Accepted refereed manuscript of: Huyben D, Grobler T, Matthew C, Bou M, Ruyter B & Glencross B (2021) Requirement for omega-3 long-chain polyunsaturated fatty acids by Atlantic salmon is relative to the dietary lipid level. *Aquaculture*, 531, Art. No.: 735805. https://doi.org/10.1016/j.aquaculture.2020.735805
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Requirement for omega-3 long-chain polyunsaturated fatty acids by Atlantic salmon is relative to the dietary lipid level

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Abstract

Requirements for omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), for Atlantic salmon are typically represented as an absolute level in the diet (e.g. g/kg or % of diet). Data for other species suggests that requirements for n-3 LC-PUFA are actually relative to dietary lipid (e.g. % of total fatty acids). A 2 x 2 factorial design of dietary lipid level x n-3 LC-PUFA level was designed to examine this question. Atlantic salmon post-smolts of 187 ± 4 g were fed one of four diets for 116 days that either had a low or high lipid level (180 or 230 g/kg) and a low or high n-3 LC-PUFA level (7 or 14 g/kg). Fish fed the diet with high-lipid + high n-3 had greater final weight and weight gain than the high-lipid + low n-3 diet, but no differences were noted between the two low-lipid diets. Significant effects of n-3 and a lipid*n-3 interaction were observed. However, no effects on feed intake, FCR and survival were found. Feeding high n-3 diets generally increased n-3 levels and retention in the whole body, especially EPA and DHA. Relative expression of lipid metabolism genes in the liver showed that fish fed high lipid + high n-3 had lower levels of expression of fatty acid synthesis genes (*fads2d5*, *fads2d6* and *elovl2*). Upregulation of lipid transcription factor (*srebp2* and *lxr*) and fatty acid beta-oxidation (*hoad* and *aco*) genes in fish fed low lipid + high n-3 further suggest that the proportion of dietary n-3 and energy level in those diets were lower than the high-lipid + high n-3 treatment. In conclusion, the significant interaction between lipid and n-3 levels on growth clearly shows that n-3 LC-PUFA requirements are relative to the lipid level in diets for Atlantic salmon. These results support the notion that requirements for this species should be defined based on a percent of total fatty acid content, implying that the absolute amount of n-3 LC-PUFA needs to increase as lipid content of the diet increases.

Keywords

Docosahexaenoic acid (DHA); Eicosapentaenoic acid (EPA); Lipid; Omega-3; Requirements

Highlights

- Highest growth for Atlantic salmon fed high lipid + high n-3 LC-PUFA diet.
- Significant interaction between lipid and n-3 levels for fish growth.
- Retention of n-3 LC-PUFA in the carcass was higher when fed high n-3 diets.
- Up-regulation of fatty acid synthesis genes in fish fed low n-3 diets.
- The n-3 LC-PUFA requirement is relative to the total lipid level in the diet.

1. Introduction

The omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are conditionally-essential dietary nutrients for Atlantic salmon (*Salmo salar*) (Glencross, 2009) (Fig. 1). Various studies have shown that n-3 LC-PUFA are required by Atlantic salmon at a level between 10 and 15 g/kg of the diet for optimal growth (Bou *et al.*, 2017, Glencross *et al.*, 2014, Ruyter *et al.*, 2000). However, this level may actually be subject to varying dietary lipid levels as there is some evidence from other species that requirements may in fact be relative not absolute (Glencross, 2009).

Throughout the literature, fatty acid requirement studies have been expressed both in terms of the amount of these nutrients in the diet (g/kg) and/or the relative proportion they represented of the total fatty acids (%TFA) (Glencross, 2009). Previous studies have indicated that fatty acid requirements are better represented relative to the level of total fatty acids in other species such as rainbow trout (*Oncorhynchus mykiss*) (Watanabe, 1982), red sea bream (*Pagrus major*) (Takeuchi *et al.*, 1992a), yellowtail (*Seriola quinqueradiata*) (Takeuchi *et al.*, 1992b) and Giant tiger shrimp (*Penaeus monodon*) (Glencross *et al.*, 2002). For example, Watanabe (1982) found that double the level of n-3 PUFA (18:3n-3) was required when feeding 100 instead of 50 g/kg total lipid to rainbow trout. The important implication of this observation is that given that it is typical to change the lipid level in diets as species grow, then relying on a single, fixed absolute level of n-3 PUFA in the diet may in fact be pushing the diets to becoming limiting in n-3 as the lipid level increases if these nutrients are not proportionally increased. However, this approach to reporting fatty acid requirements has not be fully adopted by the aquaculture nutrition community. This is in contrast to amino acid requirements that are typically represented either or both relative to protein level and/or relative to energy level.

Therefore, the objective of this study was to determine the nature of requirement responses by Atlantic salmon. To do this required a two-way factorial analysis of the effect of dietary lipid level and n-3 LC-PUFA level on the respective responses by the fish, where we present an assessment of the performance,

nutrient utilisation and transcriptomic responses of this species. We tested the hypothesis that n-3 LC-PUFA level is relative to the total lipid level, rather than absolute level, in the diet by evaluating lipid*n-3 interactions on the above response parameters.

2. Materials and Methods

2.1 Fish management

Atlantic salmon post-smolts were acquired from a commercial hatchery (Marine Harvest, Loch Ailort, Scotland) and transferred to University of Stirling's Marine Environmental Research Laboratory (Machrihanish, Scotland). Fish were sedated with MS222, weighed (187 ± 4; mean ± SD) and sorted into 12 circular tanks (500L) to achieve 32 fish per tank. Tanks contained 350 L of bag-filtered (100 μm) seawater sourced from the adjacent bay in a flow-through system. Each tank was equipped with LED lighting on a 16:8 light:dark cycle, an air stone and a probe that recorded dissolved oxygen and temperature every 10 min (Oxyguard A/S, Farum, Denmark). Fish were acclimatised to the tanks for three weeks while they were introduced to the experimental diets. Over the 17-week (116 day; 138 days including the acclimation period) experiment, temperature was 13.2 ± 0.2 ^{o}C (mean per week ± SD) and the dissolved oxygen was 92.6 ± 2.7% (8.0 ± 0.2 mg/L). The experiment was approved by the University of Stirling Animal Welfare and Ethical Review Body (reference AWERB-16/17-84) in accordance with the UK Home Office under the Animals (Scientific Procedures) Act 1986.

2.2 Experimental diets and feeding

The basic diet design was a simple 2 x 2 factorial, with high and low levels of lipid (HL and LL) and high and low levels of n-3 LC-PUFA (Hn3 and Ln3). Levels of n-3 LC-PUFA were planned to be slightly above and below reported critical optima (Glencross et al., 2014; Bou et al., 2017). Diets were formulated (Table 1) to be isoenergetic on a digestible basis. To achieve this the level of protein was increased in the low lipid diets (LL-Ln3 and LL-Hn3) to maintain the diets on this isoenergetic basis, while maintaining an equal amount of fishmeal (200 g/kg) in each diet. While clearly this changed the protein:energy ratio of the diets, this was considered less of an issue than not balancing the digestible energy content of the diets or using starch to manipulate digestible energy density. The n-3 LC-PUFA level in two of the diets (HL-Hn3 and LL-Hn3) was increased from 7 to 14 g/kg by additional inclusion of linseed and fish oils. Yttrium oxide was included as a digestibility marker in the diets. The 3 mm diets were produced by SPAROS I&D (Olhão, Portugal) using twin-screw extrusion, vacuum lipid coating, and were air-dried and stored at 4 ^{o}C.

Each tank of fish was fed one of the four extruded diets in triplicate in a randomised block design. Diets were fed twice per day for three-hour durations using automated drum feeders (Arvo-tec Oy, Huutokoski, Finland) at a rate of about 1.0 to 1.5% of fish bodyweight per day. Feeding rations were adjusted daily based on the uneaten feed from each tank to ensure satiation. Each morning, uneaten feed was collected manually from each external tank standpipe using a sieve and was weighed. A recovery and dissolution test was performed to determine a correction factor to be applied to the wet uneaten feed waste in order to calculate the daily feed intake according to (Helland *et al.*, 1996), which is included in the equation below.

2.3 Sample collection

Fish were sedated with MS222 and weighed at day 0, 21, 56 and 138 (day 116 post-acclimation period). Fish were fed until the day of sampling and faeces were stripped from all fish by gently squeezing the abdomen, pooled per tank and stored at -20 ºC. At each weighing point, eight fish per tank were euthanised by an overdose of MS222 and cervical dislocation. Four fish were measured for fork length, pooled per tank (n=3/treatment) and stored at -20 ºC. However, at the end of the trial one tank of fish developed symptoms of amoebic gill disease and was treated with freshwater, thus this tank was removed from growth performance analysis (i.e. HL-Hn3: n=2). Pooled faeces and whole carcass samples were homogenised and freeze dried overnight. The other four fish were dissected, liver and viscera weights were recorded to determine somatic indices and the liver was frozen in cryotubes on dry ice and stored at -70 ºC for gene expression analysis.

2.4 Nutritional analyses

Proximate, fatty acid and mineral composition of the diets, carcasses and faeces were performed at the Institute of Aquaculture (Stirling, UK). Moisture and ash were analysed using ovens at 105 and 550 ºC for approximately 24 and 12 hours, respectively according to the Association of Official Analytical Chemists (AOAC, 1995). Protein was analysed by digestion in sulphuric acid at 400 ºC (FOSS A/S, Hillerød, Denmark) for one hour and then addition of sodium hydroxide by a Tecator Kjeltec system (FOSS A/S) according to the Kjeldahl Method (Persson, 2008). Gross energy was measured by ballistic bomb calorimetry using a Parr 6200 bomb calorimeter (Parr Instrument Co., Moline, IL, USA). Lipid was analysed by homogenisation in 2:1 chloroform/methanol, centrifugation, aqueous layer aspiration and nitrogen evaporation (TurboVap Classic, Biotage AB, Uppsala, Sweden) according to the Folch method (Folch *et al.*, 1957). Fatty acids were analysed according to methods of the American Oil Chemists' Society (Christie, 2003). Fatty acid methyl esters (FAME) were made by acid-catalysed esterification of 1 mg of total lipid by overnight incubation at 50 ºC with an internal standard of 17:0,

sulphuric acid, methanol and toluene. A solution of 1:1 iso-hexane/diethyl ether was added and then centrifuged. The upper layer was purified through a silica cartridge, redissolved in iso-hexane and then injected onto a gas liquid chromatographer (GLC) using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm i.d. × 0.25 μm ZB-wax column (Phenomenex, Cheshire, UK), on-column injector and a flame ionisation detector. Individual FAMEs were identified by MD800 mass spectrometer (ThermoFisher Scientific, Hempstead, UK) and compared to external standards of marine oil. Data were collected and processed using Chromcard software version 2.01 (Thermoquest Italia S.p.A., Milan, Italy).

2.5 Calculations of growth performance, body indices and feed efficiency

Means for growth performance were generated based on per fish values from three replicate tanks, body indices were based on four representative fish per tank and nutrient utilisation was based on a pooled sample of four fish per tank. Weight gain, gain rate, feed intake (FI) and feed conversation ratio (FCR) were calculated using the following equations:

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

Gain rate (g $fish^{-1}$ day^{-1}) = weight gain / days

FI (g $fish^{-1}$) = [(Feed fed – (feed waste / correction factor)] / number of fish in each tank

Protein intake (g $fish^{-1}$) = FI x (diet protein % / 100)

Lipid intake (g $fish^{-1}$) = FI x (diet lipid % / 100)

FCR = FI / weight gain

Hepatosomatic index (HSI) and viscerosomatic index (VSI) were calculated according to the following equations:

HSI (%) = (liver weight / final weight) x 100

VSI (%) = (viscera weight / final weight) x 100

Nutrient retention and apparent digestibility were calculated as:

Nutrient retention (%) = [(FW x C / 100) – (SW x C / 100)] / (FI x C / 100) x 100

Apparent digestibility (%) = $[1 – (F / D \times D_i / F_i)] \times 100$

where C is % nutrient (or MJ kg^{-1} for energy) in whole body carcass or diet (D), F is % nutrient (or MJ kg^{-1} for energy) in faeces, D_i is % inert marker yttrium in diet and F_i is % inert marker yttrium in faeces.

2.6 Molecular analyses using qPCR

Liver samples were thawed on ice and approximately 50 mg of the apical tip was homogenised in 1 mL of Tri Reagent (Sigma-Aldrich, Dorset, UK) using a mini-bead beater (Biospec Products, Bartlesville,

OK, USA) for two cycles of 45 sec with 45 sec rest period. Samples were centrifuged at 12,000 *g* for 10 min and the upper layer was transferred to new tubes containing 1-bromo-3-chloropropane (Sigma-Aldrich). The RNA solution was mixed, centrifuged at 20,000 *g* for 15 min, precipitated with a solution of sodium chloride (Merck KGaA, Darmstadt, Germany), sodium citrate sesquihydrate (Sigma-Aldrich) and isopropanol. Samples were centrifuged as before and the RNA pellet was washed with two washes of 70% ethanol and then air dried in a fume hood. The RNA pellet was resuspended in RNase free water and the concentration and quality was checked using a spectrophotometer (ND-1000, Nanodrop Technologies LLC, Wilmington, DE, USA). All samples had a 260/230 nm 260/280 ratios above 2.0 and 1.8, respectively, or the extraction was redone. The quality was also checked by running denatured samples on a 1% agarose gel to verify RNA integrity of the two rRNA bands.

From two fish per tank (n=6/treatment), 6 μg was pooled and then diluted with RNase free water to 2 μg (200 ng/μL). Samples were denatured at 75 ºC for 5 min and then added to 10 μL of High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Paisley, UK) containing RT buffer, dNTP, random primers, dT oligo primers, multiscribe reverse transcriptase (50 U/μL) and nuclease free water. Non-template control (NTC) and reverse transcription negative (RT-) were included for quality control. The cDNA was synthesised in a thermocycler (T Advanced, Biometra GmbH, Göttingen, Germany) with the conditions: 25 ºC for 10 min, 37 ºC for 120 min and 85 ºC for 5 min.

The qPCR efficiency was determined for every set of primers by pooling 4 μL of each sample and then making a dilution series from 1/5 to 1/500. In duplicate, 2.5 μL of each diluted sample (1 μL for reference genes) was mixed with 5 μL of Luminaris Color HiGreen qPCR mastermix (Thermo Scientific, Paisley, UK), 0.5 μL of each primer (10 pmol) and nuclease free water in 10 μL reactions, along with a NTC. The qPCR was performed in a thermocycler (T Professional, Biometra GmbH) under conditions: 50 ºC for 2 min, 95 ºC for 10 min and 35 cycles of 95 ºC for 15 sec, 60 ºC for 30 sec and 72 ºC for 30 sec. All primer efficiencies (E) were between 90-105% and the Ct of each target gene was calibrated against the control treatment of high lipid + high n-3 (delta Ct = calibrator Ct – sample Ct). The relative gene expression was calculated based on relative quantity (RQ = E^delta Ct) between the target and the geometric mean of two reference genes (RQ target / RQ reference) (Pfaffl et al., 2000). Four reference genes (Table 2) were compared using Genorm (Vandesompele *et al.*, 2002) and *hprt* and *rps5* were selected to be the most stable genes.

2.7 Statistical analysis

Normal distribution and homogeneity of each dataset were determined using Shapiro-Wilk and Levene tests in Rstudio software version 1.0.143 (R-Core-Team, 2015). If needed, data were normalized by

log-transformation. All data are presented as means ± SE unless otherwise specified. Akaike's An Information Criterion (AIC) was used to determine the statistical model that best fitted the data. Significant differences between treatments were determined using linear models (lm) for phenomic and nutrient data and linear mixed effects (lme) models for gene expression data based on the nlme R package (Pinheiro *et al.*, 2014). Both lm and lme models included fixed effects of lipid and n-3 LC-PUFA as well as an interaction, except lme included random effect of tank since there was two pooled samples per tank for the transcriptomic data. P-values of each factor and interaction were generated using ANOVA tables and below 0.05 were considered significant and below 0.10 was considered to be a tendency. P-values among treatments were determined using Fisher's least significant difference test (LSD.test) for multiple comparisons based on the agricolae R package (de Mendiburu, 2020).

3. Results

3.1 Growth performance and feed efficiency

The levels of dietary lipid/protein (Lipid) and n-3 LC-PUFA in this experiment influenced several parameters of fish growth and feed efficiency (Table 4 and Fig. 2). Effects of n-3 and a Lipid*n-3 interaction were found for final weight, weight gain and gain rate. Growth of fish fed the HL-Hn3 diet was significantly higher than that of fish fed the HL-Ln3 diet, while growth of fish fed the LL-Ln3 or LL-Hn3 diets was similar. Protein and lipid intake, HSI and VSI were significantly affected by diet lipid level. Protein intake was higher in the LL diets and lipid intake and VSI were higher in the HL diets.

3.2 Whole body composition, nutrient retention and digestibility

Proximate composition of whole-body carcasses were only influenced by dietary lipid level, with no effects of n-3 (Table 5). Ash, lipid and energy were elevated in the HL treatments, whereas protein was reduced. Lipid and n-3 significantly affected almost every fatty acid level in the whole body represented as % of total fatty acid, but a Lipid*n-3 interaction was found for a few monoenes and n-3 PUFA (Table 5). Both lipid and n-3 levels affected total saturates, monoenes, n-3 PUFA, PUFA and LC-PUFA where low lipid and high n-3 typically increased levels found in the whole body. Total n-6 PUFA were only influenced by lipid levels, resulting in high n-6 levels when fed low lipid diets. High n-3 diets resulted in higher levels of EPA and DHA in the whole body.

Only lipid level influenced retention of protein while only n-3 level influenced the retention of fatty acids in the whole body carcass (Table 6). Retention of total saturates and monoenes were not influenced at all, whereas total n-6, n-3, PUFA and LC-PUFA were affected by the level of n-3 that typically resulted in higher retention for the high n-3 diets. EPA retention was higher in fish fed high n-3 diets, while DHA retention was unaffected.

Apparent digestibility of protein, lipid and energy were influenced by lipid and/or n-3 dietary levels, while only a few fatty acids were affected (Table 7). HL diets generally increased the digestibility of protein and energy, while Hn3 diets decreased the digestibility of lipid. A lipid*n-3 interaction existed for protein digestibility and it was significantly higher for the HL-Hn3 diet. The digestibility of total saturates were influenced by n-3 level, total monoenes were influenced by dietary lipid and no effects were found on total n-6, n-3, PUFA and LC-PUFA, including EPA and DHA.

3.3 Differential gene expression in the liver

The expression of 9 out of 13 genes related to lipid metabolism in the liver were influenced by lipid, n-3 and/or lipid*n-3 interaction (Fig. 2-4). For fatty acid synthesis, low lipid and low n-3 diets increased expression of *fads2d5* and *fads2d6* where the HL-Hn3 diet had significantly lower expression (Fig. 3). Also, LL diets tended to increase *elovl2* expression. For transcription factors, a Lipid*n-3 interaction was found for *srebp1, srebp2* and *lxr* genes that had significantly increased expression for the LL-Hn3 diet (Fig. 4). For beta-oxidation of fatty acids, HL diets increased expression of *cpt1b* and a lipid*n-3 interaction existed for *hoad* and *aco* that showed increased expression in fish fed the LL-Hn3 diet (Fig. 5).

4. Discussion

4.1 Dietary lipid and n-3 LC-PUFA on feed intake and growth performance

An interaction between lipid and n-3 LC-PUFA levels on growth performance (Table 4 and Fig. 2) provide further evidence that the level of n-3 LC-PUFA (i.e. EPA and DHA) required by Atlantic salmon is proportional/relative to the total lipid level rather than the absolute level in the diet. These results are in agreement with previous research that demonstrated that n-3 fatty acids are better represented by the proportionality of total fatty acids (Glencross *et al.*, 2002, Watanabe, 1982). In this study, both Hn3 diets had the same absolute level of n-3 LC-PUFA (i.e. 14 g/kg of diet), as did the two Ln3 diets (i.e. 7 g/kg of diet), although each of the diets differed in their relative levels of n-3 LC-PUFA (i.e. 3, 5, 7 and 9% of total fatty acids; TFA) (Table 2). Increased growth of fish fed the HL-Hn3 relative to the HL-Ln3 treatment is inline with previous studies that stipulate the required level of n-3 LC-PUFA in the diet is between 10 to 15 g/kg (Bou *et al.*, 2017, Glencross *et al.*, 2014). In comparison, the equal growth of fish fed the two LL treatments suggests that the n-3 LC-PUFA requirement is proportional and not entirely based on an absolute level between 10 to 15 g/kg. Given that the diets were formulated to be equal in terms of digestible energy in order to compare the interaction between dietary lipid and n-3 LC-PUFA levels, the lack of a difference between treatments in feed intake is perhaps not surprising (Tables 1 and 4). Because the fish were fed to satiety, the similar feed intake across treatments indicates that the fish are clearly eating to an energy demand and not an essential nutrient demand, as there was no observation that the fish were adjusting appetite to compensate for any key nutrient differences among the diets (see Fig. 1).

The higher dietary lipid level, even with a slightly lower proportion of dietary EPA and DHA (i.e. HL-Hn3 diet) resulted in a numerically better fish growth performance than the LL-Hn3 diet, suggesting that the energetic role of the dietary lipid also plays an important role beyond the n-3 LC-PUFA story. This may reflect subtle differences in the net energy value of the diets, and that Atlantic salmon metabolise energy from lipid more effectively than protein and therefore, despite that the digestible energy levels of the diets being close, the net energy values of the diets were likely more divergent (Phan *et al.*, 2019). Previous studies found that feeding higher levels of total lipid and n-3 LC-PUFA increased growth of rainbow trout and shrimp, although over-supplementation of both resulted in reduced growth (Glencross *et al.*, 2002, Watanabe, 1982). However, the proportion of n-3 LC-PUFA in the LL-Hn3 diet in the present study was similar to previous studies (i.e. 5 to 10% TFA) that resulted in optimal growth of Atlantic salmon (Glencross *et al.*, 2014, Bou *et al.*, 2017), which further supports the notion of a net energy imbalance. This would also explain similar growth of fish fed both LL diets with n-3 LC-PUFA levels of 5 and 9% TFA, respectively (see Fig. 6). However, seasonal effects, such as water temperature, have been found to effect protein, lipid and energy retentions in post-smolt salmon

fed diets based on high and low protein-lipid ratio (Dessen *et al.*, 2017). Other environmental conditions, such as hypoxia, may also play a role in dietary requirements (Glencross, 2009). In addition, life stage is known factor as Atlantic salmon fry require a lower level of dietary lipid (e.g. 80 g/kg) and hence a higher proportion of n-3 LC-PUFA (e.g. >10% TFA) (Ruyter *et al.*, 2000).

4.2 Dietary lipid and n-3 LC-PUFA on nutrient retention and digestibility

Altering the levels of protein, lipid and fatty acids in the diet had clear effects on the composition of the whole-body carcass that reflected the diet (Table 5). These results agree with previous studies that have found that feeding high levels of n-3 LC-PUFA results in higher levels in the body or muscle of salmon (Betancor *et al.*, 2014, Betancor *et al.*, 2017, Hixson *et al.*, 2017, Glencross *et al.*, 2014). The retention of n-3 PUFA, especially DHA, in the body indicates that deposition of these essential fatty acids are preferred over others (Table 6). In contrast, previous studies have found that higher levels of n-3 PUFA, such as DHA and EPA, did not result in higher retention in the whole body or flesh of salmon and can even decrease with increased dietary inclusion (Glencross *et al.*, 2014, Bell *et al.*, 2004, Bell *et al.*, 2001).

Increased (numerical, but not significant) growth of fish fed the HL-Hn3 diet (Table 4) may be explained by a higher net energy value from that diet compared to the LL-Hn3 diet (high protein), due to the lower net energy values from protein. Although digestible energy values were accounted for in the formulation, that protein has a higher heat increment of feeding than lipid may result in higher metabolic cost and subsequently result in lower net energy values from those diets (Kaushik and Médale, 1994). Higher energetic costs may also explain why fish fed the LL-Hn3 diet had numerically lower growth. In addition, higher net energy values for fish fed HL diets may have resulted in slight improvements in nutrient utilisation since fish fed HL diets had higher retention of protein as well as higher digestibilities of protein and energy (Tables 6 and 7). However, higher protein utilisation may be due to lower protein content in the HL diets and/or the quality of raw ingredients. Similar lipid, n-3 and lipid*n-3 interaction effects were found for protein digestibility of Atlantic salmon (Bendiksen *et al.*, 2003), although different lipid levels and oil type were fed to parr. In this study, the interaction between lipid and n-3 LC-PUFA on growth performance further supports the inclusion of n-3 LC-PUFA relative to lipid level, especially since lipid level can affect net energy values and feed utilisation in Atlantic salmon.

The significant effect of n-3 level on lipid digestibility agrees with previous studies on salmonids (Caballero *et al.*, 2002, Karalazos *et al.*, 2011), although no effects on specific n-3 PUFA or LC-PUFA digestibilities were found (Table 7). Previous studies have found reduced digestibility of n-3 PUFA, especially EPA, when rainbow trout were fed diets based on a mixture of vegetable oils (Caballero *et*

al., 2002). In Atlantic salmon, replacing fish oil with rapeseed oil reduced EPA (tendency) and DHA (significant) (Karalazos *et al.*, 2011). In this study, digestibility of EPA and DHA was slightly decreased (not significant) for fish fed low n-3 diets, but this lack of effect may be due to the subtle difference between the high and low n-3 diets as opposed to replacing large proportions of fish oil with vegetable oil.

4.3 Dietary lipid and n-3 LC-PUFA influences hepatic gene expression

The results indicate that both lipid and n-3 LC-PUFA levels in the diet influence the transcriptomic pathway for fatty acid synthesis, regulation and beta-oxidation in the liver of Atlantic salmon (Fig. 3-5). Reduced expression of fatty acid desaturases and elongases, such as *fads2d5* and *elolv2*, in fish fed high n-3 diets (Fig. 3) agrees with previous studies that have fed fish oil with higher n-3 LC-PUFA to salmon in comparison to vegetable oils (Zheng *et al.*, 2005, Leaver *et al.*, 2008b, Betancor *et al.*, 2014, Hixson *et al.*, 2017). Upregulation of desaturases and elongases commonly results in an increased production of intermediate products (i.e. 20:4n-3 and 22:5n-3) during EPA and DHA synthesis from 18:3n-3, which may explain the retention greater than 100% for DHA in the present study. The reduced level of expression of fatty acid elongation (*elovl2* and *elovl5a*) and desaturation (*fads2d5* and *fads2d6*) genes supports that the higher level of dietary n-3 LC-PUFA was sufficient at meeting the requirement for Atlantic salmon.

Upregulation of transcription factors, such as *srebp* and *lxr*, in fish fed the LL-Hn3 diet (Fig. 4) indicates the activation of the cholesterol and PUFA biosynthesis pathways (Leaver *et al.*, 2008a), which may be due to low levels of lipid in the diet. Previous studies have found increased expression of *srebp1* and/or *srebp2* in the liver or muscle of Atlantic salmon fed diets with low n-3 PUFA (Leaver *et al.*, 2008b, Hixson *et al.*, 2017, Betancor *et al.*, 2014). In contrast, expression of *srebp1* was not increased in fish fed the low n-3 diets in this study although differences in n-3 levels between low and high diets were considerably less than previous studies that replaced large portions of fish oil with vegetable oil (Table 1). In mammals, *srebp1* is involved in fatty acid metabolism and de novo lipogenesis, whereas *srebp2* is involved with cholesterol metabolism (Horton *et al.*, 2003). Upregulation of *srebp2* and cholesterol synthesis has been found in lean rather than fat family groups of Atlantic salmon (Morais *et al.*, 2011), which agrees with fish fed the LL-Hn3 diet in this study. In addition, *lxr* is activated by a variety of sterols, including intermediates in the synthesis of cholesterol (Horton *et al.*, 2003). Studies on the transcriptome of Atlantic salmon in response to varying DHA levels have found that sterol synthesis pathways are one of the more notable pathways affected (Glencross *et al.*, 2015). Another recent study on Atlantic salmon found that high levels of n-6 and n-3 PUFA in the diet were positively correlated to cholesterol synthesis and suggested PUFA and cholesterol were required together to maintain cell membrane fluidity (Hixson *et al.*, 2017). Therefore, significant upregulation of *srebp2* and *lxr* in the

liver of fish fed the LL-Hn3 diet in this study suggests that cholesterol synthesis pathways were activated, potentially to compensate for lower cholesterol supply due to low lipid in the diet while being stimulated by high dietary n-3 LC-PUFA.

The upregulation of the genes for *cpt1*, *hoad* and *aco* in the liver of fish fed the LL-Hn3 diet (Fig. 5) indicates that the fatty acid beta-oxidation pathway was activated to generate more energy or DHA (Leaver *et al.*, 2008a). Since this diet has high n-3 and low lipid levels, it is more likely that the upregulation of beta-oxidation genes is a catabolic response to provide fish with more energy. This is also supported by the fact that the DHA level in the whole body carcass was similar between fish fed either of the high n-3 diets (Table 5), while expression of beta-oxidation genes was only increased in the LL-Hn3 diet. Previous studies have found that feeding fish oil high in n-3 PUFA, especially EPA and DHA, resulted in upregulation of beta-oxidation genes, such as *cpt1* and *aco*, in the liver of Atlantic salmon compared with feeding vegetable oil (Stubhaug *et al.*, 2007, Jordal *et al.*, 2005). In the beta-oxidation pathway, *cpt1* activates and transports LC-PUFA into the mitochondrial matrix for catabolism, *hoad* catalyses the third step of beta-oxidation in the mitochondria and *aco* catalyses the rate-limiting step in the peroxisome (Jordal *et al.*, 2005, Leaver *et al.*, 2008b). Therefore, upregulation of beta-oxidation genes in fish fed LL-Hn3 demonstrates an increased demand for energy rather than n-3 LC-PUFA.

4.4 Conclusion

The significant interaction between dietary levels of lipid and n-3 LC-PUFA on growth in the present study provides a clear indication that the requirement for n-3 LC-PUFA by Atlantic salmon is relative to the total lipid level, rather than based on the absolute level in the diet. As such, we suggest that n-3 LC-PUFA requirements should in fact be expressed based on their proportion of the total fatty acids (i.e. %TFA). Our results agreed with previous studies that found an optimal dietary level of n-3 LC-PUFA was between 10 to 15 g/kg (Ruyter *et al.*, 2000, Bou *et al.*, 2017, Glencross *et al.*, 2014), or more precisely a relative proportion between 5 and 8% TFA (see Fig. 6). However, this requirement is based primarily on key phenomic responses under ideal conditions and further work is needed to examine growth and immunological responses of Atlantic salmon under non-ideal conditions, e.g. hypoxia or higher thermal regimes.

Additionally, this study also showed that a higher level of lipid in the high n-3 diet, despite being equal in digestible energy, allowed better growth performance. Increased growth of fish fed HL-Hn3 diet may be explained by a higher net energy value from that diet compared to the lower lipid (high protein) diets, due to the lower net energy values from protein, despite that digestible energy values were accounted for in the formulation. Levels and retentions of n-3 PUFA, especially EPA and DHA, were increased

in the whole-body carcass of fish fed the HL-Hn3 diet and indicated both energy and nutrient dependencies were met. These findings were also supported by various transcriptomic responses in the liver, which showed reduced expression of fatty acid desaturases and elongase in fish fed the high n-3 diets. In addition, elevated transcription factors and beta-oxidation in fish fed the LL-Hn3 diet further shows that the n-3 and energy levels in the diet may be insufficient, consistent with an interaction story.

Acknowledgements

Funding for this study was provided by the Norwegian Research Council (HAVBRUK2 project ES576272), Norwegian Seafood Research Fund (FHF) and the University of Stirling (UoS). The authors are especially grateful to staff at the MERL facility in Machrihanish and the Nutritional Analytical Services (NAS) in Stirling, UK. In particular, special thanks to Anna Krzyskow, Jessica Di Toro, Graeme McWhinnie and Billy Struthers at UoS/NAS as well as MSc students Tarah Mayes, Pedro Munoz and Beeke Roehe.

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Tables

Table 1. Diet formulation and proximate composition.

	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3
Formulation (g kg^{-1})				
Fishmeal[1]	200	200	200	200
Soy protein concentrate[2]	180	64	180	64
Soy protein isolate	115	300	115	300
Wheat meal	145	100	145	100
Wheat gluten	92	113	92	113
Fish oil[3]	11	11	38	38
Linseed oil	2	2	8	8
Olive oil	194	144	161	111
L-Histidine	8	8	8	8
DL-Methionine	2	3	2	3
L-Lysine	2	5	2	5
L-Taurine	4	5	4	5
Dicalcium phosphate	20	20	20	20
Vitamin & Mineral Premix[4]	10	10	10	10
Yttrium oxide	2	2	2	2
Antioxidant (Paramega™)[5]	1.5	1.5	1.5	1.5
Soy Lecithin	10	10	10	10
Astaxanthin (Carophyll Pink™)[6]	0.5	0.5	0.5	0.5
Choline chloride	1	1	1	1
Proximate composition as measured (g kg^{-1} dry matter)				
Dry matter	938	947	941	950
Protein	475	590	490	591
Digestible Protein	442	542	458	548
Lipid	241	192	222	187
Ash	85	83	85	83
Carbohydrate[7]	199	136	203	139
Gross Energy (MJ kg^{-1})	24.2	23.6	23.7	23.1
Digestible Energy (MJ kg^{-1})	21.5	20.7	21.0	20.3
Calcium (Ca)	18	17	18	18
Phosphorus (P)	13	13	13	14

HL; high lipid, Hn3; high n-3 LC-PUFA, LL; low lipid, Ln3; low n-3 LC-PUFA.

[1]Norvik LT70 (704 g kg^{-1} protein and 63 g kg^{-1} lipid; Sopropêche, France)

[2]Soycomil (624 g kg^{-1} protein and 4 g kg^{-1} lipid; ADM Animal Nutrition, Decatur, IL, USA)

[3]Savinor (10.5% EPA and 15.7% DHA; Savinor UTS, Covelas TRF, Portugal)

[4]Neovia (formerly Invivo); Vannes, France.

[5]Paramega (blend of natural mixed-tocopherols); Kemin, Herentals, Belgium.

[6]CarophyllPink (10% astaxanthin); DSM, Village-Neuf, France.

[7]Carbohydrate, calculated by difference (i.e. CHO = 1000 - protein - lipid - ash)

551 Table 2. Diet fatty acid composition (% of total fatty acids) .

Fatty acids[1]	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3
14:0	0.5	0.6	1.4	1.7
16:0	12.4	13.1	13.0	14.4
18:0	2.9	2.9	3.3	3.2
20:0	0.4	0.3	0.3	0.3
Total saturates	16.5	17.2	18.3	20.0
16:1n-9	0.2	0.2	0.2	0.2
16:1n-7	1.5	1.6	2.2	2.7
18:1n-9	61.8	58.7	56.2	48.4
18:1n-7	3.8	3.6	3.0	2.5
20:1n-9	0.6	0.7	0.7	0.9
22:1n-11	0.4	0.5	0.5	0.7
24:1n-9	0.2	0.2	0.2	0.2
Total monoenes	68.9	66.0	63.6	56.3
18:2n-6	10.5	12.0	9.2	11.5
20:4n-6	0.1	0.1	0.2	0.2
Total n-6 PUFA	10.6	12.2	9.6	11.9
18:3n-3	1.5	1.7	2.8	3.5
18:4n-3	0.2	0.2	0.5	0.6
20:5n-3	1.0	1.2	2.7	3.5
22:5n-3	0.1	0.1	0.3	0.4
22:6n-3	0.9	1.1	1.8	2.5
Total n-3 PUFA	3.7	4.3	8.3	10.8
Total PUFA	14.6	16.8	18.2	23.7
Total LC-PUFA	2.1	2.6	5.3	7.0
n-6/n-3	2.9	2.8	1.2	1.1

553 HL; high lipid, Hn3; high n-3 LC-PUFA, LL; low lipid, Ln3; low n-3 LC-PUFA.
554 [1]Fatty acids <0.2% not reported.
555

Table 3. Information on the qPCR primer pairs for reference and target genes.

Function	Gene	Full name	Primers	Length	Accession Number
Reference	*cfl2*	Cofilin-2	AGCCTATGACCAACCCACTG TGTTCACAGCTCGTTTACCG	224	TC63899[b]
	hprt	Hypoxanthine phosphoribosyl-transferase 1	GATGATGAGCAGGGATATGAC GCAGAGAGCCACGATATGG	165	XM_014212855.1[a]
	rpl2	Ribosomal protein L2	TAACGCCTGCCTCTTCACGTTGA ATGAGGGACCTTGTAGCCAGCAA	112	XM_014137227.1[a]
	rps5	Ribosomal protein S5	AACTCCATGATGATGCACGG GGTCTTGATGTTCCTGAAAGCA	284	XM_014142016.1[a]
Fatty acid synthesis	*fads2d5*	Delta-5 fatty acyl desaturase	GCCACTGGTTTGTATGGGTG TTGAGGTGTCCACTGAACCA	148	NM_001123542.2[a]
	fads2d6	Delta-6 fatty acyl desaturase	TCCTCTGGTGCGTACTTTGT AAATCCCGTCCAGAGTCAGG	163	NM_001123575.2[a]
	elovl2	Fatty acyl elongase 2	GGTGCTGTGGTGGTACTACT ACTGTTAAGAGTCGGCCCAA	190	NM_001136553.1[a]
	elovl5a	Fatty acyl elongase 5 isoform a	TGTTGCTTCATTGAATGGCCA TCCCATCTCTCCTAGCGACA	150	GU238431.1[a]
	elovl5b	Fatty acyl elongase 5 isoform b	CTGTGCAGTCATTTGGCCAT GGTGTCACCCCATTTGCATG	192	NM_001136552.1[a]
	fas	Fatty acid synthase	ACCGCCAAGCTCAGTGTGC CAGGCCCCAAAGGAGTAGC	212	CK876943[a]
Transcription factor	*lxr*	Liver X receptor	GCCGCCGCTATCTGAAATCTG CAATCCGGCAACCAATCTGTAGG	210	FJ470290[a]
	srebp1	Sterol regulatory element binding protein 1	GCCATGCGCAGGTTGTTTCTTCA TCTGGCCAGGACGCATCTCACACT	151	TC148424[a]
	srebp2	Sterol regulatory element binding protein 2	GACAGGCACAACACAAGGTG CAGCAGGGGTAAGGGTAGGT	147	DY733476[a]
Fatty acid β-oxidation	*aco*	Acyl-CoA oxidase	AAAGCCTTCACCACATGGAC TAGGACACGATGCCACTCAG	230	TC49531[a]
	cpt1a	Carnitine palmitoyl transferase 1a	TCGATTTTCAAGGGTCTTCG CACAACGATCAGCAAACTGG	166	AF327058[a]
	cpt1b	Carnitine palmitoyl transferase 1b	CCCTAAGCAAAAAGGGTCTTCA CATGATGTCACTCCCGACAG	149	AJ606076[a]
	hoad	3-hydroxyacylCoA-dehydrogenase	GGACAAAGTGGCACCAGCAC GGGACGGGGTTGAAGAAGTG	145	tcad0001a.i.15 3.1.om[c]

[a] GenBank database (http://www.ncbi.nlm.nih.gov). [b] Atlantic salmon Gene Index (http://compbio.dfci.harvard.edu/tgi). [c] Sigenae database (http://www.sigenae.org)

Table 4. Growth performance, feed efficiency and body indices of Atlantic salmon post-smolts.

	Diets					Main Effect Means					P-values[1]		
	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3	Pooled SE	LL	HL	Ln3	Hn3	Pooled SE	Lipid	n3	Lipid x n3
Initial weight (g fish^{-1})*	187.7	186.4	191.7	185.0	2.3	185.7	189.3	187.0	187.7	1.7	0.160	0.676	0.293
Final weight (g fish^{-1})	505.3a	525.3ab	552.0b	524.8ab	7.1	525.1	524.0	515.3	535.7	8.8	0.923	**0.069**	**0.043**
Weight gain (g fish^{-1})	317.6a	338.9ab	360.3b	339.8ab	8.2	339.3	334.7	328.3	348.0	8.7	0.795	**0.096**	**0.079**
Gain rate (g fish^{-1} day^{-1})	2.74a	2.92ab	3.11b	2.93ab	0.07	2.93	2.89	2.83	3.00	0.07	0.809	**0.099**	**0.086**
Feed intake (g fish^{-1})	280.6	297.0	317.6	283.5	15.2	290.2	295.4	288.8	297.1	13.0	0.757	0.650	0.226
Protein intake (g fish^{-1})	125.1	166.0	146.5	159.2	7.5	162.6	133.6	145.5	154.1	7.5	**0.017**	0.549	0.168
Lipid intake (g fish^{-1})	60.6	54.3	64.0	50.0	3.0	52.2	63.0	57.5	56.7	3.2	**0.026**	0.937	0.221
FCR (feed:gain)	0.88	0.88	0.88	0.83	0.03	0.86	0.88	0.88	0.85	0.03	0.639	0.618	0.655
Survival (%)	98.6	95.8	97.9	98.6	1.8	97.3	98.4	97.3	98.4	1.2	0.526	0.526	0.386
HSI[2]	1.13	1.06	1.13	1.02	0.04	1.04	1.13	1.10	1.06	0.03	**0.079**	0.649	0.612
VSI[2]	8.45bc	6.83a	8.67c	6.98ab	0.36	6.90	8.54	7.64	7.65	0.38	**0.010**	0.715	0.944

*Initial weight is the weight at the end of the three-week acclimation period. FCR; feed conversion ratio, HSI; hepatosomatic index; HL; high lipid, Hn3; high n-3 LC-PUFA, LL; low lipid, Ln3; low n-3 LC-PUFA;VSI; viscerosomatic index.

[1]P-values from linear model with lipids and n-3 LC-PUFA (n3) as fixed effects and a lipid x n3 interaction. P-values in bold are <0.10.

[2]n=12, 4 fish were sampled in each triplicate tank.

Table 5. Whole body proximate (g kg^{-1} wet matter basis) and fatty acid (% of total fatty acids) composition of Atlantic salmon (n=3, pooled per tank)

	Diets					Pooled	Main Effect Means				Pooled	P-values		
	Initial	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3	SE	LL	HL	Ln3	Hn3	SE	Lipid	n3	Lipid x n3
Dry matter	304	315	310	315	313	3.7	312	315	313	314	2.5	0.408	0.707	0.779
Ash	18	16ab	18b	15a	18b	0.6	18	16	17	17	0.5	**0.021**	0.430	0.537
Protein	167	185a	195b	182a	195b	2.2	195	184	190	189	2.3	**0.002**	0.634	0.481
Lipid	101	92ab	83a	102b	89ab	4.1	86	97	88	95	3.5	**0.040**	0.124	0.655
Energy (MJ kg^{-1})	8.1	8.6	8.3	8.7	8.4	1.5	8.3	8.6	8.4	8.6	0.1	**0.094**	0.472	0.700
Fatty acids[2]														
14:0	3.7	1.4a	1.6b	1.9c	2.1d	0.02	1.8	1.6	1.5	2.0	0.1	**<0.001**	**<0.001**	0.376
16:0	13.5	12.4a	13.1b	12.8ab	13.8c	0.15	13.4	12.6	12.8	13.3	0.2	**0.001**	**0.008**	0.278
18:0	3.1	3.4a	3.6ab	3.4a	3.7b	0.05	3.6	3.4	3.5	3.6	0.0	**0.007**	0.274	0.532
20:0	0.2	0.3a	0.2bc	0.2ab	0.2c	0.01	0.2	0.2	0.2	0.2	0.0	**0.002**	**0.077**	0.696
Total saturates	21.2	17.7a	18.7b	18.6b	20.1c	0.18	19.4	18.2	18.2	19.3	0.3	**<0.001**	**<0.001**	0.220
16:1n-9	0.3	0.5b	0.5b	0.4a	0.4a	0.02	0.5	0.5	0.5	0.4	0.0	0.662	**<0.001**	0.773
16:1n-7	4.4	2.1a	2.3b	2.6c	2.9d	0.03	2.6	2.4	2.2	2.8	0.1	**<0.001**	**<0.001**	**0.078**
18:1n-9	29.9	53.1c	49.4b	49.1b	43.6a	0.18	46.5	51.1	51.2	46.4	1.1	**<0.001**	**<0.001**	**0.002**
18:1n-7	3.8	3.0	3.0	2.9	2.8	0.10	2.9	2.9	3.0	2.9	0.1	0.844	0.244	0.936
20:1n-9	4.2	3.5b	3.4b	3.2a	3.1a	0.05	3.2	3.3	3.4	3.2	0.1	0.103	**0.001**	0.855
22:1n-11	3.7	0.9a	1.0ab	1.0a	1.1b	0.04	1.1	0.9	1.0	1.0	0.0	**0.022**	0.173	0.449
22:1n-9	0.6	0.4a	0.4b	0.4a	0.4ab	0.01	0.4	0.4	0.4	0.4	0.0	**0.014**	0.253	0.803
24:1n-9	1.3	0.3a	0.4ab	0.4bc	0.4c	0.01	0.4	0.4	0.4	0.4	0.0	**0.026**	**0.005**	0.641
Total monoenes	48.9	64.5c	61.0b	60.6b	55.6a	0.24	58.3	62.5	62.8	58.1	1.0	**<0.001**	**<0.001**	**0.022**
18:2n-6	9.8	7.9a	9.4b	8.2a	9.8c	0.11	9.6	8.1	8.7	9.0	0.2	**<0.001**	**0.022**	0.575
18:3n-6	0.2	0.3b	0.4c	0.2a	0.2a	0.01	0.3	0.2	0.3	0.2	0.0	**0.008**	**<0.001**	0.493
20:2n-6	0.7	0.8a	1.0c	0.8b	1.1d	0.02	1.0	0.8	0.9	1.0	0.0	**<0.001**	**0.001**	0.482
20:3n-6	0.2	0.7c	0.8d	0.4a	0.5b	0.02	0.7	0.6	0.8	0.5	0.0	**0.001**	**<0.001**	0.629
20:4n-6	0.4	0.4b	0.5c	0.3a	0.3ab	0.01	0.4	0.3	0.4	0.3	0.0	**0.006**	**0.001**	0.406
Total n-6 PUFA	11.5	10.2a	12.2b	10.0a	12.1b	0.11	12.1	10.1	11.2	11.0	0.3	**<0.001**	0.370	0.708
18:3n-3	3.0	1.2a	1.4b	2.0c	2.4d	0.03	1.9	1.6	1.3	2.2	0.1	**<0.001**	**<0.001**	**0.004**
18:4n-3	1.1	0.4a	0.4b	0.5c	0.5d	0.01	0.5	0.4	0.4	0.5	0.0	**<0.001**	**<0.001**	0.123
20:4n-3	0.8	0.3a	0.3b	0.4c	0.5d	0.01	0.4	0.4	0.3	0.5	0.0	**<0.001**	**<0.001**	**0.008**
20:5n-3	3.7	1.3a	1.3a	1.9b	1.9b	0.03	1.6	1.6	1.3	1.9	0.1	0.441	**<0.001**	0.406
22:5n-3	1.3	0.5a	0.6b	0.8c	0.9d	0.01	0.7	0.6	0.5	0.8	0.0	**0.001**	**<0.001**	0.157
22:6n-3	6.9	3.4a	3.7a	4.5b	5.0b	0.17	4.3	4.0	3.6	4.7	0.2	**0.078**	**<0.001**	0.416
Total n-3 PUFA	17.4	7.3a	7.8a	10.4c	11.7d	0.20	9.7	8.8	7.5	11.0	0.5	**0.003**	**<0.001**	**0.098**
Total PUFA	29.9	17.8a	20.3b	20.8b	24.4c	0.29	22.3	19.3	19.0	22.6	0.8	**<0.001**	**<0.001**	0.151
Total LC-PUFA	14.8	7.6a	8.4b	9.5c	10.8d	0.20	9.6	8.6	8.0	10.2	0.4	**0.001**	**<0.001**	0.258

HL; high lipid, Hn3; high n-3 LC-PUFA, LL; low lipid, Ln3; low n-3 LC-PUFA, SE; pooled standard error of the mean.
[1]P-values from linear model with lipids and n-3 LC-PUFA (n3) as fixed effects and a lipid x n3 interaction. P-values in bold are <0.10.
[2]Fatty acids ≤0.2 not detailed.

Table 6. Retention (%) of macronutrients and fatty acids in the whole-body carcass of Atlantic salmon (n=3, pooled per tank).

	Diets				Pooled	Main Effect Means				Pooled	P-values[1]		
	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3	SE	LL	HL	Ln3	Hn3	SE	Lipid	n3	Lipid x n3
Protein	50.2b	43.0a	44.3ab	44.8ab	2.2	43.9	47.2	46.6	44.5	1.7	0.230	0.474	0.170
Lipid	46.4	48.2	53.4	57.4	5.4	52.8	49.9	47.3	55.4	4.0	0.650	0.228	0.865
Energy (MJ kg^{-1})	45.1	42.8	43.1	47.1	3.1	45.0	44.1	44.0	45.1	2.0	0.781	0.719	0.323
Fatty acids[2]													
14:0	38.5	39.1	47.1	50.0	8.2	44.6	42.8	38.8	48.6	5.6	0.847	0.296	0.904
16:0	52.9	51.1	56.3	64.0	6.4	57.6	54.6	52.0	60.2	4.5	0.666	0.258	0.501
18:0	66.4	68.3	63.8	81.4	6.8	74.8	65.1	67.3	72.6	5.2	0.220	0.494	0.312
20:0	30.2	25.6	33.6	32.7	4.2	29.2	31.9	27.9	33.2	2.9	0.550	0.265	0.686
Total saturated	53.8	52.7	56.1	64.5	6.5	58.6	54.9	53.2	60.3	4.6	0.602	0.328	0.498
16:1n-9	184.4	178.1	131.8	140.8	23.2	159.4	158.1	181.2	136.3	16.4	0.954	**0.091**	0.751
16:1n-7	39.6	41.2	49.2	55.1	7.0	48.2	44.4	40.4	52.1	5.0	0.621	0.150	0.778
18:1n-9	56.9	52.2	58.7	68.2	5.7	60.2	57.8	54.6	63.4	4.3	0.697	0.174	0.265
18:1n-7	40.3a	38.1a	49.3ab	64.8b	6.7	51.4	44.8	39.2	57.1	5.6	0.372	**0.034**	0.244
20:1n-9	268.7	220.7	223.4	197.1	24.0	208.9	246.0	244.7	210.2	18.1	0.202	0.233	0.695
22:1n-11	-41.7a	-31.0ab	-15.6bc	-6.0c	6.5	-18.5	-28.7	-36.4	-10.8	6.0	0.186	**0.006**	0.939
24:1n-9	-41.0a	-32.5ab	6.0ab	12.0b	12.6	-10.3	-17.5	-36.8	9.0	11.1	0.641	**0.015**	0.936
Total monounsaturated	57.4	52.7	59.6	69.0	6.1	60.8	58.5	55.1	64.3	4.5	0.728	0.184	0.300
18:2n-6	37.3a	39.6a	47.4ab	56.6c	4.5	48.1	42.3	38.5	52.0	3.8	0.252	**0.020**	0.483
20:4n-6	258.8b	289.8b	79.8a	92.9a	27.5	191.4	169.3	274.3	86.4	32.8	0.529	**0.001**	0.797
Total n-6 PUFA	49.4a	53.0ab	56.8ab	68.7b	5.7	60.8	53.1	51.2	62.7	4.4	0.222	**0.084**	0.501
18:3n-3	14.9a	17.0a	33.4b	39.7b	2.8	28.3	24.2	16.0	36.5	3.6	0.245	**<0.001**	0.549
18:4n-3	7.8ab	7.0a	24.7bc	25.9c	5.1	16.4	16.2	7.4	25.3	4.3	0.970	0.862	0.590
20:4n-3	48.9a	54.8a	141.1b	155.1b	15.3	105.0	95.0	51.9	148.1	17.6	0.590	**0.001**	0.824
20:5n-3	10.8ab	3.2a	24.6b	20.9b	4.1	12.1	17.7	7.0	22.8	3.8	0.242	**0.008**	0.675
22:5n-3	72.8a	81.0a	119.2b	125.4b	14.8	103.2	96.0	76.9	122.3	12.6	0.678	**0.026**	0.955
22:6n-3	113.8	99.1	109.4	109.2	11.3	104.2	111.6	106.4	109.3	7.8	0.550	0.813	0.559
Total n-3 PUFA	39.4ab	35.3a	52.7ab	55.3b	5.5	45.3	46.1	37.3	54.0	4.5	0.895	**0.02**	0.586
Total PUFA	46.0	47.4	54.6	60.8	5.7	54.1	50.3	46.7	57.7	4.1	0.537	**0.098**	0.697
Total LC-PUFA	111.5	103.3	81.0	84.4	11.1	93.8	96.3	107.4	82.7	8.3	0.838	**0.065**	0.627
Total Fatty Acids	55.2	51.8	58.1	66.1	6.1	59.0	56.6	53.5	62.1	4.4	0.720	0.210	0.393

HL; high lipid, Hn3; high n-3 LC-PUFA, LL; low lipid, Ln3; low n-3 LC-PUFA, SE; pooled standard error of the mean.

[1]P-values from linear model with lipids and n-3 LC-PUFA (n3) as fixed effects and a lipid x n3 interaction. P-values in bold are <0.10.

[2]Fatty acids <0.2% in the diet not detailed.

Table 7. Apparent digestibility (%) of macronutrients and fatty acids for Atlantic salmon (n=3, pooled per tank).

	Diets				Pooled	Main Effect Means				Pooled	P-value[1]		
	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3	SE	LL	HL	Ln3	Hn3	SE	Lipid	n3	Lipid x n3
Protein	93.2c	92.0a	93.5d	92.8b	0.1	92.4	93.4	92.6	93.2	0.2	**<0.001**	**<0.001**	**0.032**
Lipid	98.3b	97.3ab	95.6a	96.2a	0.6	96.8	97.2	97.8	96.1	0.5	0.517	**0.025**	0.424
Energy (MJ kg^{-1})	89.0b	87.7a	88.9b	88.0ab	0.3	87.9	88.9	88.3	88.5	0.2	**0.010**	0.718	0.492
Fatty acids[2]													
14:0	95.0	94.8	86.7	92.0	1.7	93.1	90.9	94.9	89.4	1.8	0.393	0.104	0.418
16:0	97.8	97.1	92.8	94.4	1.1	95.5	95.3	97.5	93.6	1.0	0.725	**0.043**	0.497
18:0	97.1	96.1	92.0	92.9	1.3	94.2	94.6	96.7	92.5	1.2	0.988	**0.045**	0.616
20:0	97.3	95.7	93.0	92.8	1.4	94.0	95.2	96.7	92.9	1.1	0.613	**0.051**	0.684
Total saturates	97.5	96.7	92.1	93.8	1.2	95.0	94.8	97.2	92.9	1.1	0.732	**0.045**	0.500
16:1n-9	99.7	100.0	100.0	99.8	0.1	99.9	99.8	99.8	99.9	0.1	0.889	0.689	0.264
16:1n-7	99.6	99.3	99.1	99.3	0.1	99.3	99.4	99.5	99.2	0.1	0.823	0.153	0.202
18:1n-9	99.7	99.3	99.6	99.5	0.1	99.4	99.7	99.5	99.6	0.1	**0.043**	0.749	0.365
18:1n-7	99.6b	99.3ab	99.2ab	98.9a	0.1	99.1	99.4	99.5	99.0	0.1	0.137	**0.042**	0.898
20:1n-9	98.8	97.9	98.3	98.3	0.3	98.1	98.6	98.5	98.3	0.2	0.212	0.648	0.219
22:1n-11	98.9	98.0	98.3	98.4	0.3	98.3	98.6	98.5	98.4	0.2	0.481	0.718	0.209
24:1n-9	96.0	92.7	90.0	92.7	1.5	96.7	96.1	96.5	96.3	1.0	0.986	0.165	0.205
Total monoenes	99.6	99.3	99.5	99.4	0.1	99.3	99.6	99.5	99.4	0.1	**0.063**	0.797	0.386
18:2n-6	99.4	99.2	99.2	99.1	0.2	99.1	99.3	99.3	99.1	0.1	0.522	0.556	0.774
20:4n-6	97.1a	96.5a	99.6b	98.9ab	0.7	97.9	98.3	96.9	99.2	0.6	0.457	**0.017**	0.928
Total n-6 PUFA	99.3	99.1	99.1	99.0	0.2	99.1	99.2	99.2	99.1	0.1	0.565	0.569	0.740
18:3n-3	99.6	99.4	99.7	99.7	0.1	99.6	99.6	99.5	99.7	0.1	0.416	0.195	0.435
18:4n-3	99.2	99.1	99.4	99.6	0.2	99.4	99.3	99.1	99.5	0.1	0.726	**0.072**	0.485
20:5n-3	99.7	99.6	99.8	99.8	0.1	99.7	99.7	99.6	99.8	0.1	0.707	0.155	0.774
22:5n-3	99.3	98.6	99.8	99.3	0.3	99.0	99.6	99.0	99.6	0.3	0.209	0.180	0.845
22:6n-3	98.5	98.1	99.2	99.0	0.4	98.6	98.8	98.3	99.1	0.3	0.529	0.129	0.823
Total n-3 PUFA	99.3	99.1	99.6	99.5	0.2	99.3	99.4	99.2	99.5	0.1	0.527	0.134	0.701
Total PUFA	99.3	99.1	99.3	99.3	0.2	99.2	99.3	99.2	99.3	0.1	0.552	0.646	0.676
Total LC-PUFA	98.8	98.5	99.5	99.3	0.3	99.0	99.2	98.7	99.4	0.2	0.607	0.100	0.807
Total	99.2	98.8	98.1	98.2	0.3	98.5	98.7	99.1	98.2	0.3	0.767	**0.070**	0.532

HL; high lipid, Hn3; high n-3 LC-PUFA, LL; low lipid, Ln3; low n-3 LC-PUFA, SE; pooled standard error of the mean.

[1]P-values from linear model with lipids and n-3 LC-PUFA (n3) as fixed effects and a lipid x n3 interaction. P-values in bold are <0.10.

[2]Fatty acids <0.2% in the diet not detailed.

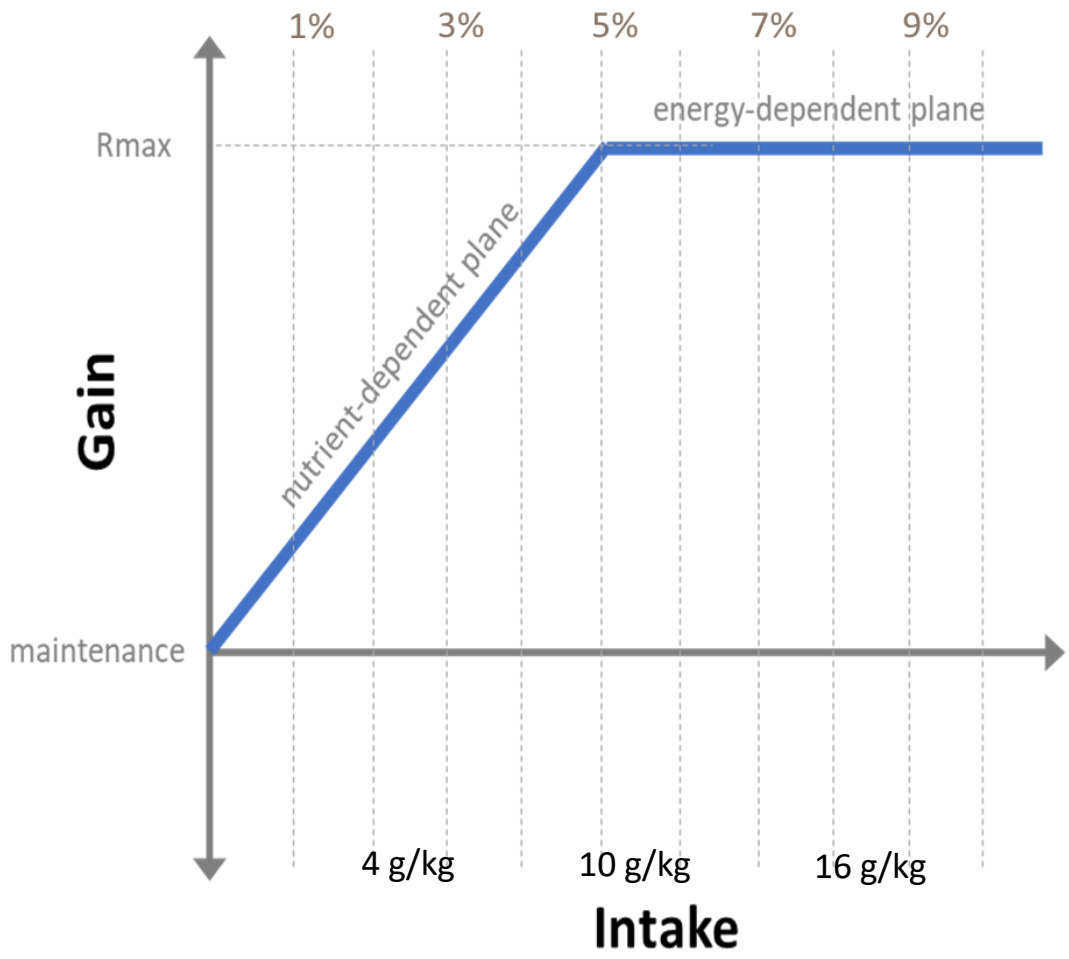

Figure1. General nutrient response schematic showing the approximate relative levels at which responses by Atlantic salmon to variable n-3 LC-PUFA supply were observed based on the results from Glencross et al. (2014). In this example performance is projected to decline to a maintenance point consistent with the needs for conditionally-essential nutrients. Across the top of the figure are the relative levels of n-3 LC-PUFA (% of total fatty acids; TFA) in a diet with 200 g/kg of lipid, whereas along the bottom of the figure are the commensurate absolute n-3 LC-PUFA levels (g/kg).

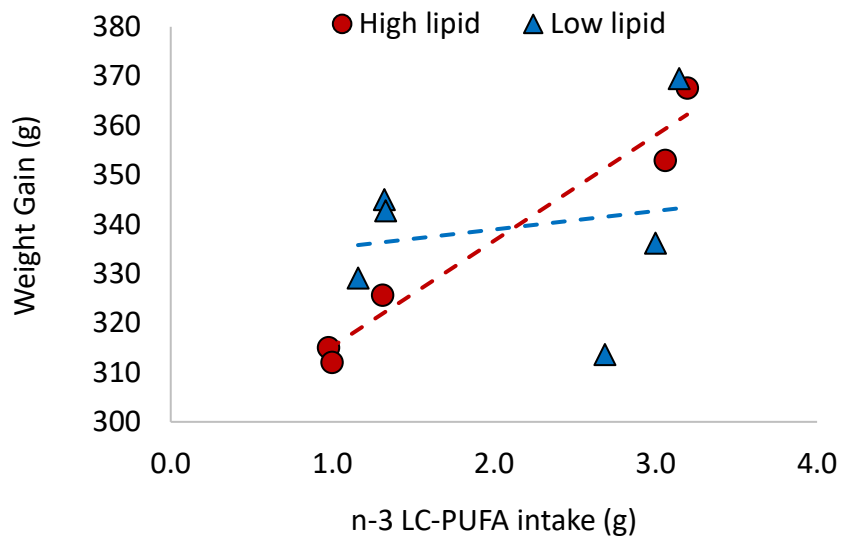

Figure 2. Interaction plot of mean weight gain based on total n-3 LC-PUFA intake of each tank of fish fed either high-lipid (red) or low-lipid (blue) diets. The cross-over of slopes indicate an interaction between dietary lipid and n-3 levels, where weight gain was more increased for fish fed high than low lipid diets at a similar n-3 intake. The higher slope of the high-lipid data shows that these diets are on a nutrient-dependent plane, whereas the low-lipid data are more closely representing responses on an energy-dependent plane (see Fig. 5).

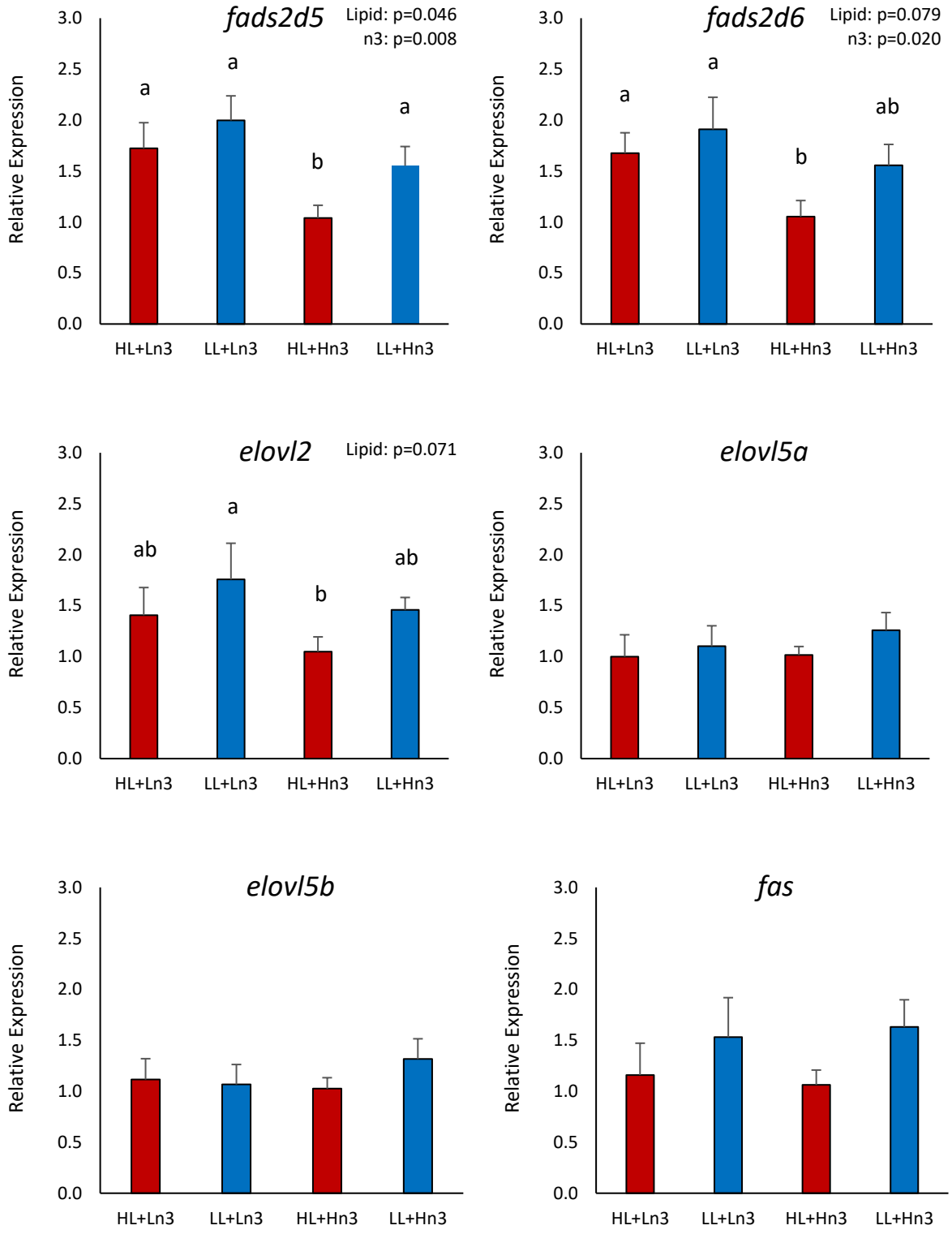

Figure3. Expression of genes (mean ± SE, n=6/treatment) relative to the geometric mean of two reference genes (*hprt* and *rps5*) involved in fatty acid synthesis in the liver of Atlantic salmon fed low and high levels of lipids and/or n-3 LC-PUFA (i.e. EPA and DHA).

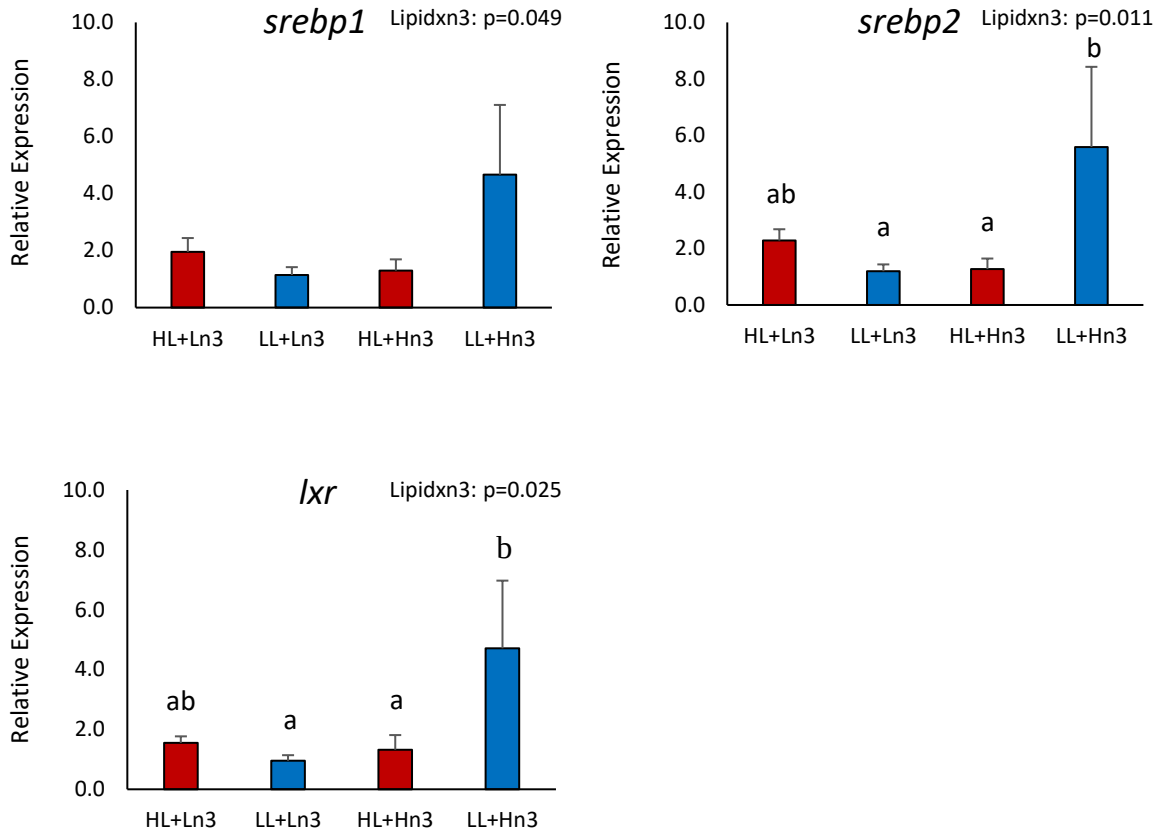

Figure 4. Expression of genes relative to the geometric mean of two reference genes (*hprt* and *rps5*) involved in transcription factors in lipid regulation in the liver of Atlantic salmon fed low and high levels of lipids and/or n-3 LC-PUFA (i.e. EPA and DHA).

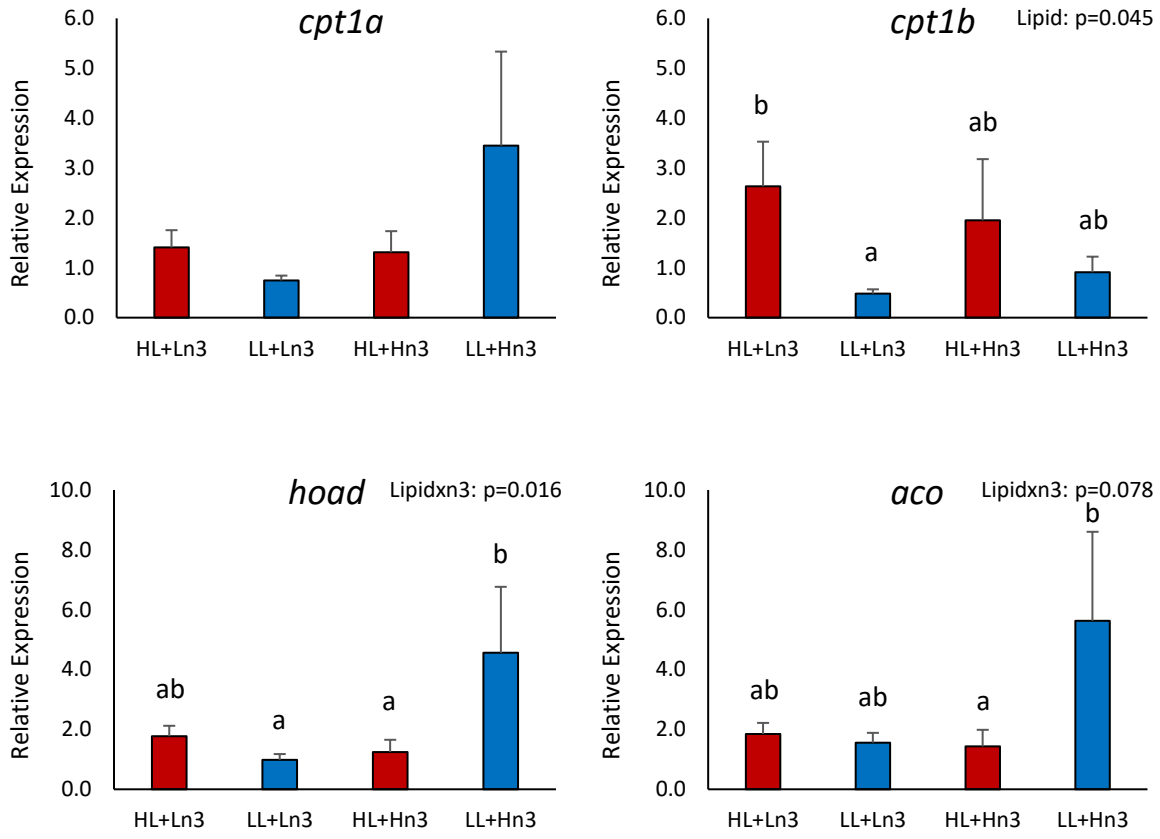

Figure 5. Expression of genes relative to the geometric mean of two reference genes (*hprt* and *rps5*) involved in beta oxidation of fatty acids in the liver of Atlantic salmon fed low and high levels of lipids and/or n-3 LC-PUFA (i.e. EPA and DHA).

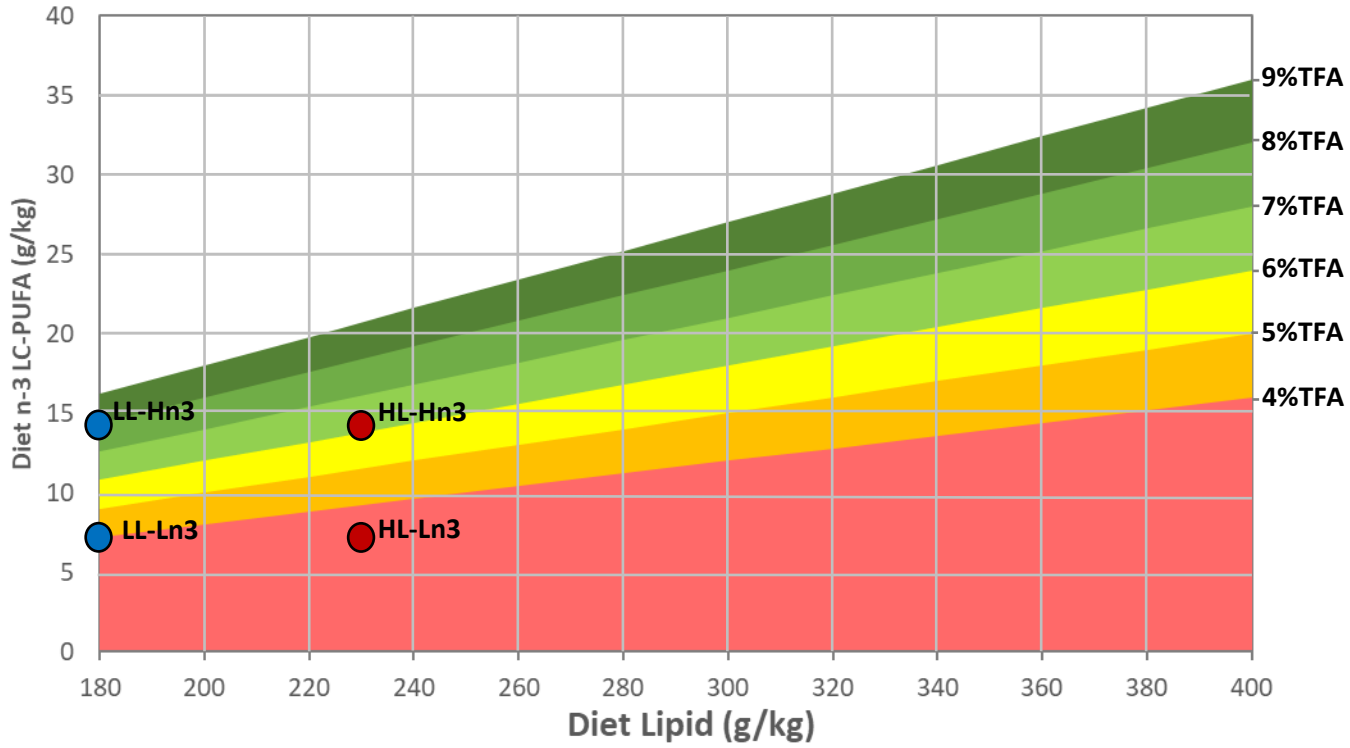

Figure 6. Expression of the relativity of n-3 LC-PUFA requirements by Atlantic salmon overlaid on to variable absolute dietary lipid levels (x-axis), absolute n-3 levels (left y-axis) and n-3 levels relative to lipid level (right y-axis). The four diets (i.e. LL-Ln3, HL-Ln3, LL-Hn3 and HL-Hn3) from this study are overlaid and show the n-3 level in relation to low (blue) and high (red) lipid level. The lower boundary between the orange and red is commensurate with an n-3 level at 4% of total fatty acids (TFA; marginal level), followed by the yellow and orange boundary at 5% TFA (threshold level), light-green and yellow boundary at 6% TFA (optimal level) and so on up to a 9%TFA level. Notable is how the actual (g/kg) level of n-3 in the diet needs to increase as dietary lipid level increases. An optimal level of 10 g/kg of n-3 in a diet with 200 g/kg of lipid being equivalent to an optimal level of 15 g/kg of n-3 in a diet with 300 g/kg of lipid.