1.1
Scale damage	2 [a]	6 [ab]	12 [bc]	0 [a]	4 [a]	19 [c]	2 [a]	0 [a]	2.3
Eye damage	2 [ab]	0 [a]	2 [ab]	4 [b]	0 [a]	2 [ab]	0 [a]	2 [ab]	0.6
AGD gill score	1.3 [ab]	1.6 [ab]	1.1 [a]	1.7 [b]	1.4 [ab]	1.5 [ab]	1.5 [ab]	1.4 [ab]	0.064

AGD : Amoebic Gill Disease. Score based on that reported in Taylor et al., 2009. All other data are reported as percentage of fish showing the particular clinical sign.
Different superscripts indicate significant differences between means among treatments ($P<0.05$). Lack of a superscript implies that there were no significant differences.

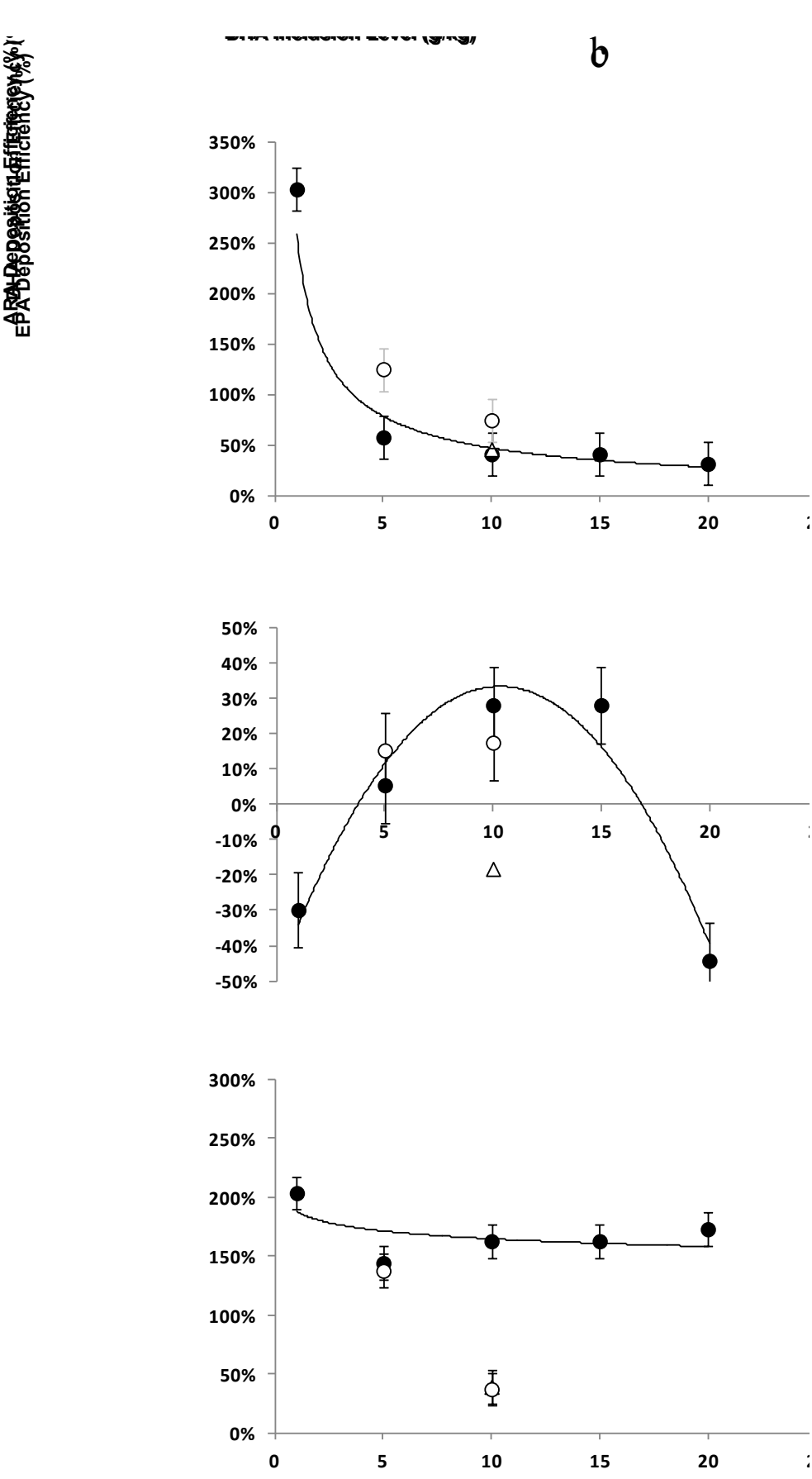

b
350%
300%
250%
200%
150%
100%
50%
0%
0
5
10
15
20
50%
40%
30%
20%
10%
0%
-10%
-20%
-30%
-40%
-50%
300%
250%
200%
150%
100%
50%
0%

Figure 1

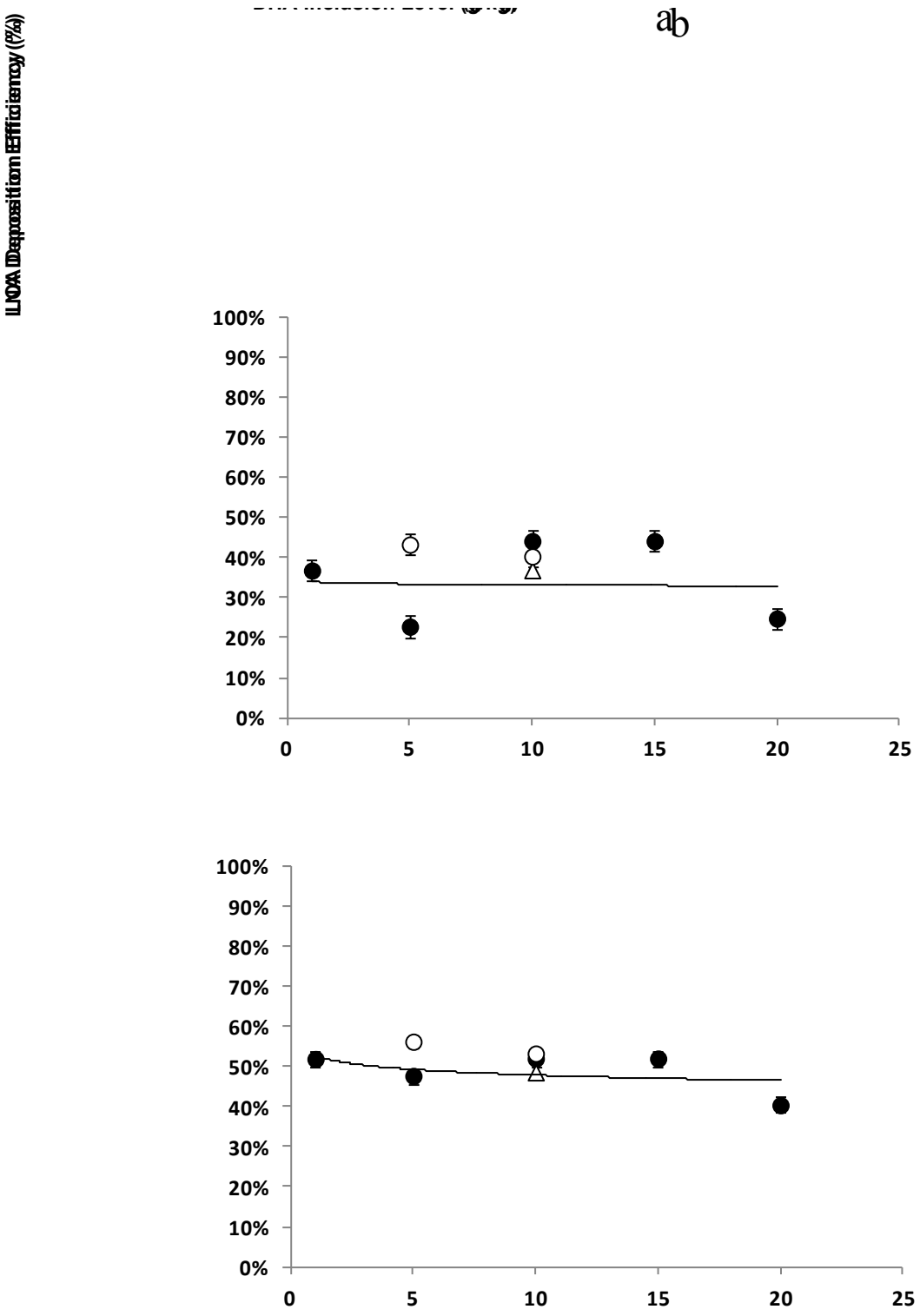
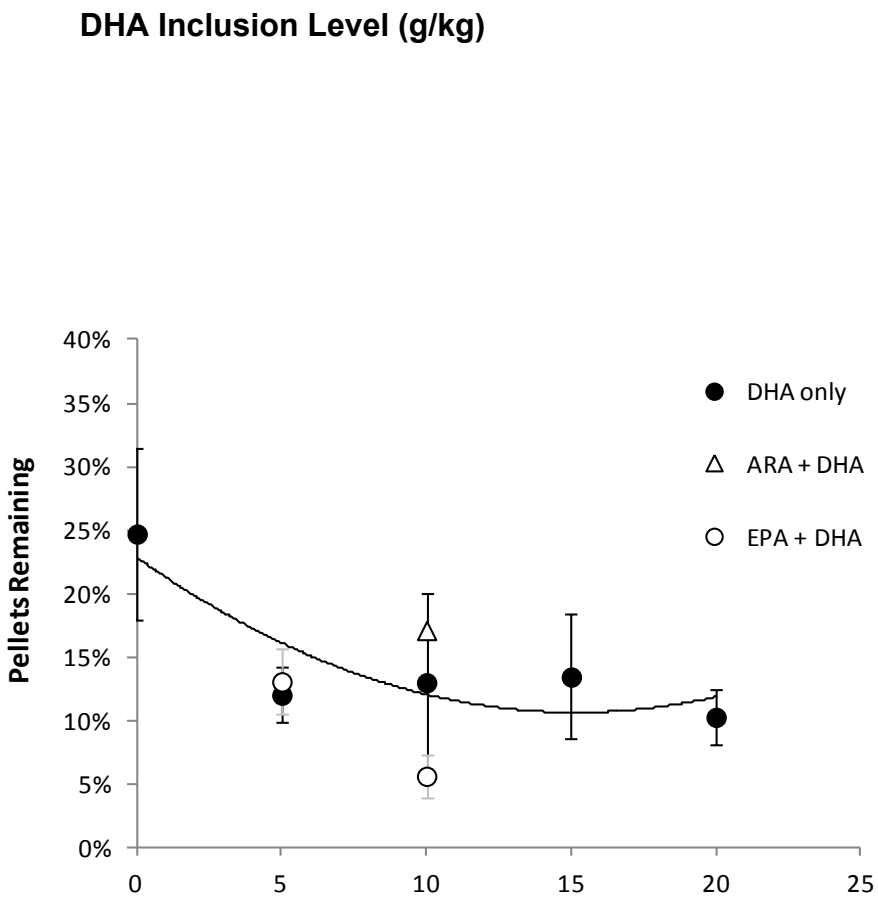


Figure 2


DHA Inclusion Level (g/kg)
Pellets Remaining
40%
35%
30%
25%
20%
15%
10%
5%
0%
0
5
10
15
20
25
DHA only
ARA + DHA
EPA + DHA

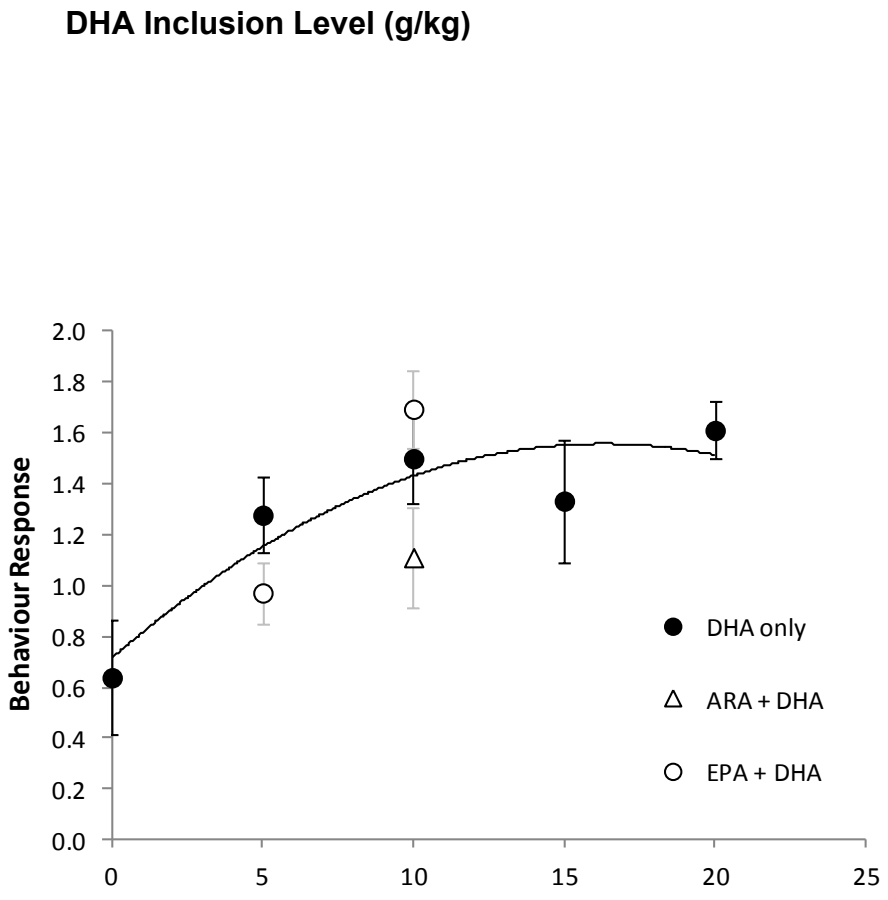

DHA Inclusion Level (g/kg)
Behaviour Response
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
DHA only
ARA + DHA
EPA + DHA

Figure 3.