Hypoxia does not change responses to dietary omega-3 long-chain polyunsaturated fatty acids, but rather reduces dietary energy demand by Atlantic salmon

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Abstract

Over 116 days, Atlantic salmon post-smolts of 183 ± 5 g were fed diets with high or low levels of lipid (230 or 180 g/kg) with high or low levels of omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA; 14 or 7 g/kg). Fish were reared under hypoxic and normoxic conditions (6.7 or 8.0 mg/L), as an environmental stressor. Higher levels of lipid and n-3 in the diet increased final weight and weight gain, although no lipid x n-3 interaction was found across both environments. Hypoxia led to reduced growth, which can be explained by a reduced feed intake, with no effects on FCR being observed. High lipid diets improved protein and energy retention in the fish carcass as well as improved the digestibility of lipid and LC-PUFA, including EPA and DHA. High n-3 diets also improved retention and digestibility of LC-PUFA, and transcriptomic analysis showed that the liver had reduced levels of expression of fatty acid synthesis genes, e.g. *fads2d5* and *elovl2* in those treatments. A clear relationship between performance and energy intake, independent of n-3 LC-PUFA intake, shows that energy demand under hypoxia was an over-riding feature of the nutritional responses in this study.

Keywords

Docosahexaenoic acid (DHA); Eicosapentaenoic acid (EPA); Hypoxia; Environmental conditions; Lipids, Energy demand

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*). Recent studies have shown that between 10 to 15 g of EPA and DHA kg^{-1} of diet are required for optimal growth of Atlantic salmon (Bou *et al.*, 2017, Glencross *et al.*, 2014). Although previous studies have found that fatty acid requirements are better represented relative to the level of total fatty acids in Atlantic salmon (Huyben *et al.*, 2021), 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). Huyben *et al.* (2021) suggested that the EPA and DHA requirement for Atlantic salmon should be between 5 and 8% of total fatty acids in the diet. However, this requirement was only based on phenomic responses, such as growth, under ideal conditions and further work is needed to examine responses under non-ideal conditions, e.g. hypoxia (reduced dissolved oxygen).

Most previous studies have evaluated Atlantic salmon under normoxic conditions, while hypoxia is a common stressor in the commercial aquaculture systems. Net-pens of fish routinely experience daily fluctuations in dissolved oxygen caused by changes in tides, climate and seasons. Hypoxic events may become more frequent due to the impact of climate change on warming waters, thus coping strategies are needed. Bou *et al.* (2017) suggested that there may be a higher demand for n-3 LC-PUFA in challenging conditions, thus previous studies are under-estimating this requirement since most experiments are performed under ideal conditions. These authors also suggested that nutrient retention and transcriptomic pathways should be investigated, not only phenomic responses. A higher inclusion of n-3 LC-PUFA in the diet may have a positive effect on coping with chronic stress of hypoxia to the extent that similar growth performance might be achieved compared with fish under normoxic conditions. In addition, salmonids under hypoxia are known have reduced feed intake (Glencross, 2009, Vikeså *et al.*, 2017), thus a higher level and proportion of n-3 LC-PUFA may be needed in the diet. A study by Vikeså *et al.* (2017) found that higher dietary energy had no effect on fish growth and nutrient retention under either normoxic or hypoxic conditions. However, this study as well as others have confounding factors of varying lipid without maintaining iso-energetic diets.

The objective of this study was to determine the effect of dietary lipid, n-3 LC-PUFA and dissolved oxygen levels on the growth performance, feed utilisation and hepatic gene expression of Atlantic salmon post-smolts. This study builds on from one previously published (Huyben *et al.*, 2021), which examined the relationship between LC-PUFA demands and lipid levels, by exploring aspects of that relationship when dissolved oxygen levels are compromised.

Materials and Methods

Fish management

Atlantic salmon post-smolts were acquired from a Marine Harvest hatchery (Loch Ailort, Scotland) and transferred to University of Stirling's Marine Environmental Research Lab (Machrihanish, Scotland). Fish were sedated with MS222, weighed (190.2 ± 28.4; mean ± SD) and sorted into 15 circular tanks (800L) to achieve 40 fish per tank. Tanks contained 500 L of drum-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 four weeks while they were introduced to the experimental diets while the dissolved oxygen was lowered in 12 random tanks by restricting water flow rates in order to expose the fish to hypoxic stress. Over the 16 week (110 day) experiment, temperature was 13.15 ± 0.19 ^{o}C (mean per week ± SD) and the dissolved oxygen was 92.56 ± 2.66% (8.00 ± 0.23 mg/L) for the high oxygen tanks and 77.96 ± 2.32% (6.71 ± 0.20 mg/L) for the low oxygen tanks. This experiment was approved by the Animal Welfare and Ethical Review Body (reference AWERB/1617/84) in accordance with the UK Home Office under the Animals (Scientific Procedures) Act 1986.

Experimental diets

Diets were formulated (Table 1) to be isoenergetic and isonitrogenous and were produced by SPAROS I&D (Olhão, Portugal). Four diets were formulated based on a 2 x 2 factorial design. Two diets had high lipid (HL; 230 g/kg) and the other two had low lipid (LL; 180 g/kg). One of each HL and LL diets had high n-3 LC-PUFA (Hn3; 14 g/kg) and the other had low n-3 LC-PUFA (Ln3; 7 g/kg). Each diet had an equal level of fishmeal (200 g/kg) and digestible energy (21 MJ/kg). Yttrium oxide was included as a digestibility marker in the diets. The 3 mm diets were made by twin-screw extrusion, vacuum coated with oils, and stored at 4 ^{o}C.

Each of the four dietary treatments were randomly assigned, in triplicate, to the tanks for the entire experiment, including an additional control group fed the HL-Hn3 diet under normoxia. Therefore, there were 15 tanks total where 12 were hypoxic and 3 were normoxic. Diets were fed twice daily over separate three-hour periods using automated drum feeders (Arvo-tec Oy, Huutokoski, Finland) at a rate of ~1.0-1.5% of fish bodyweight per day. Feeding rations were adjusted based on the amount of uneaten feed collected from each tank in order to ensure that a slight excess of feed was fed. Each morning, the uneaten feed was collected manually from each external tank standpipe and was weighed. A recovery

test was performed to determine a correction factor for the wet uneaten feed in order to calculate the daily feed intake according to (Helland *et al.*, 1996).

Sample collection

Fish were sedated with MS222 and weighed at day 0, 35 and 116. 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 ^{o}C. At each weighing point, eight fish per tank were euthanised by an overdose of MS222 and cervical dislocation. Four fish were pooled per tank (n=3) and stored at -20 ^{o}C for whole body composition analysis. One replicate tank in the high oxygen group was excluded from growth performance analysis due to high incidence of amoebic gill disease (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 tissue samples frozen in cryotubes on dry ice and stored at -70 ^{o}C for subsequent gene expression analysis.

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 ^{o}C for approximately 24 hours according to the Association of Official Analytical Chemists (AOAC, 1995). Protein was analysed by digestion in sulphuric acid at 400 ^{o}C (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). 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 the American Oil Chemists' Society (Christie, 2003). First, fatty acid methyl esters (FAME) were made by acid-catalysed esterification of 1 mg of total lipid by overnight incubation at 50 ^{o}C 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 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, Hemel Hempstead, UK) and compared to external standards and published data (Connell, 1980). Data were collected and processed using Chromcard for Windows (version 2.01; Thermoquest Italia S.p.A., Milan, Italy).

Calculations of growth performance, body indices and feed utilisation

Weight gain, specific growth rate (SGR), feed conversation ratio (FCR), feed intake (FI), crude protein (CP) intake were calculated using the following equations:

Weight gain (g) = ((FW – SW) / SW) x 100

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

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

FCR = FI / (FW – SW)

where FW is the final weight (g) of the fish, SW is the initial weight of the fish (g), T is the duration of the experiment (days), feed intake is the total feed intake (g) minus the feed waste (g) as corrected according to Helland *et al.* (1996) dependent on the number of fish per tank. CP intake is the total feed intake (g) multiplied by the CP% in each diet. Means were generated based on per fish values from the three replicate tanks.

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

HSI (%) = (W_{Liv} / FW) x 100

VSI (%) = (W_{Vis} / FW) x 100

where W_{Liv} is the weight of liver (g), W_{Vis} is the weight of viscera (g) and FW is fish weight.

Nutrient retention and apparent digestibility coefficients (ADC) were calculated as:

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

Diet ADC (%) = [1 – (F / D × D_i / F_i)] x 100

where C is % nutrient (or MJ kg^{-1} for energy) in whole body carcass, 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.

Transcriptomic analyses

Liver samples were thawed on ice and approximately 50 mg of the apex 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. 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 identify the two bands.

From two fish per tank (n=6/treatment), 6 μg were pooled and then diluted with RNase free water to 2 μg (200 ng/μL). Samples were denatured at 75 °C for 5 min and then added in 10 μL was 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 °C for 10 min, 37 °C for 120 min and 85 °C 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 °C for 2 min, 95 °C for 10 min and 35 cycles of 95 °C for 15 sec, 60 °C for 30 sec and 72 °C 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, n3 and oxygen (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 3) were compared using Genorm (Vandesompele *et al.*, 2002) and *hprt* and *rps5* were selected to be the most stable genes.

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 fit the data. Differences between treatments were determined using either linear models (lm) or linear mixed effects

models (lme) based on the *stats* and *nlme* R packages (Pinheiro *et al.*, 2014) with lipids, LC-PUFA and oxygen as fixed effects and fish tank as a random effect for the lme. Interactions between lipids and LC-PUFA were also included in the model (y = lipids x LC-PUFA + oxygen). Oxygen was included or excluded from the model based on the AIC score. P-values of each factor and interaction from the models were generated using ANOVA tables and below 0.05 were considered significant. P-values between treatments were determined using Fisher's Least Significant Difference test.

Results

Growth performance and feed efficiency

Fish growth and feed intake were influenced by the level of dietary lipid, n-3 LC-PUFA and dissolved oxygen, although no interactions were found (Table 4, Fig. 1). Final weight was significantly affected ($p<0.05$) by all three factors where fish fed the HL-Hn3 diet had the highest growth under hypoxic conditions. In the present study, all three factors had a tendency ($p<0.10$) to alter feed intake, which was lowest for fish fed the LL-Ln3 diet. Level of n-3 affected protein intake, which was higher in fish fed the high n-3 diets. No effects on FCR, survival, HSI and VSI were found ($p>0.10$).

An additional data analysis was performed that included three additional treatments under normoxic conditions in order to put the present study into a larger context. Weight gain data from a parallel study (Huyben *et al.*, 2021) were merged and showed that oxygen, lipid and n-3 were significant ($p<0.05$) when including both normoxic and hypoxic conditions (Fig. 2).

Water quality

Dissolved oxygen fluctuated in both normoxic and hypoxic groups over the 116-day experiment, but remained at least 1.0 mg L^{-1} difference (Fig. 3). Ammonia in the water decreased before or at each feeding event and then increased afterward, which was slightly higher for fish under hypoxic compared to normoxic conditions (Fig 4).

Whole body composition, nutrient retention and digestibility

Lipid level significantly ($p<0.05$) affected dry matter, ash, lipid and energy content in the whole body carcasses, while n-3 and oxygen had no effect (Table 5). High lipid diets increased content of dry matter, lipid and energy in the carcass while ash was decreased. In regard to fatty acids, level of lipid and n-3 had significant effects on almost every individual and group of fatty acids, including total n-3 and total

LC-PUFA. Total saturates, n-6, n-3, PUFA and LC-PUFA were highest in fish fed the LL-Hn3 diet while monoenes were the lowest in this treatment. In addition, a lipid x n-3 interaction existed for three n-3 PUFAs where levels were elevated in the carcass of fish fed the LL-Hn3 diet. Oxygen had no effects on carcass composition, except on 18:0.

Retention of protein and energy in the whole body carcass was affected by dietary lipid level ($p<0.05$), which was elevated in fish fed the high lipid diets (Table 6). No effect of n-3 or oxygen was found. In regard to the fatty acids, level of lipid and n-3 had significant effects on approximately half the fatty acids including a lipid x n3 interaction on two n-6 PUFAs. High lipid diets increased the retention of total n-3 and LC-PUFA, including EPA and DHA. High n-3 diets decreased the retention of LC-PUFA, including EPA but not DHA. The retention of total n-3, EPA and DHA was the highest in fish fed the HL-Hn3 diet but no significant lipid x n-3 interaction was found.

Digestibility of lipid was affected by lipid level in the diet, whereas energy was affected by n-3 (Table 7). High lipid diets increased lipid digestibility while high n-3 diets decreased energy digestibility. In regard to fatty acids, level of lipid affected every fatty acid digestibility except 14:0 where high lipid diets increased the digestibility of total saturates, monoenes, n-6, n-3, PUFA and LC-PUFA, including EPA and DHA. Level of n-3 affected fewer fatty acids where high n-3 diets increased the digestibility of total n-3 and LC-PUFA, including DHA and EPA. Significant lipid x n-3 interactions existed for DHA, total n-3 and LC-PUFA where fish fed the HL-Hn3 diet has the highest digestibilities.

Differential gene expression in the liver

The expression of *fads2d5, fads2d6* and *elovl2* involved in fatty acid synthesis were the only genes impacted in this study (Fig. 5-7). Compared to all other treatments, fish fed the HL-Hn3 diet had significantly reduced expression of *fads2d5* and *elovl2* ($p<0.05$) as well as a reduced tendency of *fads2d6* ($p=0.057$). Fish fed high lipid diets had lower expression of genes related to lipid transcription factors (Fig. 6) and beta-oxidation of fatty acids (Fig. 7), although no significant differences were found. No significant effects of oxygen were found as well ($p>0.05$).

Discussion

Dietary lipid, n3 and dissolved oxygen on growth performance

Under hypoxia, we found significant effects of both lipid and n-3 LC-PUFA dietary levels on fish growth (Table 4), although no lipid x n-3 LC-PUFA interaction was found. This may suggest that n-3

is required as a proportion of the diet rather than proportion of lipid. However, any requirement responses are likely to have been constrained by the reduced feed intake induced by the hypoxia stressor, thus fish responses were predominantly constrained by energy intake rather than any potential interaction lipid and n-3 LC-PUFA. Previous studies have found that PUFA are better represented as a proportion of total lipid rather than a proportion of diet for optimal growth of rainbow trout (Watanabe, 1982) and Giant tiger prawn (*Penaeus monodon*) (Glencross *et al.*, 2002). In a parallel study, Atlantic salmon post-smolts had the highest weight gain when fed higher levels of lipid and n-3 LC-PUFA at normoxia, which included a lipid x n-3 LC-PUFA interaction indicating that the n-3 requirement was based on the proportion of dietary lipid (Huyben *et al.*, 2021). These authors also noted that a slight difference in net energy levels between high and low lipid diets may have influenced growth. Particularly in the present study, lower feed intake would have exacerbated the difference in net energy between fish fed high and low lipid diets and may explain higher growth of fish fed the HL diets.

These results also show the importance of high lipid diets as a preferred energy source when hypoxia results in lower feed intake. Other studies have shown that the effect of lipid is less than that of protein on oxygen demand, so under hypoxic conditions it suggests that high lipid diets might perform better even when the diets were formulated to be isoenergetic on a digestible basis (Cho and Bureau, 1995). Rainbow trout have been found to have an increased preference and intake of diets with higher n-3 LC-PUFA (Roy *et al.*, 2020), which may have occurred in the present study due to tendency for higher intake when fed both high n-3 and lipid diets (Table 4). In a parallel study, hypoxia had only minor effects on the gut microbiome, while high lipid and high n-3 LC-PUFA diets significantly influenced bacterial diversity and composition (Huyben *et al.*, 2020). Previous studies have found that reductions in dissolved oxygen reduce feed intake and growth of Atlantic salmon (Vikeså *et al.*, 2017, Hansen *et al.*, 2015, Remen *et al.*, 2014, Remen *et al.*, 2012). In contrast, Vikeså *et al.* (2017) found similar final weights of large Atlantic salmon under normoxia and hypoxia fed diets with different levels of digestible energy. However, low energy diets resulted in higher FCR and numerically lower growth. These authors also included a treatment group of fish that were pair fed the same amount as the hypoxia group and found that reduced feed intake only explained 50% of the poor growth. Previous studies have linked reduced growth under hypoxic conditions to lower appetite rather than reduced feed efficiency (Thetmeyer *et al.*, 1999, Foss *et al.*, 2002). Chronic stressors, such as hypoxia, can reduce appetite and supress several other physiological pathways in fish that can lead to poor growth (Segner *et al.*, 2012). The present study is in agreement since FCR was not affected by lipid, n-3 or oxygen level and no significant effects were found under hypoxic conditions.

Nutrient retention and digestibility

Under both normoxic and hypoxic conditions, high lipid diets improved protein, lipid and energy retention in the fish carcass as well as lipid digestibility (Tables 6 and 7). In rainbow trout, Glencross (2009) found that lower oxygen correlated with lower feed intake but had no effect on the marginal utilization efficiency of digestible protein and energy. Vikeså *et al.* (2017) found that dissolved oxygen reduced the retention of digestible protein and energy in Atlantic salmon, although fish were under lower levels of dissolved oxygen (5.5 mg/L) compared to the present study (6.7 mg/L). These authors suggested that hypoxia resulted in a higher use of nutrients as energy rather than deposition due to higher energetic cost related to low oxygen, e.g. accelerated gill ventilation (Perry *et al.*, 2009). (Remen *et al.*, 2014) found that cyclical decreases from 80% to 50% saturation in dissolved oxygen reduced lipid retention in Atlantic salmon, but again these authors attributed this to reduced feed intake since FCR was unaffected.

Retention of protein and energy were affected by dietary levels of lipid, whereas both lipid and n-3 affected the retention of LC-PUFA (Table 6). In Atlantic salmon, diets high in n-3 LC-PUFA have been previously shown to reduce retention of n-3 in the whole body carcass and fillet (Huyben *et al.*, 2021, Glencross *et al.*, 2014, Bou *et al.*, 2017). It is common to find lower retention of a nutrient when it has a higher inclusion in the diet than its biosynthetic needs, although information on the effects of other nutrients, such as lipid, on n-3 LC-PUFA retention is lacking. Dessen *et al.* (2017) found that high protein diets did not affect energy retention while lipid and protein retention were only affected during specific times of the year, indicating a seasonal or life-stage influence. Vikeså *et al.* (2017) found no effects of low or high energy diets on the retention of digestible protein or lipid in Atlantic salmon. This is in contrast to the present study, although the diets in our case were isoenergetic.

Both dietary levels of lipid and n-3 influenced digestibility of LC-PUFA, with a notable improvement for fish fed the high lipid diet with high n-3 (Table 7). A lipid x n-3 interaction on digestibility of LC-PUFA, including DHA, was found and may provide further support to the notion that the n-3 requirement is proportional to the level of lipid in the diet. The extent of this interaction may have been masked by the reduced feed intake induced by the hypoxia stressor, thus affecting some nutrient utilisation parameters at lower levels of intake without significant effects on growth performance. In a parallel study, lipid and/or n-3 levels significantly affected the digestibility of protein, lipid and energy for Atlantic salmon reared under normoxic conditions, while the digestibility of LC-PUFA was unaffected (Huyben *et al.*, 2021). In contrast, studies have found that DHA digestibility was lower for Atlantic salmon fed diets with vegetable oils containing less DHA (Karalazos *et al.*, 2011, Betancor *et al.*, 2015). Lower feed intake under hypoxia conditions may have accentuated the effects of these two dietary factors on LC-PUFA digestibility in the present study.

The impact of lipid level on the digestibilities of several minerals were noted in the study (Table 8), although it may be correlated with fish growth performance. Significant interactions between dietary lipid x n-3 for Mg and Zn digestibilities may be a result of fish size since highest values were found for fish with the largest final weight (Table 4). Larger fish need to deposit more minerals for skeletal development (NRC, 2011). Mineral content in the diets were not suspected of influencing digestibility since the contents were similar between diets, except for K (Table 1). Negative values for Ca and Mg retention were expected since fish can absorb Ca and Mg from the water column (NRC, 2011), even to the degree where dietary supplementation is not necessary for Atlantic salmon in sea water (Lall and Bishop, 1977). More research is needed to investigate a possible link between dietary lipid and n-3 levels on mineral digestibility.

Dietary lipid and hypoxia on hepatic gene expression

The results from the present study indicate that dietary lipid level and hypoxia had little effect on the transcriptomic pathways for fatty acid synthesis, regulation and beta-oxidation whereas dietary n-3 only affected fatty acid synthesis (Figs. 5-7). Reduced expression of *fads2d5*, *fads2d6* and *elovl2* in fish fed the high n-3 diets agrees with previous findings that fed fish oil with higher n-3 LC-PUFA content to Atlantic salmon (Zheng *et al.*, 2004, Leaver *et al.*, 2008, Betancor *et al.*, 2017). In contrast, previous studies have found that feeding different levels of n-3 results in altered expression of lipid transcription factors and fatty acid beta-oxidation genes in salmon (Leaver *et al.*, 2008). Gene expression of other lipid metabolic pathways may not have been affected due to the slight reduction in dissolved oxygen (i.e. 8.0 to 6.7 mg/L or 93 to 78% saturation) and therefore only a slight reduction in feed intake (Table 4). Similar to growth, reduced feed intake may have masked effects on gene expression since a parallel study found a significant lipid x n-3 interaction on the expression of lipid transcription factors and beta-oxidation of fatty acids (Huyben *et al.*, 2021).

Conclusion

This study was novel in evaluating the growth, feeding and transcriptomic responses of Atlantic salmon post-smolts under hypoxic conditions that are more relevant to commercial aquaculture systems. Contrasting previous results from other studies (Huyben *et al.*, 2021, Vikeså *et al.*, 2017, Bou *et al.*, 2017), fish growth was driven predominantly by energy intake, rather than n-3 LC-PUFA intake, with this intake response being caused by the environmental stressor. Higher retention of protein, energy, EPA and DHA in fish fed high lipid diets may be a result of increased growth while dietary n-3 level had little to no apparent effect. However, a lipid x n-3 interaction was prominent regarding the digestibility of LC-PUFA, including DHA, which was highest for those fish fed the high lipid diet with high n-3. This interaction may not have been evident at the phenomic level due to the overarching

effects of the reduced feed intake and being masked by a drive for higher energy intake. In terms of transcriptomic responses, there was a generalised reduction in the expression of genes regulating fatty acid synthesis in fish fed high n-3 diets, while there was no apparent effect of hypoxia or dietary lipid. These results support that dietary intake of energy is prioritised over lipids and n-3 LC-PUFA for Atlantic salmon under hypoxic conditions.

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Conflict of Interest

The authors declare that there is no competing or financial interests.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

AOAC (1995) *Official Methods of Analysis of AOAC International*, Association of Official Analytical Chemists, Washington, DC.

Betancor, M.B., Li, K., Sprague, M., Bardal, T., Sayanova, O., Usher, S., Han, L., Måsøval, K., Torrissen, O., Napier, J.A., Tocher, D.R. & Olsen, R.E. (2017) An oil containing EPA and DHA from transgenic Camelina sativa to replace marine fish oil in feeds for Atlantic salmon (Salmo salar L.): Effects on intestinal transcriptome, histology, tissue fatty acid profiles and plasma biochemistry. *PLOS ONE,* **12,** e0175415.

Betancor, M.B., Sprague, M., Sayanova, O., Usher, S., Campbell, P.J., Napier, J.A., Caballero, M.J. & Tocher, D.R. (2015) Evaluation of a high-EPA oil from transgenic Camelina sativa in feeds for Atlantic salmon (Salmo salar L.): Effects on tissue fatty acid composition, histology and gene expression. *Aquaculture,* **444,** 1-12.

Bou, M., Berge, G.M., Baeverfjord, G., Sigholt, T., Østbye, T.-K., Romarheim, O.H., Hatlen, B., Leeuwis, R., Venegas, C. & Ruyter, B. (2017) Requirements of n-3 very long-chain PUFA in Atlantic salmon (*Salmo salar* L): effects of different dietary levels of EPA and DHA on fish performance and tissue composition and integrity. *British Journal of nutrition,* **117,** 30-47.

Cho, C.Y. & Bureau, D. (1995) Determination of the energy requirements of fish with particular reference to salmonids. *Journal of Applied Ichthyology*, **11,** 141-163.

Christie, W. (2003) *Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids*, The Oily Press, Bridgwater, UK.

Connell, J.J. (1980) *Advances in fish science and technology*, Fishing News Books.

Dessen, J.-E., Weihe, R., Hatlen, B., Thomassen, M.S. & Rørvik, K.-A. (2017) Different growth performance, lipid deposition, and nutrient utilization in in-season (S1) Atlantic salmon post-smolt fed isoenergetic diets differing in protein-to-lipid ratio. *Aquaculture*, **473,** 345-354.

Folch, J., Lees, M. & Stanley, G.S. (1957) A simple method for the isolation and purification of total lipides from animal tissues. *Journal of biological chemistry*, **226,** 497-509.

Foss, A., Evensen, T.H. & Øiestad, V. (2002) Effects of hypoxia and hyperoxia on growth and food conversion efficiency in the spotted wolffish *Anarhichas minor* (Olafsen). *Aquaculture Research*, **33,** 437-444.

Glencross, B., Smith, D., Thomas, M. & Williams, K. (2002) Optimising the essential fatty acids in the diet for weight gain of the prawn, *Penaeus monodon*. *Aquaculture*, **204,** 85-99.

Glencross, B.D. (2009) Reduced water oxygen levels affect maximal feed intake, but not protein or energy utilization efficiency of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture Nutrition*, **15,** 1-8.

Glencross, B.D., Tocher, D.R., Matthew, C. & Bell, J.G. (2014) Interactions between dietary docosahexaenoic acid and other long-chain polyunsaturated fatty acids on performance and fatty acid retention in post-smolt Atlantic salmon (*Salmo salar*). *Fish Physiology and Biochemistry*, **40,** 1213-1227.

Hansen, T.J., Olsen, R.E., Stien, L., Oppedal, F., Torgersen, T., Breck, O., Remen, M., Vågseth, T. & Fjelldal, P.G. (2015) Effect of water oxygen level on performance of diploid and triploid Atlantic salmon post-smolts reared at high temperature. *Aquaculture*, **435,** 354-360.

Helland, S., Grisdale-Helland, B. & Nerland, S. (1996) A simple method for the measurement of daily feed intake of groups of fish in tanks. *Aquaculture*, **139,** 157-163.

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**.

Huyben, D., Roehe, B.K., Bekaert, M., Ruyter, B. & Glencross, B. (2020) Dietary Lipid:Protein Ratio and n-3 Long-Chain Polyunsaturated Fatty Acids Alters the Gut Microbiome of Atlantic Salmon Under Hypoxic and Normoxic Conditions. *Frontiers in Microbiology*, **11**.

Karalazos, V., Bendiksen, E. & Bell, J.G. (2011) Interactive effects of dietary protein/lipid level and oil source on growth, feed utilisation and nutrient and fatty acid digestibility of Atlantic salmon. *Aquaculture*, **311,** 193-200.

Lall, S. & Bishop, F. (1977) Studies on mineral and protein utilization by Atlantic salmon (*Salmo salar*) grown in seawater (Technical Report No. 68) In *Fish and Marine Science*.

Leaver, M.J., Villeneuve, L.A., Obach, A., Jensen, L., Bron, J.E., Tocher, D.R. & Taggart, J.B. (2008) Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (Salmo salar). *Bmc Genomics*, **9,** 299.

NRC (2011) *Nutrient Requirements of Fish and Shrimp*, National Academy Press, Washington, DC, USA.

Perry, S.F., Jonz, M.G. & Gilmour, K.M. (2009) Chapter 5 Oxygen Sensing And The Hypoxic Ventilatory Response In *Fish Physiology* (Richards, J.G., *et al.* eds.), Vol. 27, pp. 193-253. Academic Press.

Persson, J.-Å. (2008) *Handbook for Kjeldahl Digestion: a recent review of the classical method with improvements developed by FOSS*, FOSS.

Pinheiro, J., Bates, D., DebRoy, S. & Sarkar, D. (2014) R Package nlme: linear and nonlinear mixed effects models. *R package version 3.1-117*, **http://CRAN.R-project.org/package=nlme**.

R-Core-Team (2015) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Remen, M., Aas, T.S., Vågseth, T., Torgersen, T., Olsen, R.E., Imsland, A. & Oppedal, F. (2014) Production performance of Atlantic salmon (*Salmo salar* L.) postsmolts in cyclic hypoxia, and following compensatory growth. *Aquaculture Research,* **45,** 1355-1366.

Remen, M., Oppedal, F., Torgersen, T., Imsland, A.K. & Olsen, R.E. (2012) Effects of cyclic environmental hypoxia on physiology and feed intake of post-smolt Atlantic salmon: Initial responses and acclimation. *Aquaculture,* **326-329,** 148-155.

Roy, J., Mercier, Y., Tonnet, L., Burel, C., Lanuque, A., Surget, A., Larroquet, L., Corraze, G., Terrier, F., Panserat, S. & Skiba, S. (2020) Rainbow trout prefer diets rich in omega-3 long chain polyunsaturated fatty acids DHA and EPA. *Physiology & Behavior,* **213,** 112692.

Segner, H., Sundh, H., Buchmann, K., Douxfils, J., Sundell, K.S., Mathieu, C., Ruane, N., Jutfelt, F., Toften, H. & Vaughan, L. (2012) Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. *Fish Physiology and Biochemistry,* **38,** 85-105.

Takeuchi, T., Shiina, Y. & Watanabe, T. (1992a) Suitable levels of n-3 highly unsaturated fatty acids in diet for fingerlings of red sea bream. *Nippon Suisan Gakkaishi,* **58,** 509-514.

Takeuchi, T., Shiina, Y., Watanabe, T., Sekiya, S. & Imaizumi, K. (1992b) Suitable levels of n-3 highly unsaturated fatty acid in diet for fingerlings of yellowtail. *Nippon Suisan Gakkaishi,* **58,** 1341-1346.

Thetmeyer, H., Waller, U., Black, K.D., Inselmann, S. & Rosenthal, H. (1999) Growth of European sea bass (*Dicentrarchus labrax* L.) under hypoxic and oscillating oxygen conditions. *Aquaculture,* **174,** 355-367.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. & Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology,* **3,** research0034. 0031.

Vikeså, V., Nankervis, L. & Hevrøy, E.M. (2017) High dietary energy level stimulates growth hormone receptor and feed utilization in large Atlantic salmon (*Salmo salar* L.) under hypoxic conditions. *Aquaculture Nutrition,* 1-11.

Watanabe, T. (1982) Lipid nutrition in fish. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* **73,** 3-15.

Zheng, X., Tocher, D.R., Dickson, C.A., Bell, J.G. & Teale, A.J. (2004) Effects of diets containing vegetable oil on expression of genes involved in highly unsaturated fatty acid biosynthesis in liver of Atlantic salmon (*Salmo salar*). *Aquaculture,* **236,** 467-483.

Figures

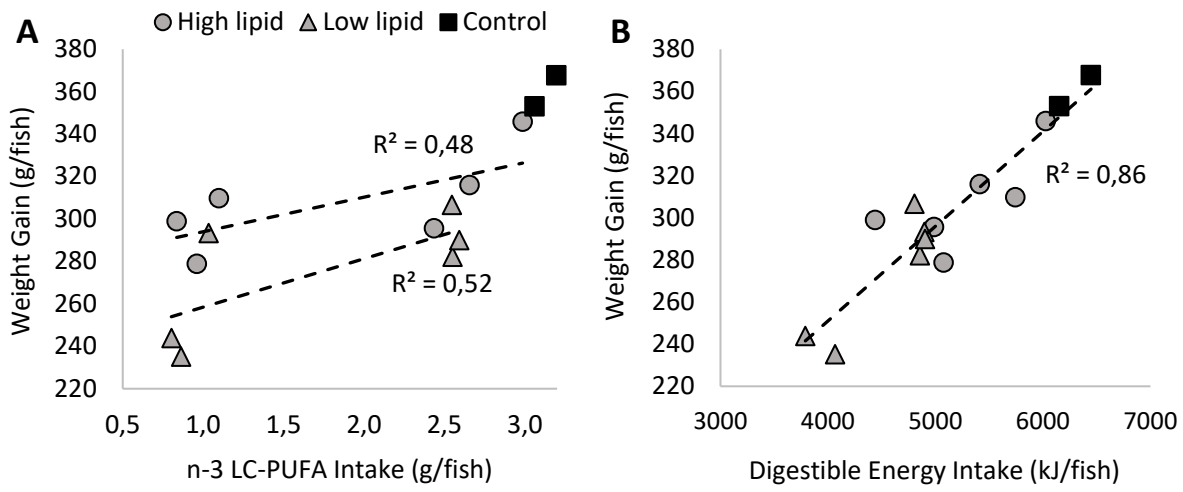

Figure 1. Weight gain of Atlantic salmon fed high and low levels of total lipid under hypoxia (grey symbols) compared to a control diet under normoxia (black symbols) for 116 days plotted as a function of A) n-3 LC-PUFA intake, and B) digestible energy intake. The parallel trendlines indicate there was no lipid x n-3 interaction on weight gain, whereas weight gain had a linear relationship with digestible energy intake.

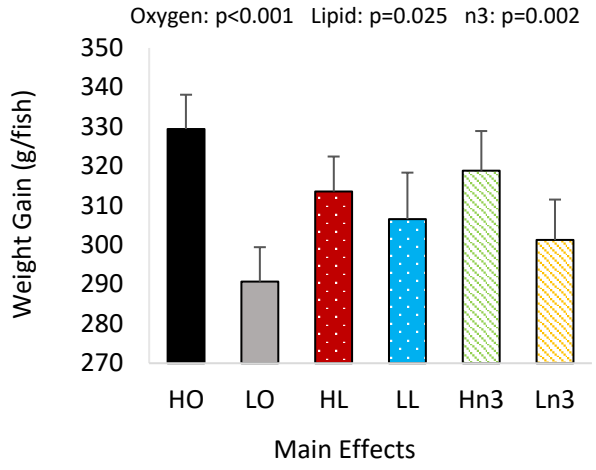

Figure 2. Main effect means of weight gain of Atlantic salmon relative to each of the high (H) and low (L) factorial parameters (O; oxygen, L; lipid and n3; n-3 LC-PUFA) of the full study (this study plus data from Huyben et al., 2021). An interaction effect between lipid level x n-3 LC-PUFA was found in Huyben et al. (2021) under normoxic conditions, but not in the present study under hypoxic conditions (see Fig. 1).

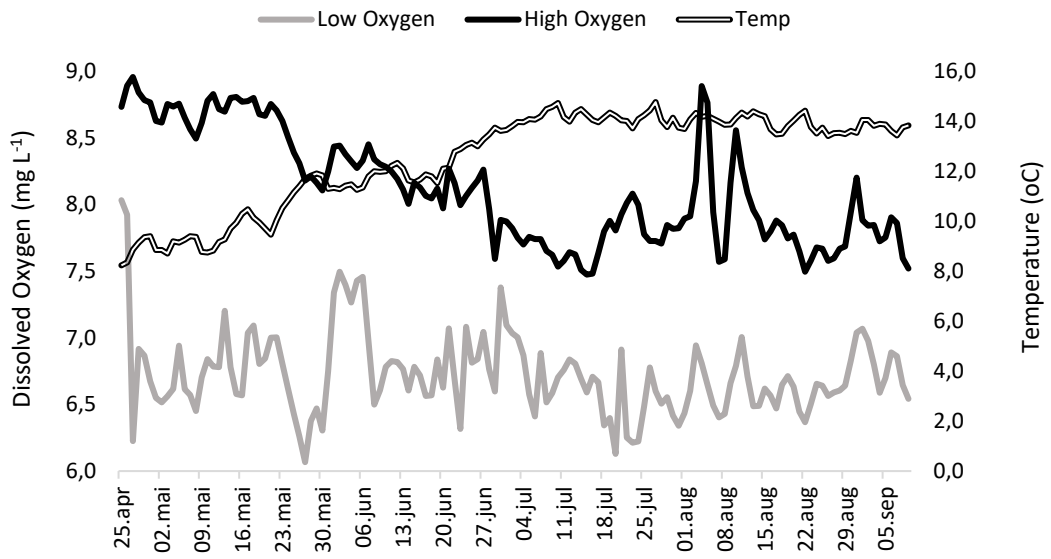

Figure 3. Fluctuations in dissolved oxygen (mg L^{-1}) between the high and low oxygen treatments over four months along with changes in water temperature.

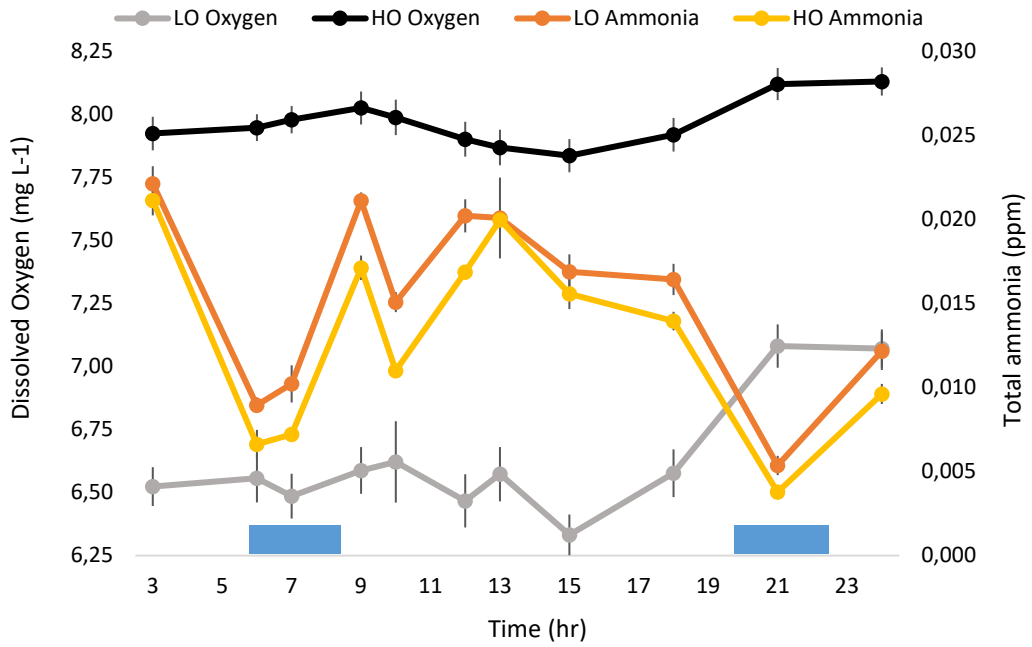

Figure 4. Temporal varition of total ammonia and dissolved oxygen levels mean (±SE) from the discharged tank water over a 24 hour period and pooled across dietary groups. Feeding times (indicated by the blue bars) were from 5.5-8.5 and 19.5-22.5 hours.

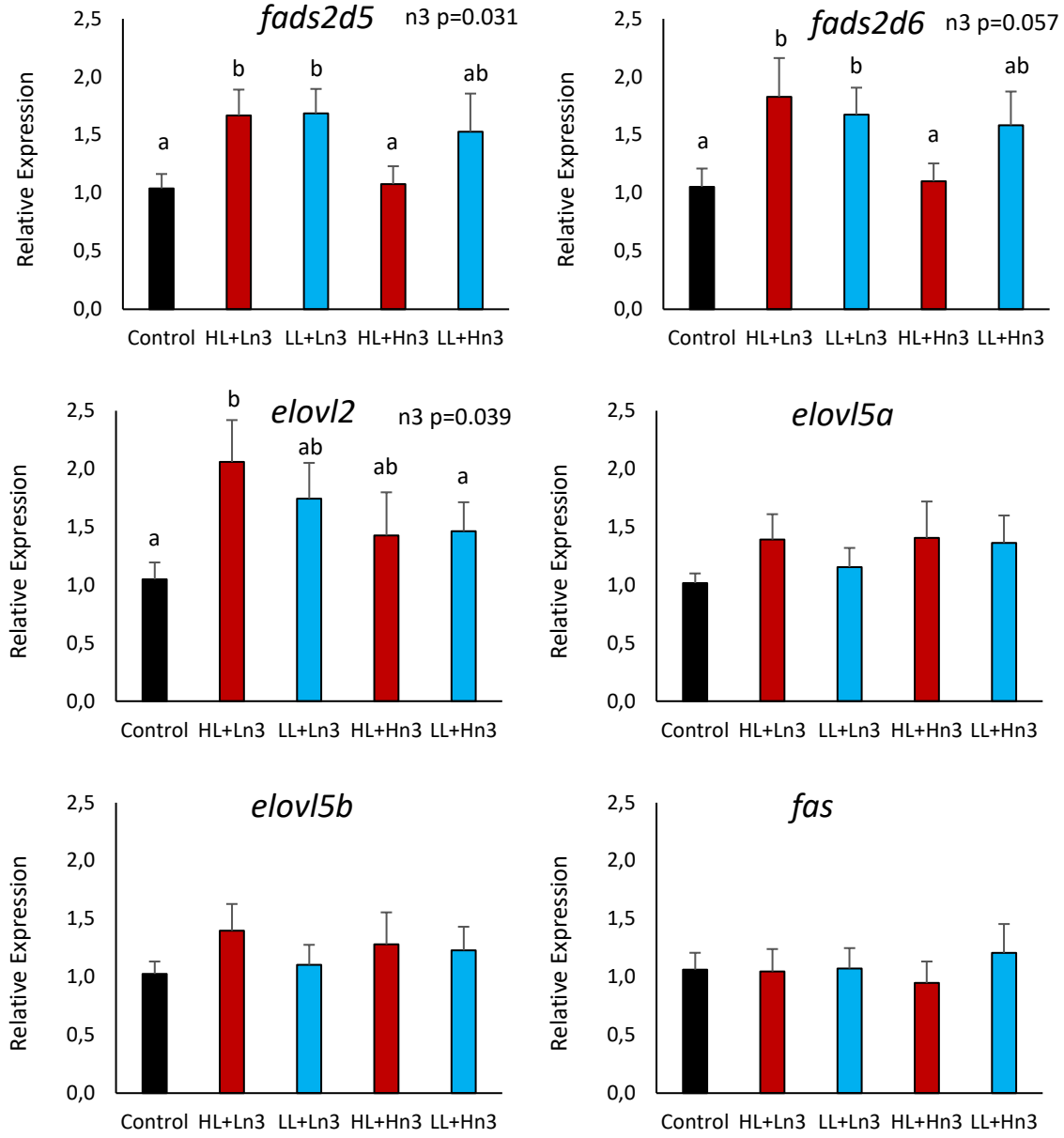

Figure 5. Expression of genes (mean ± SE, n=6) relative to the geometric mean of two reference genes (*hprt* and *rps5*) involved in fatty acid synthesis in the liver of Atlantic salmon fed high or low levels of lipid (HL or LL) and high or low levels of n-3 LC-PUFA (Hn3 or Ln3) under hypoxia compared to a control diet (HL-Hn3) under normoxia (Ct=1.0). Different letters indicate significant difference ($p<0.05$).

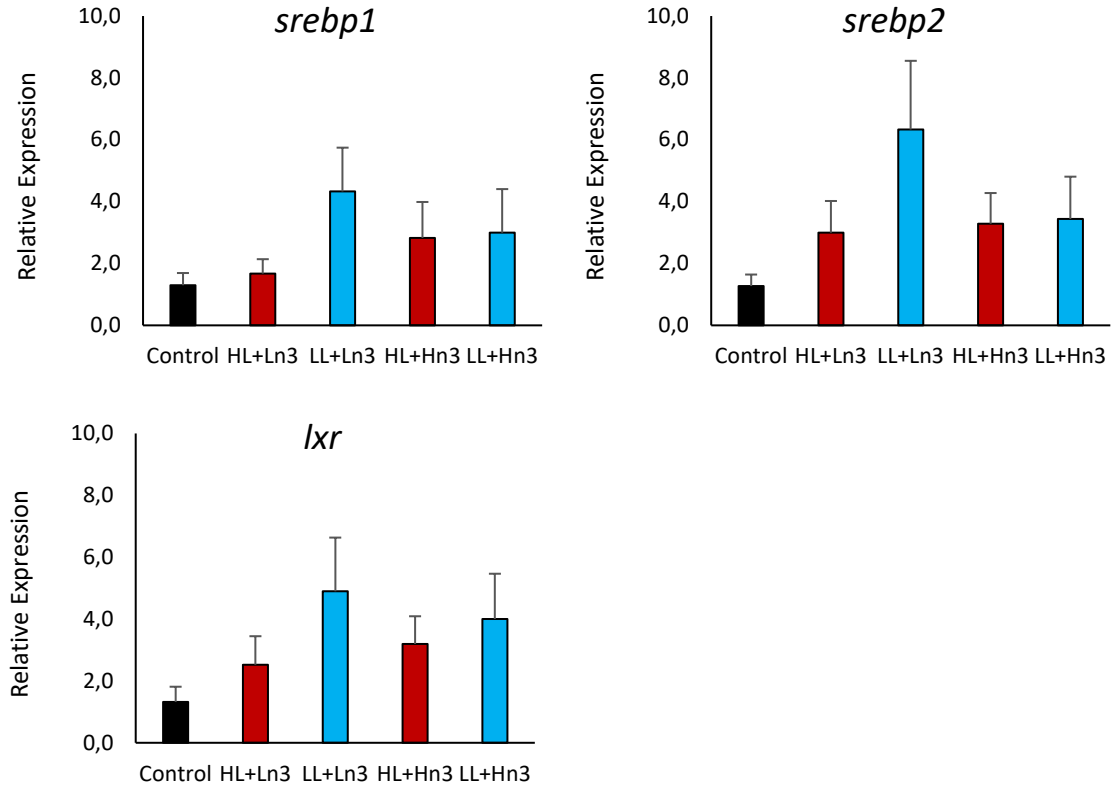

Figure 6. Expression of genes (mean ± SE, n=6) relative to the geometric mean of two reference genes (*hprt* and *rps5*) involved in transcription factors in lipid regulation in the liver of Atlantic salmon fed high or low levels of lipid (HL or LL) and high or low levels of n-3 LC-PUFA (Hn3 or Ln3) under hypoxia compared to a control diet (HL-Hn3) under normoxia (Ct=1.0).

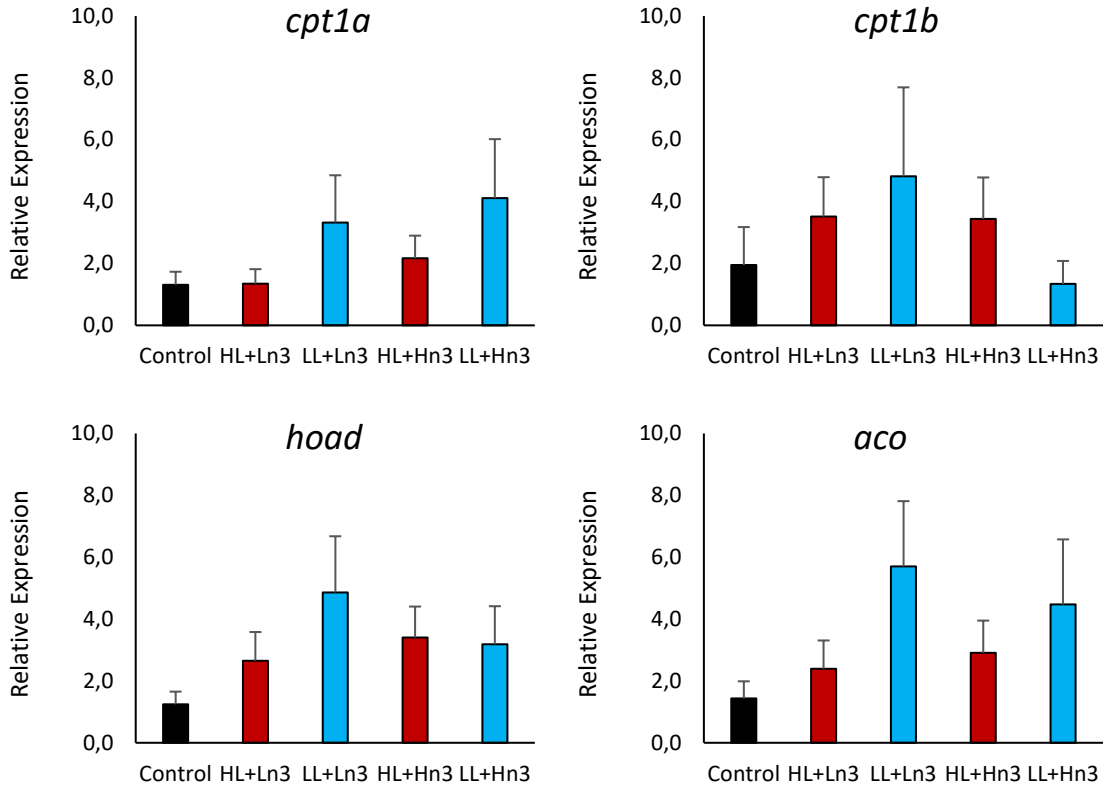

Figure 7. Expression of genes (mean ± SE, n=6) relative to the geometric mean of two reference genes (*hprt* and *rps5*) involved in beta oxidation of fatty acids in the liver of Atlantic salmon fed high or low levels of lipid (HL or LL) and high or low levels of n-3 LC-PUFA (Hn3 or Ln3) under hypoxia compared to a control diet (HL-Hn3) under normoxia (Ct=1.0).

Tables

Table 1. Diet formulation, proximate composition and mineral content.

	Diets			
Formulation (g kg^{-1} wet matter)	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3
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	1.5	1.5	1.5	1.5
Soy Lecithin	10	10	10	10
Carophyll Pink	0.5	0.5	0.5	0.5
Choline chloride	1	1	1	1
Proximate composition (g kg^{-1} dry matter)				
Dry matter	938	947	941	950
Protein	475	590	490	591
Lipid	241	192	222	187
Ash	85	83	85	83
CHO[5]	199	136	203	139
Energy (MJ kg^{-1})	24.2	23.6	23.7	23.1
Mineral content (g kg^{-1} dry matter)				
Calcium (Ca)	17.6	17.3	18.1	17.6
Magnesium (Mg)	1.7	1.4	1.7	1.4
Phosphorus (P)	13.0	13.3	13.1	13.9
Potassium (K)	8.4	5.2	8.5	5.3
Sodium (Na)	5.1	6.7	5.2	6.9
Zinc (Zn)	0.4	0.4	0.4	0.4

HL; high lipid, LL; low lipid, L/Hn3; high or low n-3 long chain polyunsaturated fatty acids
[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]CHO; carbohydrate, calculated by difference (i.e. CHO = 1000 - protein - lipid - ash)

12 Table 2. Fatty acid (% total fatty acids) of the diets.

	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

13 Fatty acids <0.2% not reported.

14

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

Function	Gene	Full name	Primers	Bp	Accession
Reference	*cfl2*	Cofilin-2	AGCCTATGACCAACCCACTG TGTTCACAGCTCGTTTACCG	224	TC63899[b]
	hprt	Hypoxanthine phosphor-ribosyl-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.153.1.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 (n=3, tanks per treatment) fed low and high levels of lipids and/or n3 LC-PUFA under low and high oxygen conditions.

	High Oxygen	Low Oxygen					p-value[1]			
	HL-Hn3 (control)	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3	SE	Lipid	n3	Oxygen	Lipid x n3
Initial weight (g)	191.7a	181.8b	178.5b	181.5b	178.5b	1.8	0.087	0.954	**0.003**	0.919
Final weight (g)	552.0a	477.6b	435.8c	500.6b	471.4bc	10.3	**0.015**	**0.036**	**0.023**	0.611
Weight gain (g)	360.3a	295.8bc	257.4c	319.1ab	292.9bc	11.3	**0.028**	**0.042**	0.065	0.636
Gain rate (g $fish^{-1}$ day^{-1})	3.11a	2.55bc	2.22c	2.75ab	2.52bc	0.1	0.347	0.118	**0.001**	0.462
Feed intake (g $fish^{-1}$)	317.6a	247.2bc	210.3c	273.4ab	246.8bc	12.1	0.054	0.057	0.083	0.728
Protein intake (g $fish^{-1}$)	146.5a	110.2c	117.6bc	126.1abc	138.6ab	5.8	0.187	**0.026**	0.097	0.724
Lipid intake (g $fish^{-1}$)	66.7a	53.4b	38.5c	57.4ab	43.5c	2.5	**0.001**	0.155	0.076	0.871
FCR	0.88	0.84	0.82	0.86	0.84	0.02	0.621	0.492	0.661	0.921
Survival (%)	97.9	100.0	97.2	100.0	100.0	0.7	0.143	0.143	0.163	0.143
HSI[2]	1.13	1.14	1.23	1.07	1.07	0.02	0.694	0.301	0.639	0.737
VSI[2]	8.67	9.69	9.05	8.54	7.96	0.04	0.454	0.189	0.921	0.971

FCR; feed conversion ratio, HSI; hepatosomatic index; n3 LC-PUFA; n-3 long chain polyunsaturated fatty acids (i.e. EPA and DHA), VSI; viscerosomatic index.

[1]P-values from linear model with lipids, n3 LC-PUFA (n3) and oxygen as fixed effects with a lipid-n3 interaction. Differing letters indicate $p<0.05$. P-values in bold are <0.05.

[2]n=12, four fish were sampled from each triplicate tank.

Table 5. Whole body proximate (g kg^{-1} wet matter basis) and fatty acid (% of total) composition of Atlantic salmon (n=3, pooled per tank) fed low and high levels of lipids and n-3 LC-PUFA under low and high oxygen conditions.

	High Oxygen	Low Oxygen					p-value[1]			
	HL-Hn3 (control)	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3	SE	Lipid	n3	Oxygen	Lipid x n3
Dry matter	315a	317a	295b	315a	300b	3.5	**<0.001**	0.694	0.993	0.360
Ash	15b	18ab	19a	16b	18a	0.7	**0.030**	0.146	0.732	0.472
Protein	182	186	186	184	192	2.5	0.190	0.526	0.663	0.193
Lipid	102a	97a	76b	97a	77b	3.7	**0.001**	0.940	0.444	0.976
Energy (MJ kg^{-1})	8.7a	8.6a	7.7b	8.7a	7.9b	0.1	**<0.001**	0.322	0.999	0.553
Fatty acids[2]										
14:0	1.9b	1.4d	1.6c	1.8b	2.1a	0.03	**<0.001**	**<0.001**	0.418	0.406
16:0	12.8ab	12.1c	12.5b	12.7b	13.3a	0.11	**0.005**	**<0.001**	0.688	0.918
18:0	3.4a	3.3c	3.4a	3.3bc	3.4ab	0.02	**0.002**	0.292	**0.038**	0.224
Total saturates	18.6b	17.2d	18.0c	18.4bc	19.3a	0.13	**<0.001**	**<0.001**	0.351	0.881
16:1n-9	0.4bc	0.5a	0.4ab	0.4bc	0.4c	0.01	0.119	**0.002**	0.715	0.589
16:1n-7	2.6b	2.1d	2.3c	2.6b	2.9a	0.02	**<0.001**	**<0.001**	0.533	0.679
18:1n-9	49.1b	53.5a	48.7b	48.9b	42.9c	0.23	**<0.001**	**<0.001**	0.589	**0.038**
18:1n-7	2.9	2.9	2.9	3.0	2.9	0.11	0.437	0.886	0.498	0.759
20:1n-9	3.2b	3.5a	3.5a	3.1b	3.2b	0.04	0.127	**<0.001**	0.120	0.412
22:1n-11	1.0c	1.0c	1.1ab	1.0bc	1.2a	0.03	**0.001**	0.146	0.392	0.585
22:1n-9	0.4bc	0.4bc	0.4a	0.4c	0.4ab	0.01	**0.003**	0.279	0.333	0.928
24:1n-9	0.4ab	0.3b	0.4ab	0.4ab	0.5a	0.02	**0.040**	0.192	0.992	0.839
Total monoenes	60.6b	64.8a	60.6b	60.5b	55.1c	0.20	**<0.001**	**<0.001**	0.874	0.051
18:2n-6	8.2b	8.1b	9.8a	8.4b	10.2a	0.10	**<0.001**	**0.019**	0.269	0.779
20:2n-6	0.8c	0.8d	1.0b	0.8cd	1.1a	0.02	**<0.001**	**0.001**	0.122	0.058
20:3n-6	0.4d	0.7b	0.9a	0.5d	0.6c	0.01	**<0.001**	**<0.001**	0.348	0.554
20:4n-6	0.3c	0.4b	0.5a	0.3c	0.4ab	0.01	**<0.001**	**0.002**	0.262	0.171
Total n-6 PUFA	10.0c	10.4b	12.7a	10.2bc	12.7a	0.11	**<0.001**	0.421	0.231	0.501
18:3n-3	2.0b	1.3d	1.5c	2.1b	2.5a	0.03	**<0.001**	**<0.001**	0.148	**0.024**
18:4n-3	0.5b	0.4c	0.4bc	0.5b	0.6a	0.01	**0.004**	**<0.001**	0.247	0.478
20:4n-3	0.4b	0.3d	0.3c	0.4b	0.5a	0.01	**<0.001**	**<0.001**	0.806	**0.002**
20:5n-3	1.9b	1.3d	1.4c	1.9b	2.1a	0.05	**0.006**	**<0.001**	0.919	0.797
22:5n-3	0.8b	0.5d	0.6c	0.8b	0.9a	0.01	**<0.001**	**<0.001**	0.847	**0.013**
22:6n-3	4.5b	3.4d	3.8c	4.4b	5.2a	0.11	**0.001**	**<0.001**	0.689	0.212
Total n-3 PUFA	10.4b	7.3d	8.3c	10.4b	12.2a	0.16	**<0.001**	**<0.001**	0.966	0.081
Total PUFA	20.8b	18.0c	21.4b	21.1b	25.5a	0.22	**<0.001**	**<0.001**	0.098	0.098
Total LC-PUFA	9.5b	7.6c	8.8b	9.4b	11.3a	0.14	**<0.001**	**<0.001**	0.062	0.062

n3 LC-PUFA; n-3 long chain polyunsaturated fatty acids (i.e. EPA and DHA), SE; pooled standard error of the mean.

[1]P-values from linear model with lipids, n-3 LC-PUFA (n3) and oxygen as fixed effects with a lipid-n3 interaction. Differing letters indicate $p<0.05$. P-values in bold are <0.05.

[2]Fatty acids ≤0.2 not detailed.

Table 6. Retention (% wet matter basis) of macronutrients and fatty acids (% of total) in the whole body carcass of Atlantic salmon (n=3, pooled per tank) fed high and low levels of lipids and/or n-3 LC-PUFA under low and high oxygen conditions.

	High Oxygen	Low Oxygen					p-value[1]			
	HL-Hn3 (control)	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3	SE	Lipid	n3	Oxygen	Lipid x n3
Protein	44.3bc	54.2a	43.5c	49.9ab	44.4bc	1.8	**0.022**	0.493	0.064	0.258
Lipid	53.4	55.4	44.1	55.6	44.3	4.3	0.056	0.970	0.775	0.990
Energy (MJ kg^{-1})	43.1ab	47.7a	40.2b	47.1a	42.0b	1.9	**0.040**	0.820	0.304	0.663
Fatty acids[2]										
14:0	47.1a	42.1a	5.0b	44.7a	31.1ab	8.4	**0.019**	0.144	0.851	0.223
16:0	56.3	57.0	44.1	57.2	46.5	4.5	**0.048**	0.813	0.904	0.843
18:0	63.8	70.6	61.5	63.1	58.0	4.7	0.212	0.329	0.931	0.714
20:0	33.6a	35.7a	20.2b	33.7a	21.3b	3.3	**0.003**	0.909	0.996	0.676
Total saturates	56.1	58.1	44.7	56.5	46.2	4.7	0.051	0.996	0.957	0.776
16:1n-9	131.8b	182.5a	151.0ab	138.5ab	112.7b	15.0	0.096	**0.025**	0.767	0.855
16:1n-7	49.2a	45.1a	23.6b	50.9a	35.3b	6.0	**0.019**	0.219	0.861	0.668
18:1n-9	58.7	64.2	52.1	60.3	54.6	4.3	0.091	0.890	0.817	0.515
18:1n-7	49.3ab	46.9ab	32.5b	54.2a	50.2ab	6.1	0.183	0.080	0.605	0.436
20:1n-9	223.4ab	302.8a	208.2b	217.7b	152.0b	24.3	**0.012**	**0.023**	0.883	0.597
22:1n-11	-15.6a	-30.3a	-66.7b	-9.7a	-29.3a	10.9	**0.030**	**0.026**	0.715	0.463
24:1n-9	6.0a	-39.0ab	-69.9b	2.8a	-28.2ab	16.3	0.097	**0.033**	0.896	0.996
Total monoenes	59.6	64.9	51.3	61.4	54.3	4.6	0.071	0.962	0.813	0.544
18:2n-6	47.4	44.0	40.2	50.6	46.7	3.7	0.374	0.142	0.592	0.989
20:4n-6	79.8b	373.8a	318.3a	97.8b	109.2b	27.0	0.559	**<0.001**	0.734	0.382
Total n-6 PUFA	56.8	58.4	54.2	60.6	57.8	4.9	0.521	0.597	0.621	0.896
18:3n-3	33.4a	20.3bc	12.5c	36.2a	31.1ab	3.8	0.143	**0.002**	0.639	0.749
18:4n-3	24.7a	21.8a	-14.8b	30.3a	17.4a	6.5	**0.005**	**0.014**	0.572	0.116
20:5n-3	24.6a	11.2b	-10.3c	25.2a	14.6ab	3.2	**0.002**	**<0.001**	0.915	0.189
22:5n-3	119.2a	109.5a	39.8b	121.0a	96.3ab	19.3	**0.049**	0.138	0.954	0.310
22:6n-3	109.4ab	128.9a	72.8b	109.7ab	84.1b	11.2	**0.007**	0.753	0.989	0.240
Total n-3 PUFA	52.7a	47.6a	19.8b	54.3a	42.0a	6.2	**0.012**	0.052	0.867	0.265
Total PUFA	54.6	54.7	44.0	57.5	48.8	5.3	0.117	0.516	0.729	0.857
Total LC-PUFA	81.0b	131.8a	84.9b	82.4b	66.9b	10.8	**0.026**	**0.018**	0.218	0.218
Total	58.1	62.3	48.9	59.8	51.4	4.7	0.064	0.997	0.821	0.647

n3 LC-PUFA: n-3 long chain polyunsaturated fatty acids (i.e. EPA and DHA), SE; pooled standard error of the mean.

[1]P-values from linear model with lipids, n-3 LC-PUFA (n3) and oxygen as fixed effects with a lipid-n3 interaction. Differing letters indicate $p<0.05$.. P-values in bold are <0.05.

[2]Fatty acids ≤0.2 in the diets are not detailed.

Table 7. Apparent digestibility coefficients (dry matter basis) of macronutrients and fatty acids (% of total) for Atlantic salmon (n=3, pooled per tank) fed low and high levels of lipids and/or n-3 LC-PUFA under low and high oxygen conditions.

	High Oxygen	Low Oxygen					p-value[1]			
	HL-Hn3 (control)	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3	SE	Lipid	n3	Oxygen	Lipid x n3
Protein	93.6	94.3	93.8	94.0	94.0	0.2	0.272	0.887	0.198	0.199
Lipid	96.1a	97.6a	93.6b	96.9a	93.0b	0.5	**<0.001**	0.242	0.147	0.710
Energy (MJ kg^{-1})	88.9b	90.6a	90.4a	89.8a	89.8a	0.2	0.731	**0.038**	0.077	0.835
Fatty acids[2]										
14:0	86.7	95.3	91.2	92.8	86.8	2.2	0.114	0.262	0.177	0.768
16:0	92.8bc	97.6a	93.5abc	95.3ab	88.9c	1.2	**0.005**	**0.042**	0.250	0.463
18:0	92.0ab	96.6a	91.0b	94.4ab	85.7c	1.4	**0.002**	**0.046**	0.330	0.366
20:0	93.0ab	96.4a	86.1bc	93.4ab	84.7c	2.1	**0.002**	0.380	0.907	0.736
Total saturates	92.1bc	97.3a	92.7abc	94.9ab	88.0c	1.3	**0.006**	0.054	0.253	0.501
16:1n-9	100.0a	99.7b	98.7c	100.0a	100.0a	0.1	**0.001**	**<0.001**	0.999	**0.001**
16:1n-7	99.1abc	99.5a	98.6bc	99.3ab	98.4c	0.2	**0.006**	0.431	0.590	0.918
18:1n-9	99.6a	99.6a	98.7b	99.6a	98.5b	0.2	**<0.001**	0.569	0.874	0.718
18:1n-7	99.2ab	99.5a	98.5b	99.3a	97.3c	0.2	**<0.001**	**0.010**	0.768	0.060
20:1n-9	98.3a	98.5a	94.7b	98.2a	95.3b	0.5	**<0.001**	0.786	0.882	0.467
22:1n-11	98.3a	98.5a	95.5b	98.5a	95.9b	0.6	**0.002**	0.783	0.810	0.767
24:1n-9	90.0ab	95.9a	90.3ab	92.6a	85.1b	1.9	**0.014**	0.081	0.424	0.665
Total monoenes	99.5a	99.6a	98.5b	99.5a	98.3b	0.2	**<0.001**	0.446	0.960	0.697
18:2n-6	99.2a	99.3a	98.4b	99.2a	98.2b	0.1	**<0.001**	0.435	0.817	0.804
20:4n-6	99.6a	97.5b	90.5c	99.1a	97.3b	0.4	**<0.001**	**<0.001**	0.516	**<0.001**
Total n-6 PUFA	99.1a	99.3a	98.2b	99.2a	98.1b	0.2	**<0.001**	0.563	0.757	0.983
18:3n-3	99.7a	99.5ab	99.0c	99.7a	99.3bc	0.1	**0.001**	**0.036**	0.755	0.628
18:4n-3	99.4a	99.2a	98.7b	99.7a	99.3a	0.1	**0.008**	**0.006**	0.255	0.735
20:5n-3	99.8a	99.6b	99.1c	99.8a	99.5b	0.0	**<0.001**	**<0.001**	0.913	0.080
22:5n-3	99.8a	99.8a	98.1b	99.4ab	98.7ab	0.4	**0.036**	0.769	0.483	0.278
22:6n-3	99.2a	98.5b	95.5d	99.1ab	97.6c	0.2	**<0.001**	**<0.001**	0.696	**0.003**
Total n-3 PUFA	99.6a	99.3a	98.0c	99.6a	98.9b	0.1	**<0.001**	**<0.001**	0.972	**0.024**
Total PUFA	99.3a	99.3a	98.1b	99.4a	98.5b	0.1	**<0.001**	0.122	0.884	0.331
Total LC-PUFA	99.5a	98.9b	96.6c	99.4a	98.5b	0.1	**<0.001**	**<0.001**	0.790	**0.001**
Total	98.1ab	99.2a	97.5bc	98.6ab	96.3c	0.4	**0.001**	**0.067**	0.404	0.451

n3 LC-PUFA; n-3 long chain polyunsaturated fatty acids (i.e. EPA and DHA), SE; pooled standard error of the mean.

[1]P-values from linear model with lipids, n-3 LC-PUFA (n3) and oxygen as fixed effects with a lipid-n3 interaction. Differing letters indicate $p<0.05$. P-values in bold are <0.05.

[2]Fatty acids ≤0.2 in the diets are not detailed.

Table 8. Apparent digestibility coefficients (dry matter basis) of minerals for Atlantic salmon (n=3, pooled per tank) fed low and high levels of lipids and/or n-3 LC-PUFA under low and high oxygen conditions.

	High Oxygen	Low Oxygen					p-value[1]			
	HL-Hn3 (control)	HL-Ln3	LL-Ln3	HL-Hn3	LL-Hn3	SE	Lipid	n3	Oxygen	Lipid x n3
Calcium (Ca)	-15.3	-24.1	-29.4	-22.2	-27.7	2.2	0.064	0.506	0.095	0.966
Magnesium (Mg)	-274.6a	-279.3a	-423.4c	-336.7ab	-364.5bc	22.8	**0.005**	0.977	0.101	**0.038**
Phosphorus (P)	22.0c	25.6bc	34.1a	25.7bc	33.3ab	2.3	**0.010**	0.900	0.329	0.870
Potassium (K)	94.4a	94.5a	87.8b	94.2a	88.1b	0.2	**<0.001**	0.919	0.714	0.273
Sodium (Na)	19.9	13.1	8.5	14.2	16.4	3.3	0.741	0.224	0.267	0.351
Zinc (Zn)	38.7ab	30.2c	36.6b	40.1a	31.2c	0.9	0.168	**0.024**	0.259	**<0.001**

n3 LC-PUFA; n-3 long chain polyunsaturated fatty acids (i.e. EPA and DHA), SE; pooled standard error of the mean.

[1]P-values from linear model with lipids, n-3 LC-PUFA (n3) and oxygen as fixed effects with a lipid-n3 interaction. Differing letters indicate $p<0.05$. P-values in bold are <0.05.