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Steroidogenic and innate immune responses in Atlantic salmon are influenced by dietary total lipid, long chain polyunsaturated fatty acids and dissolved oxygen

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Abstract

Recent studies have found that feeding high levels of long chain polyunsaturated fatty acids (LC-PUFA) influenced the steroid biosynthesis pathway of Atlantic salmon on a transcriptomic level, although research observed on a metabolic level is lacking. Dietary lipid and chronic stress have also been suggested to play a role in steroidogenesis. A study was performed on Atlantic salmon post-smolts (184 g) fed with diets of high and low levels of lipid with and without high levels of LC-PUFA under hypoxic (stressed) or normoxic (unstressed) conditions. Liver, head kidney and blood samples were collected after 35 and 116 days to determine short- and long-term effects. On day 35, dissolved oxygen affected the levels of the steroid hormones 11-deoxycortisol, cortisone and testosterone as well as the relative expression of *cyp11a* involved in steroidogenesis and *ud2a2* involved in sex steroid metabolism. On day 116, an interaction between lipid x LC-PUFA x oxygen was found for *cyp11a*, *hsd3b* (steroidogenesis) and *ud2a2* genes, whereas LC-PUFA and/or oxygen affected the steroid levels of 11-dehydrocorticosterone, corticosterone, cortisol, dehydroepiandrosterone (DHEA), pregnenolone and testosterone. The observation that both LC-PUFA and oxygen affected *cyp11a* expression and the levels of pregnenolone, provides evidence for the influence of both parameters on the rate-limiting point of steroid production, which can take >35 days to establish. The 3-way interaction between lipid, LC-PUFA and dissolved oxygen on the expression of steroid genes suggests that the chronic stressor of hypoxia may be over-riding dietary influences of the lipid and LC-PUFA, though the relative proportion of LC-PUFA within the lipid may play a role as well. In terms of fatty acid composition, low dietary lipid and high LC-PUFA increased the levels of total n-6 and n-3 PUFA in the liver, particularly EPA and DHA, and the head kidney to a lesser extent. In terms of immune pathways, hypoxia influenced cytokine gene expression (*tgfb, il10*), cellular repair gene expression (*gr, hsp70* and *hsp90*) and

eicosanoid levels (PGE2 and LTB5) in the head kidney, while dietary lipid and LC-PUFA influenced cytokines (*ifnγ* and *il1β*) and eicosanoids (PGE2 and LTB5). These findings demonstrate that feeding different levels of LC-PUFA significantly alters steroidogenesis and innate immune response in Atlantic salmon post-smolts with additional interactions from dietary lipid and hypoxia stress. These results will significantly impact the aquaculture industry since it demonstrates salmon fed high levels of LC-PUFA are likely more resilient to cope with chronic stressors (e.g. prolonged hypoxia) due to lower corticosteroid levels and higher immune response.

Keywords

Cytokines; Fatty acids; Gene expression; Haematology; Hypoxia; LC-MS/MS; Salmonids; Steroids; Stress

1. Introduction

Omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are important not only for fish growth, but also as factors to support reproduction, immunity and fillet quality (Glencross, 2009). Levels of EPA and DHA have been consistently demonstrated to be conditionally essential dietary nutrients for Atlantic salmon (Ruyter et al., 2000; Glencross et al., 2014; Bou et al., 2017). The dietary LC-PUFA requirement for Atlantic salmon has been reported to be in range of 10 to 15 g/kg (Glencross et al., 2014; Bou et al., 2017). However, recent studies have found that the relative proportion of LC-PUFA to lipid, rather than the absolute lipid level, to be a better metric for optimal growth performance (Huyben et al., 2021b; Huyben et al., 2021a). Although effects on growth are generally nominal, and can take a considerable time to amortise, a reduction in the levels of n-3 LC-PUFA in the diet significantly reduces the content of LC-PUFA in various organs of the animal and has a range of impacts on different sub-clinical responses in the fish, e.g. gene expression (Rosenlund et al., 2016; Betancor et al., 2017). One such sub-clinical observation was that plasma levels of cholesterol were also reduced when the levels of n-3 LC-PUFA in the diet were reduced (Glencross, 2009; Betancor et al., 2014). Concurrent with that study were the observations of significant perturbations in the hepatic gene expression of various regulatory points in steroidogenic pathways when n-3 LC-PUFA levels were low (Glencross et al., 2015). These concomitant changes in plasma cholesterol levels and steroidogenic regulatory genes raised questions about the role of dietary n-3 LC-PUFA on steroidogenic activity and levels of various steroids, including corticosteroids (glucocorticoids, mineralocorticoids), sex steroids (androgens, estrogens and progestogens) and calciferols (vitamin D_3) (Stocco, 2001; Miller and Bose, 2011).

Steroidogenesis is usually regulated at the first step in the steroid biosynthesis pathway, through the conversion of cholesterol to pregnenolone by the cytochrome P450 side chain cleavage enzyme

(P450scc, gene named *cyp11a*), with the supply of cholesterol to the P450scc enzyme controlled by the steroidogenic acute regulatory protein (StAR) (Stocco, 2001; Geslin and Auperin, 2004; Miller and Bose, 2011). The primary organ in steroidogenesis in teleost fishes is widely recognised as the head kidney (Tokarz et al., 2015), although recent transcriptomic research has suggested the liver plays a significant role as well (Glencross et al., 2015). The liver is the primary organ involved in fatty acid synthesis and metabolism (Monroig et al., 2010), and also a major target for cortisol action due to the ability to produce glucose and provide metabolic energy for stress adaptation (Aluru and Vijayan, 2009). However, steroid hormone responses are complex, and usually no physiological process can be attributed to the actions of a single steroid hormone (Tokarz et al., 2015). Thus, measuring the levels of steroid hormones and their associated genes throughout various steroidogenic pathways will allow for a better understanding of this complex system.

Originally, it was thought that LC-PUFA only influenced lipid metabolism in fish. However, recent research has found that increased levels of dietary LC-PUFA, specifically DHA, down-regulated the steroid biosynthesis pathways in the liver of Atlantic salmon based on a microarray study by Glencross et al. (2015). Betancor et al. (2015) also found similar results in the pyloric caeca of Atlantic salmon. However, both of these findings were at a transcriptomic level and research is lacking on any assessment on a metabolomic level of steroid hormones in direct response to differences in the levels of dietary LC-PUFA fed to Atlantic salmon.

In addition to lipid metabolism and steroidogenesis, LC-PUFA have also been implicated in pro and anti-inflammatory processes and innate immune responses in fish. In the same microarray study, Glencross et al. (2015) reported a general upregulation of immune pathways with increasing levels of dietary DHA, suggested to be an indirect effect linked more closely to EPA levels in the diet. A previous study on Atlantic salmon found that dietary n-3 PUFA significantly altered the level of B-lymphocytes in the blood (Thompson et al., 1996). In mice, dietary n-3 PUFAs reduce intestinal inflammation by decreasing pro-inflammatory cytokine synthesis, reducing myeloid cell recruitment and activation, and enhancing epithelial barrier function (Whiting et al., 2005). LC-PUFA, especially arachidonic acid (ARA) and to a lesser extent EPA and DHA, act as precursors to eicosanoid metabolites such as prostaglandins, leukotrienes, thromboxanes, docosanoids, maresins and resolvins, which regulate inflammatory and immune response processes (Calder et al., 2009).

High dissolved oxygen (normoxia) is important for optimal feed intake, fish growth, aerobic metabolism and immune function (Burt et al., 2013; Claireaux and Chabot, 2016). Changes to environmental conditions, such as reduced dissolved oxygen levels (hypoxia), are known to lower metabolic rate, inhibit growth and decrease immunity while increasing the concentration of steroids in fish plasma (Pickering and Pottinger, 1989; Pottinger and Moran, 1993; Burt et al., 2013). In addition

to hypoxia, changes to the levels of n-3 LC-PUFA intake have been reported to affect the levels of cortisol in plasma (Jutfelt et al., 2007; Montero et al., 2015), suggesting that stress responses are modulated by dietary n-3 LC-PUFA. Long-term stress due to chronic stressors, such as poor nutrition and water quality, can lead to decreased muscle growth through increased protein catabolism, poorer amino acid utilisation and decreased feed intake, as well as immunosuppression through a reduction in circulating leukocytes and lymphocytes (Fast et al., 2008). Exploring the potential of LC-PUFA, and its proportion to dietary lipid, to modulate hepatic genes involved in the stress response can provide an insight into the interaction between nutrition and stress mediation in Atlantic salmon.

The objective of this study was to investigate the main and interactive effects of dietary lipid, LC-PUFA and dissolved oxygen on the steroidogenesis and innate immune response of post-smolt Atlantic salmon. Several tissues, including the liver, head kidney and blood, were collected and analysed using differential gene expression by RT-qPCR, liquid chromatography with tandem mass spectrometry (LC-MS/MS) of steroid hormones, haematology and plasma biochemistry profiling and ultra-high performance liquid chromatography of fatty acid methyl esters. This research evaluated the transcriptomic and metabolomic responses and builds on parallel studies that investigated these same three factors, albeit on the microbiome, phenomic and lipidomic responses of Atlantic salmon (Huyben et al., 2020; Huyben et al., 2021b; Huyben et al., 2021a).

2. Materials and Methods

2.1 Fish and facilities

Post-smolt Atlantic salmon were acquired from a commercial producer (Mowi UK Ltd, Loch Ailort, Scotland) and transferred to the Marine Environmental Research Lab (MERL; Machrihanish, UK), where 40 fish were stocked into each of 24 circular tanks (960 fish total) with a volume of 1 m^3 equipped with individual LED lights set at a 16:8 light-dark regime. The flow rate and aeration were decreased for half of the tanks to expose fish to hypoxic conditions as a chronic stressor. Seawater was filtered from Machrihanish bay in a flow-through system with a mean temperature of 13.1 ± 0.19 ºC (mean per week ± SD). Dissolved oxygen was 92.6 ± 2.66% (8.0 ± 0.23 mg/L) for the high oxygen tanks and 78.0 ± 2.32% (6.7 ± 0.20 mg/L) for the low oxygen tanks. Water turnover in the high oxygen tanks was 10-15 L/min to achieve 90-100% saturation (normoxia), while turnover in the low oxygen tanks was adjusted to achieve 70-80% saturation (hypoxia), thereby potentially lowering metabolic rate in an 'oxygen conforming' species like Atlantic salmon (Barnes et al., 2011). Fish were acclimated for three weeks under these conditions based on previous research that found 21-34 days of acclimation did not change the threshold for anaerobiosis and stress (Remen et al., 2012; Remen et al., 2014). Fish were

sedated with MS-222 and individually weighed (184 ± 5 g) before the start of the experiment. 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.

2.2 Experimental diets

Four iso-energetic diets (A, B, C and D) were formulated to satisfy the nutritional requirements of Atlantic salmon and were produced as 3 mm pellets using expansion-extrusion (SPAROS I&D, Ohlão, Portugal). The diets contained 20% fishmeal (FM) with an energy level of 21.0 MJ/kg and were formulated to provide either a high level of total lipid (230 g/kg for diets A and C) or a low level (180 g/kg for diets B and D) (Table 1). Conversely, the high lipid diets (A and C) were formulated to have a low level of protein (464 g/kg) or a high level (569 g/kg) in the low lipid diets (B and D). To investigate omega-3 long chain polyunsaturated fatty acid (n-3 LC-PUFA), further referred to as PUFA, requirements and its effects on salmon, each diet was then prepared to provide either a low level of PUFA (7 g/kg for diets A and B) or high level (14 g/kg for diets C and D). Levels of PUFA were planned to be slightly above and below reported critical optima (Glencross et al., 2014; Bou et al., 2017). The four diets and the two oxygen conditions allowed for a 2 x 2 x 2 factorial comparison of total lipid content, PUFA level and dissolved oxygen for a total of eight treatments, in triplicate, using 24 tanks.

Experimental feeds were fed in excess using automated feeders (Arvo-tec Oy, Huutokoski, Finland) twice per day, over a three-hour period each time. Uneaten feed was collected each morning, weighed and corrected for moisture in order to calculate daily feed intake (Helland et al., 1996). Feed rations were increased incrementally over the duration of the study to ensure daily rations were always marginally in excess.

2.3 Sample collection

At days 35 and 116, fish were starved overnight (14-18 hours) and four fish from each tank were euthanized with an overdose of MS-222. Approximately 3 mL of blood was collected from the caudal vein using a 5 mL heparinised (150 IU) syringe (n=12 per treatment). A 200 µL aliquot of whole blood was stored on ice while the remainder was centrifuged at 1,000 × *g* for 2 minutes. Plasma was transferred to a sterile tube, frozen on dry ice and stored at -70 °C. Whole blood and plasma was submitted to the Central Analytical Laboratory at SAC Consulting Veterinary Services (Edinburgh, UK) for blood haematology and plasma biochemistry analyses, respectively. The cervical vertebrae of each fish were severed, the abdomen dissected, the apical tip of each liver and the first 2 cm of the head

kidney collected, frozen on dry ice and stored at -70 °C. In addition, each diet was collected and stored at -20 °C.

2.4 Proximate, fatty acid and lipid class analyses

Each diet was analysed for dry matter, protein, lipid and ash at the Institute of Aquaculture (Stirling, UK), according to Huyben et al. (2021b). In brief, moisture and ash were analysed using ovens at 105 and 550 ºC for approximately 24 and 12 hours (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) (Persson, 2008). Gross energy was measured by ballistic bomb calorimetry (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) (Folch et al., 1957).

The liver and head kidney were analysed for lipid class and fatty acid composition (Christie, 2003). Diets were analysed for fatty acid composition in addition to the proximate analysis. Fatty acid methyl esters (FAME) were made by acid-catalysed esterification of 1 mg of total lipid by overnight incubation at 50ºC 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).

Lipid classes were identified based on separation of polar and neutral lipids. Separation of main lipid classes was conducted in 10 × 10-cm plates (VWR, Lutterworth, UK) by double development high-performance thin-layer chromatography (HPTLC), according to Tocher, Harvie (1988). Firstly, plates were pre-run in diethyl ether and then activated at 120 °C for one hour. Lipid classes were visualised after spraying with 3% (w/v) copper acetate, containing 8% (v/v) phosphoric acid by charring at 160 °C for 20 minutes. Quantification was made by densitometry using a CAMAG-3 TLC scanner version 1.14.16 (CAMAG, Muttenz, Switzerland) with winCATS Planar Chromatography Manager.

2.5 RNA extraction and quantitative real time PCR (qPCR)

Liver and head kidney tissues were analysed to determine the effect of each treatment on the relative steroidogenic and immune, respectively, gene expression using RNA extraction and quantitative real time PCR (qPCR) according to Betancor et al. (2014). A 100 mg piece of tissue from each fish was homogenized in 1 mL Tri Reagent® RNA extraction buffer (Sigma-Aldrich, Dorset, UK) following the manufacturer's instructions. A 100 µL aliquot of the phase separation reagent 1-bromo-3-chloropropane (Sigma-Aldrich) was added and the precipitate pellets were washed twice with 75% ethanol before resuspension in Milli-Q water. Quality and concentration of the isolated total RNA was measured by spectrophotometry with an ND-1000 NanoDrop (Labtech Int., East Sussex, UK) and agarose gel electrophoresis. A 6 µg sample of total RNA from two individual fish from the same tank were pooled together and analysed as a single replicate (three replicates per tank, n=6 fish per treatment) following the methodology from Glencross et al. (2015). The cDNA was synthesized with 2 µg pooled RNA in 20 µL reactions with the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Paisley, UK). The resulting cDNA was diluted 1:20 with Milli-Q water.

Primers for genes involved in cholesterol biosynthesis, immune response and cellular repair were based on those from previous studies (Olsvik et al., 2013; Betancor et al., 2014; Gajardo et al., 2017) (Table 3). Genes involved in steroidogenesis were chosen for qPCR based on changes in expression from the study by Glencross et al. (2015) and primers were designed using Primer-BLAST and GenBank® (https://www.ncbi.nlm.nih.gov/genbank). Genes involved in steroid hormone biosynthesis and sex hormone metabolism were analysed in the liver and genes involved in cytokine production, oxidative stress and cellular repair were analysed in the head kidney. The efficiencies of all primers were evaluated to ensure they were between 90-105%. Four potential reference genes were compared using GeNorm analysis to determine the most stable reference genes for each tissue (Vandesompele et al., 2002). The qPCR was performed in 96-well plates in duplicate 10 µL reactions using a Biometra thermocycler (Analytic Jena, Toettingen, Germany). A 2.5 µL aliquot of cDNA was used with 5 µL Luminaris Color HiGreen qPCR Master Mix (Thermo Scientific) and 0.5 µL of each forward and reverse primer (10 pmol). Reference genes were determined with the same components, although 1 µL of cDNA was used. Amplifications were carried out with a no template control (NTC) containing no cDNA to ensure that no nucleic acid contamination was present. The cycle threshold (Ct) value of each target gene was calibrated against the control treatment of HC (i.e. high lipid, high PUFA and normoxia), where 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 three reference genes (RQ target/RQ reference) (Pfaffl, 2001).

2.6 Steroid analysis

Steroid analysis was performed by extraction of samples through automated supported liquid extraction (SLE) on an Extrahera liquid handling robot (Biotage, Uppsala, UK) followed by targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Mass Spectrometry Core, Centre for Cardiovascular Sciences, University of Edinburgh (Edinburgh, UK). Equal volumes of plasma from three fish were pooled into one sample per tank (n=3 per treatment), enabling extraction of 200 µL of sample. Analysis was performed using an I-class Acquity UPLC (Waters, Wilmslow, UK) interfaced to a QTRAP 6500+ (Sciex, Warrington, UK) mass spectrometer. Instrument control and data acquisition were achieved using Sciex Analyst 1.6.3 software and data were integrated using MultiQuant 2.3.1 software (Framingham, MA, USA).

Chromatographic separation was achieved on a Kinetex C18 (3 x 150 mm; 2.6 µm particle size), column fitted with a KrudKatcher Ultra In-Line Filter with 0.5 µm porosity (Phenomenex, UK). The mobile phase system was water and methanol with ammonium fluoride (50 µM) as modifier at a flow rate of 0.3 mL/minute over 16 minutes, starting at 55% B for 2 minutes, rising to 100% B over 6 minutes, held for 2 minutes, before returning to 55% B over 0.1 minutes and equilibrating for 4.9 minutes, all held at a temperature of 50 ^{o}C. The solvent flow was diverted to waste from 0-2 minutes and 11-16 minutes.

The mass spectrometer was operated in electrospray ionisation (ESI) mode with polarity switching using a TurboIonSpray source and data were collected in unit resolution (0.7 *m/z* full width at half maximum). The source was operated at 600 ^{o}C with an IonSpray voltage of 5.5 kV/-4.5 kV, a Curtain Gas of 30 psi, nitrogen nebuliser ion source gas 1 (GS1) and heater ion source gas 2 (GS2) of 40 psi and 60 psi, respectively. Multiple reaction monitoring (MRM) transitions are detailed in Table 4 with chromatographic retention time for each compound also listed.

Additionally, pregnenolone was quantified in plasma from two fish per tank at day 35 (n= 6 per treatment) with an ELISA kit (BioSite, Täby, Sweden), according to the manufacturer's instructions. Briefly, samples were incubated for 45 minutes at 37 °C and then incubated with HRP-Streptavidin conjugate for 30 minutes at 37 °C before terminating the reaction. Pregnenolone concentration was determined by comparing the optical density (OD) at 450 nm of the samples to standard curves.

2.7 Eicosanoid analysis

Eicosanoid analysis was performed as described in Hundal et al. (2021), with slight modifications to the extraction protocol. Homogenisation of the samples was performed using a 6875D Large Freezer/Mill (SPEX® SamplePrep, Metuchen, NJ, USA) supplied with liquid nitrogen to keep the samples cool. Samples were weighed and successive aliquots (500 µL) of acetonitrile (ACN), containing equal concentrations of the internal standards PGE2-d4 and LTB4-d4 (20 ng/mL), and pure

chloroform was added before vortex mixing for 30 seconds. A second extraction was performed, but with pure ACN and chloroform. The samples were centrifuged for one minute at 3000 *g* and filtered. The filtrate was evaporated under vacuum at 30 °C with a RapvidVap (Labconco, Kansas City, MO, USA). Thereafter, the samples were diluted in 50 µL methanol, vortexed and centrifuged at 3000 *g* for one minute before injection into an LC-MS/MS system (Agilent 6495 QQQ triple quadropole, Agilent technologies, Waldbronn, Germany). Further details of the LC-MS/MS fragmentation can be found in the abovementioned study.

2.8 Statistical analysis

All data are presented as means ± SE unless otherwise specified. Normal distribution and variance of homoscadicity of each dataset were determined using Shapiro-Wilk and Levene tests in RStudio version 1.3.1093 (R-Core-Team, 2015). Data were normalised using log transformation if needed and a three-way analysis of variance (ANOVA; *aov*) was performed to determine effects of total lipid, PUFA, oxygen and interactions between all combinations of factors (y = Lipid x PUFA x Oxygen). A generalised linear model (*glm*) was performed on non-normal data since they did not pass the assumptions required for an ANOVA. P-values of each treatment were determined using Least Square Means test (*lsmeans*) with Tukey adjustment for multiple comparisons. A $P<0.05$ value was considered significant.

3. Results

3.1 Steroid levels in the plasma

Steroid levels in the plasma were clearly different between day 35 and day 116, where hypoxia initially dominated influences on steroid levels, but later these were more influenced by dietary PUFA with several interactions with hypoxia (Table 5, Fig. 1). In the early parts of the study (day 35), low oxygen levels significantly increased 11-deoxycortisol ($P=0.008$), 11-ketoandrostenedione ($P=0.004$) and cortisone ($P=0.047$), while dehydroepiandrosterone ($P=0.001$) and testosterone ($P=0.022$) were decreased. However, the effects of PUFA were more notable after a longer term (day 116), with high PUFA levels significantly decreased 11-dehydrocorticosterone ($P=0.003$), corticosterone ($P=0.002$), cortisol ($P=0.008$), dehydroepiandrosterone-sulphate ($P=0.011$), pregnenolone ($P=0.002$) and testosterone ($P=0.010$). Oxygen had no significant effect as a sole factor on day 116, but there was a significant interaction between PUFA x oxygen for 11-dehydrocorticosterone ($P=0.005$), dehydroepiandrosterone-sulphate ($P=0.035$) and pregnenolone ($P=0.025$). No significant effects of lipid level were found ($P>0.05$), except for androstenedione on day 35.

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332 3.2 Gene expression in the liver and head kidney

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334 There were no significant effects of lipid or PUFA on steroid biosynthesis gene expression, whereas
335 several effects of oxygen and interactions involving all three factors were found (Fig. 1-2). On day 35,
336 low oxygen levels significantly downregulated expression of *cyp11a* (P=0.002), whereas *ud2a2* was
337 upregulated (P<0.001). On day 116, low oxygen upregulated *hsd3b* (P=0.018) and downregulated
338 *hsd17b2* (P=0.033), in addition to a lipid x PUFA x oxygen interaction on *cyp11a*, *hsd3b* and *ud2a2*
339 (P=0.035, 0.023 and 0.033).

340

341 There were only two significant effects of dietary components on the expression of immune and
342 oxidative stress genes, while oxygen induced many effects (Fig. 3). High lipid diets upregulated *ifnγ*
343 (P=0.015) and there was a PUFA x oxygen interaction on *il1β* (P=0.020). Low oxygen significantly
344 upregulated *il1β, il10, tgfβ, gr, hsp70* and *hsp90* (P=0.020, 0.049, <0.001, <0.001, 0.013 and <0.001,
345 respectively).

346

347 3.3 Eicosanoid production

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349 Eicosanoid production on day 116 was significantly different between the liver and head kidney
350 (P<0.05), where more effects were found in the head kidney (Fig. 4). Low oxygen levels significantly
351 increased PGE_3 and PGD_2 levels in the liver (P=0.038 and 0.010), while PGE_2 and LTB_5 levels were
352 decreased in the head kidney (P=0.039 and 0.006). High PUFA levels significantly decreased PGE2 in
353 the head kidney, whereas it increased PGD_2 in the liver and LTB_5 in the liver and head kidney. A lipid
354 x oxygen interaction was found for PGE_2, PGE_3 and LTB_5 in the head kidney (P=0.035, 0.044 and
355 0.037).

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357 3.4 Plasma biochemistry and haematology

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359 No significant effects of PUFA were found, although both lipid and oxygen levels influenced a few
360 parameters of plasma biochemistry (Table 6). High lipid level increased levels of amylase, cholesterol
361 and creatinine (P<0.001, 0.001 and 0.049) on day 35 and increased cholesterol (P=0.005) again on day
362 116. Low oxygen decreased cholesterol and lipase (P=0.029 and <0.001) with increased creatinine
363 (P<0.001) on day 35 while on day 116 creatinine increased (P=0.001) and total protein decreased
364 (P=0.012). A lipid x PUFA interaction and an interaction between all three factors was found for
365 amylase (P=0.017 and 0.047) at day 35. A lipid x PUFA interaction was found for alanine transaminase
366 (P=0.027) at day 116.

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3.5 Lipid class and fatty acid composition of the liver and head kidney

The three factors of lipid, PUFA and oxygen had a few significant effects on lipid classes in the liver (Table 7). High lipid level increased total lipid, wax/sterol esters and total neutral lipids (P=0.027, 0.018 and 0.022), whereas and total polar lipids decreased (P=0.022). High PUFA levels decreased phosphatidylethanolamine (P=0.016), whereas lysophosphatidylcholine increased (P<0.001). Low oxygen levels decreased wax/sterol esters (P=0.004), whereas cholesterol increased (P=0.001).

Dietary lipid and PUFA levels affected most fatty acids in the liver (Table 7). High lipid increased total monounsaturates (P<0.001), whereas total n-6 PUFA, total n-3 PUFA and docosahexaenoic acid (DHA) were decreased (P<0.001, 0.011 and 0.008). High PUFA levels decreased monosaturates and total n-6 PUFA (P=0.014 and <0.001), whereas total n-3 PUFA, DHA and eicosapentaenoic acid (EPA) were increased (P<0.001, <0.001 and <0.001). A lipid x PUFA interaction was found for EPA (P=0.026).

In the head kidney, fewer effects of dietary lipid and PUFA levels were found compared to the liver (Table 8). High lipid levels increased total lipid and total neutral lipid in the head kidney (P=0.009 and 0.021), whereas phosphatidylserine and total polar lipids decreased (P=0.030 and 0.021). High PUFA levels had no effect on total lipid or lipid class (P>0.05). Low oxygen levels decreased total lipid, triacylglycerols and phosphatidic acid (P=0.041, 0.029 and 0.021), whereas cholesterol, phosphatidylethanolamine and phosphatidylcholine increased (P<0.001, 0.005 and 0.007). Of the head kidney fatty acids, high lipid level increased total monounsaturates (P=0.005), whereas total n-6 PUFA decreased (P<0.001). High PUFA levels decreased total n-6 PUFA (P<0.001), whereas total n-3 PUFA and EPA increased (P=0.001 and <0.001). Low oxygen had no effect on fatty acids, except for increased 18:0 (P=0.021).

3.6 Fish performance

Final weight was highest for fish fed the high lipid diet with high PUFA under high oxygen (treatment HC; 552 ± 4 g/fish; mean ± SE) with a feed intake of 318 ± 7 g/fish. The lowest final weight was for fish fed the low lipid diet with low PUFA under low oxygen (treatment LA) at 436 ± 17 g/fish with a feed intake of 210 ± 16 g/fish. Further details on the phenomic responses of the trial have been published by Huyben et al. (2021b); Huyben et al. (2021a).

4. Discussion

Steroid hormone responses are complex and because one steroid is often the precursor to another, are often interrelated (Fig. 1). In this study one of our objectives was to determine dietary and stress related effects on the steroidogenic responses by examining the transcriptomic and metabolomic responses in Atlantic salmon. To do this, we looked at the effects of dietary total lipid and LC-PUFA combined with a variable of water oxygen levels on steroid, immune and fatty acids levels in several tissues. This comprehensive analysis was based on previous observations that showed increasing levels of LC-PUFA in the diet significantly down-regulated the expression of hepatic genes involved in the steroid biosynthesis pathway in Atlantic salmon (Glencross et al., 2015). The authors suggested that LC-PUFA may have key functional roles in other pathways in addition to the usual lipid metabolism pathways that are typically studied.

4.1 Steroid production and gene expression

Hypoxia resulted in higher production of cortisone while cortisol levels were unaffected early on (day 35) and the opposite was found later (day 116) in the study (Table 5 and Fig. 1-2). These results may be explained by the inactivation of cortisol to cortisone after fish adapted to the hypoxic stressor on day 116. In addition, a previous study found that plasma cortisone levels increased after coho salmon (*Oncorhynchus kisutch*) were exposed to an acute stressor and suggests both cortisol and cortisone are indicators of stress (Patiño et al., 1987). Previous studies on salmonids have found that cortisol levels eventually return to basal levels after prolonged exposure to stress (Patiño et al., 1987; Fast et al., 2008). However, this does not explain why cortisone was unaffected while cortisol levels were elevated subsequently. One explanation could be due to the delayed conversion of cortisol to cortisone and slow clearance rate of cortisone from the blood (Pottinger and Moran, 1993).

Elevated gene expression of *hsd3b* and *ud2a2* (Fig. 2) were also indicative of hypoxia stress affecting the steroidogenesis pathway. The *hsd3b* gene codes for a catalytic enzyme responsible for the biosynthesis of pregnenolone, progesterone, 17α-hydroxyprogesterone and 17α-hydroxypregnenolone, which are precursors to the stress hormone cortisol (Fig. 1). The *ud2a2* gene codes for an enzyme responsible for glucuronidation of estrone and estradiol, which has been found to correlate with elevated cortisol levels (stress) in salmonid fishes (Pottinger and Moran, 1993). Similarly, a previous study showed increased expression of *ud2a2* protein in the liver of adult Atlantic salmon when exposed to an environmental stressor of elevated temperature (Nuez-Ortín et al., 2018). Despite this gene upregulation, the levels of estrone and estradiol in the plasma were below the detection level of the LC-MS/MS method of this study (Table 5). However, these steroids may have already been converted to estrogen and estrone glucuronides, thus were not detected (Fig. 1). Future research should include estrogen and estrone glucuronides in their LC-MS/MS analysis or use β-glucuronidase to determine the degree of conjugation.

Higher levels of stress hormones and reduced weight gain under hypoxia indicated that the fish were chronically stressed throughout the trial despite being exposed to only a moderate level of hypoxia, with dissolved oxygen saturation of 78% (6.7 mg/L) cf. 92.6% (8.0 mg/L) over the 116-day period. Fish are known to acclimate to the new environmental conditions, albeit with a reduced level of performance. Prolonged activation of the stress response has been linked to damaging side effects, e.g. growth suppression, and explains many of the features we observed in this study (Pickering and Pottinger, 1995). Early in the study (day 35), there was a clear effect where hypoxia increased the stress regulatory steroids (e.g. 11-deoxycortisol + cortisone) and reduced growth by reducing some of the anabolic steroids (e.g. DHEA + testosterone) (Table 5). Continual up-regulation of the stress pathways and down-regulation of anabolic pathways no doubt contributed to reduced feed intake observed under the hypoxic treatments of this study, each of which most likely contributed to the reduced weight gain of the fish as previously reported (Huyben et al., 2021a). Furthermore, fish fed the low lipid and low LC-PUFA diet under hypoxic conditions had the lowest final weight. All the diets were formulated to have equal gross energy (isoenergetic), although lipid is more energy dense than protein and provides higher digestible energy (NRC, 2011). Therefore, fish fed high lipid diets were better able to meet energy requirements than fish fed low lipid (high protein) diets, which has been found previously in Atlantic salmon under hypoxia (Vikeså et al., 2017). Dietary protein would be metabolized for energy rather than lipid (opposite of protein-sparing effect) (Schrama et al., 2018), which may influence downstream pathways, such as sterol metabolism and steroidogenesis, that rely on regulatory proteins (Alves Martins et al., 2013).

This study confirmed previous findings that feeding different levels of LC-PUFA to Atlantic salmon influences the synthesis of fatty acids as well as steroids, such as cortisol, at both transcriptomic and metabolomic levels (Betancor et al., 2015; Glencross et al., 2015). Increased use of vegetable oils in fish diets, resulting in reduced LC-PUFA intake, has been reported to alter the post-stress levels of plasma cortisol (Jutfelt et al., 2007; Oxley et al., 2010). In later stages of the study (day 116), a range of steroids (11-dehydrocorticosterone, corticosterone, cortisol, dehydroepiandrosterone-sulphate, pregnenolone and testosterone) were all lower in the plasma of fish fed high LC-PUFA diets (Table 5). Lower pregnenolone reduced the level of corticosterone and its deactivation to 11-dehydrocorticosterone as well as decreased cortisol and testosterone (Fig. 1b). Low dietary LC-PUFA may be influencing steroid production due to down regulation of prostaglandin F2α (PGF2α) production, which in turn reduced steroidogenesis acute regulatory protein (StAR) (Alves Martins et al., 2013). In addition, LC-PUFA (e.g. DHA) has the capacity to modulate steroidogenesis via its influence on peroxisome proliferator-activated receptor (PPAR), which in turn can also modulate genes such as StAR (Kowalewski et al., 2009).

In addition to higher levels of plasma steroids in fish fed the low LC-PUFA diets, this effect was enhanced when fish were exposed to a hypoxic stressor. Therefore, meeting the dietary LC-PUFA requirement for Atlantic salmon is even more important under chronic stress. Interestingly, an LC-PUFA x oxygen interaction existed for several steroids (pregnenolone, DHEA and 11-dehydrocorticosterone), while high LC-PUFA levels decreased the stress hormones corticosterone and cortisol (Table 5). In early stages of the study, hypoxia induced a stress response but by later stages (day 116) there was little effect of hypoxia, suggesting the fish had acclimated to that stressor to some extent, while dietary LC-PUFA continued to influence most stress pathways. Stimulation of each of these pathways indicates that salmon fed diets deficient in LC-PUFA are not as resilient to cope with a chronic stressor. In European sea bass larvae, Montero et al. (2015) found that increased dietary levels of n-6 LC-PUFA, specifically arachidonic acid (ARA), down-regulated of genes related to cortisol synthesis, such as *StAR* and *cyp11β,* and up-regulated genes related to glucocorticoid receptor complex, e.g. *hsp70* and *gr*. *In vitro*, supplementation of LC-PUFA to interrenal cells from gilthead seabream (*Sparus aurata*) reduced cortisol production (Ganga et al., 2006). However, this has not previously been observed *in vivo* for fish, but has been reported from studies in rodents and humans where chronic stress has been linked to a release of glucocorticoids that induce hippocampal atrophy and dendritic hypertrophy (Pérez et al., 2013; Hennebelle et al., 2014). In rats fed diets enriched with LC-PUFA and subjected to chronic restraint stress, elevated plasma corticosterone levels and anxiety behaviours were reduced (Pérez et al., 2013). *In vitro* studies examining the exposure of canine kidney cells to LC-PUFA, to mimic properties of the blood–brain barrier, suggested that these fatty acids affect the permeability of the blood–brain barrier to cortisol (Navarro et al., 2011).

4.2 Immune response, signalling and gene expression

Hypoxia was the main influencer on the expression of immune genes in the present study, with higher expression of *il1β, il10* and *tgfβ*, which are involved in both pro- and anti-inflammatory cytokine pathways, observed in fish from the hypoxic treatments (Fig. 3). These results agree with another study on Atlantic salmon that found increased expression of *il1β* after a four week exposure to a chronic stressor of daily handling (Fast et al., 2008). In common carp, an acute netting stressor was found to increase the gene expression of *il1β, il10* and *tgfβ,* although expression was down-regulated over time (Shimon-Hophy and Avtalion, 2017). In contrast, rainbow trout exposed to a higher water temperature showed reduced expression of *il1β, il8, il17, ifnγ* and *tgfβ* (Huyben et al., 2019), suggesting that temperature and oxygen stressors may impact the immune system differently.

Hypoxia also led to increased expression of *gr*, *hsp70* and *hsp90* in the head kidney (Fig. 3), and indicates activation of oxidative stress and cellular repair pathways. Similarly, Atlantic salmon exposed to increased temperature had increased expression of *hsp90* in the liver, although *gr* decreased (Olsvik

et al., 2013). The present study did not find an effect of dietary LC-PUFA on gene expression in the head kidney, which agrees with a similar study that found no effect of feeding high plant oil inclusion diets (low n-3 PUFA) on expression of *cat* and *sod* genes in the liver (Betancor et al., 2016).

A significant LC-PUFA x oxygen interaction on the expression of *il1β* (Fig. 3), suggests that the pro-inflammatory response is overwhelmed under hypoxia while dietary LC-PUFA plays a role under normoxic conditions. This agrees with a study on mice that found reduced expression of *il1β* when n-3 PUFA was fed to mice (Whiting et al., 2005). Previous research on Atlantic salmon found that increased levels of dietary LC-PUFA down-regulated the pathway involved in N-glycan biosynthesis (Glencross et al., 2015), which is important for glycan-binding receptors (lectins) secreted or found on the surface of immune cells (van Kooyk and Rabinovich, 2008). Glencross et al. (2015) also found that dietary LC-PUFA may influence the innate immune response since they found dietary DHA content had a tendency to affect chemokine signalling, such as high-affinity IgE receptor signalling and Natural Killer cell mediated cytotoxicity pathways in the liver. In addition, high lipid levels upregulated the expression of *ifnγ* (Fig. 3). These findings suggest levels of lipid, LC-PUFA and oxygen play a role in the expression of pro-inflammatory cytokines.

LC-PUFA, especially ARA and to a lesser extent EPA and DHA, act as precursors to eicosanoid metabolites such as prostaglandins, leukotrienes, thromboxanes, docosanoids, maresins and resolvins which regulate inflammatory and immune response processes (Calder et al., 2009). In the present study, hypoxia resulted in reduced eicosanoid levels of PGE_2 and LTB_5 in the head kidney (Fig. 4), which may be due to lower feed intake and consequently lower PUFA intake. Conversely, eicosanoid levels were increased in both liver and head kidney when fish were fed diets high in PUFA, which can be explained by 2-3x higher levels of arachidonic acid (ARA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3) in the diet (Table 2), being precursors for PGE_2 and LTB_5, respectively (Calder, 2009; Calder et al., 2009). In previous studies, changes in the fatty acid composition of the diet, especially the n-6 to n-3 ratio of PUFA, have been found to alter eicosanoid levels and expression of regulatory genes in the liver and head kidney of Atlantic salmon (Martinez-Rubio et al., 2013; Holen et al., 2018; Katan et al., 2020). In the present study, high PUFA levels were found to increase production of eicosanoids in the head kidney (Table 8 and Fig. 4), except for ARA which was lower than fish fed low PUFA diets. This may be explained by the higher conversion of ARA to eicosanoids. PGE_2 is generally related with pro-inflammatory and some immunosuppressive functions (e.g. inhibiting leukotrienes and resolvins), while LTB_4 is an important chemotactic agent for leukocytes, enhancing the proliferation of T and B cells, increasing the production of *tnf□, il1* and *il6*, and inducing NK cell activity (Tafalla et al., 2002; Calder, 2009; Calder et al., 2009). LTB_4 derives from ARA while LTB_5 derives from EPA (Calder, 2009), thus the significant increase in LTB_5 instead of LTB_4 in the liver and head kidney may be due to higher levels of EPA found in these tissues and the diets (Tables 2, 7 and 8).

4.3 Lipid composition in the liver and head kidney

This study found that high lipid levels in the diet decreased n-6 and n-3 PUFA in the liver and head kidney as an effect of fatty acid dilution, whereas high LC-PUFA levels decreased n-6 PUFA with increased n-3 PUFA (Tables 7 and 8). These results agree with previous studies that have found that feeding high levels of LC-PUFA resulted in higher levels in tissues of Atlantic salmon (Betancor et al., 2014; Glencross et al., 2014; Betancor et al., 2017; Hixson et al., 2017). Ruyter et al. (2000) showed that increasing the inclusion of EPA and DHA in the diets for Atlantic salmon fry led to increased percentages of these fatty acids in the liver. In the liver, there was a lipid x LC-PUFA inteaction on EPA content, which was more concentrated in fish fed the low lipid diet with high LC-PUFA (diet D). This was similar for DHA and shows that the proportion of LC-PUFA to lipid is important to consider, as reported previously (Huyben et al., 2021b; Huyben et al., 2021a).

There was a clear difference in the responsiveness of the liver to changes in diet and dissolved oxygen, whereas fewer effects were found in the head kidney, especially to hypoxia. Previous studies have found that the LC-PUFA in the liver, rather than the head kidney, better reflects the content in the diet (Bell and Sargent, 2003; Betancor et al., 2014). This is probably due to the metabolic role of the liver whereas the head kidney is mainly involved in immune function. Betancor et al. (2014) found that the liver most strongly reflected diet followed by the head kidney, brain and gill when Atlantic salmon were fed increasing levels of DHA as well as ARA and EPA.

5. Conclusion

This study confirmed via qPCR transcriptomic findings from a microarray in Glencross et al. (2015) and further demonstrated novel metabolomic findings that dietary LC-PUFA influenced the steroidogenesis and innate immune responses of Atlantic salmon. Initially on day 35, there were no significant effects of dietary LC-PUFA on steroid gene and hormone production in the liver and plasma, respectively. However, when fish were sampled on day 116 these effects were apparent. The high LC-PUFA diets reduced the levels of cortisol, corticosterone, pregnenolone and testosterone under both hypoxic and normoxic conditions, but this influence was only found for fish fed high lipid diets under normoxic conditions at the transcriptomic level. The 3-way interaction between lipid, LC-PUFA and dissolved oxygen on the expression of steroid genes suggests that the chronic hypoxia stressor may be over-riding dietary influences and the lipid to LC-PUFA proportion may play a role as well. Reductions in n-6 and n-3 PUFA in the liver and head kidney in fish fed high lipid diets also confirmed with previous studies that LC-PUFA in the diet should be proportional to lipid content rather than an absolute level in the diet. High dietary lipid and LC-PUFA was found to upregulate pro-inflammatory cytokines

and increase levels of eicosanoids, further demonstrating their importance in modulating the immune response. These findings demonstrate that feeding different levels of LC-PUFA significantly alters steroidogenesis in Atlantic salmon post-smolts with additional interactions from dietary lipid and hypoxia stress. These results will significantly impact the aquaculture industry since it demonstrates salmon fed high levels of LC-PUFA are likely more resilient to cope with chronic stressors (e.g. prolonged hypoxia) due to lower corticosteroid levels and higher immune response.

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Data Availability

Data is available upon reasonable request.

Authors' Contributions

All co-authors contributed equally to the planning and writing of the manuscript. DH, TC, CM and BG contributed to sampling fish and managing the trial. DH, TC and KLB contributed to the qPCR analysis. TC and NZMH contributed to steroid analysis. NHS and BKH contributed to eicosanoid analysis. The experiment was designed by BG and BR, as part of a project grant coordinated by BR.

Ethical Approval

This experiment was approved by the Animal Welfare and Ethical Review Body (reference AWERB/1617/84) of the University of Stirling in accordance with the UK Home Office regulations under the Animals (Scientific Procedures) Act 2013.

Consent for Publication

All co-authors have seen and agreed to publish this manuscript.

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818

819

820 **Tables**

821

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

	HL-LP A	LL-LP B	HL-HP C	LL-HP D
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	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
Calcium (Ca)	18	17	18	18
Phosphorus (P)	13	13	13	14

823 High; H, Lipid; L, Low; L, PUFA; P.
824 [1]Norvik LT70 (704 g/kg protein and 63 g/kg lipid; Sopropêche, France)
825 [2]Soycomil (624 g/kg protein and 4 g/kg lipid; ADM Animal Nutrition, Decatur, IL, USA)
826 [3]Savinor (10.5% EPA and 15.7% DHA; Savinor UTS, Covelas TRF, Portugal)
827 [4]Neovia (formerly Invivo; Vannes, France)
828 [5]CHO; carbohydrate, calculated by difference (i.e. CHO = 1000 - protein - lipid - ash)
829

830

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

	HL-LP A	LL-LP B	HL-HP C	LL-HP D
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 saturated	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 monounsaturated	68.9	66.0	63.6	56.3
18:2n-6	10.5	12.0	9.2	11.5
20:4n-6 (ARA)	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 (EPA)	1.0	1.2	2.7	3.5
22:5n-3	0.1	0.1	0.3	0.4
22:6n-3 (DHA)	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

832 Fatty acids <0.2 were excluded

833

834 Table 3. Primers for expression of steroid, immune and oxidative stress genes used in qPCR.

Function	Gene	Full name	Primers	Length (bp)	E	Accession Number
Reference	*cfl2*	Cofilin-2	AGCCTATGACCAACCCACTG TGTTCACAGCTCGTTTACCG	224	1.93	TC63899
	hprt	Hypoxanthine phosphoribosyl-trans1	GATGATGAGCAGGGATATGAC GCAGAGAGCCACGATATGG	165	1.99	XM_014212855.1
	rpl2	Ribosomal protein L2	TAACGCCTGCCTCTTCACGTTGA ATGAGGGACCTTGTAGCCAGCAA	112	1.95	XM_014137227.1
	rps5	Ribosomal protein S5	AACTCCATGATGATGCACGG GGTCTTGATGTTCCTGAAAGCA	284	2.07	XM_014142016.1
Steroid hormone biosynthesis	*cyp11a*	Cholesterol side chain cleavage cytochrome p450	TGGAGTCCTGCTCAAGAATG TTATGTACTCGGGCCACAAA	141	2.00	XM_014126110
	hsd3β	3 beta-hydroxysteroid dehydrogenase delta 5	TATGGGAGACGGGATTCGGA GTCAGAGTAGCTGACAGGCG	202	1.99	XM_014174054.1
Sex steroid metabolism	*hsd17β2*	Hydroxysteroid 17-beta dehydrogenase 2	GCGAGAGGGAGTTGTGATAGGG ACCACATAGCACACCAGAGG	133	2.00	XM_014125589.1
	ud2a2	UDP-glucoronosyltransferase 2A2	CCAGAAGGTGGTGTGGAGAC GCGCACCATGTTGTCAAACT	220	2.01	XM_014135997.1
Pro-inflammatory cytokines	*ifny*	Interferron gamma	CTAAAGAAGGACAACCGCAG CACCGTTAGAGGGAGAAATG	159	2.14	NM_001171804
	il1b	Interleukin 1 beta	GCTGGAGAGTGCTGTGGAAGA TGCTTCCCTCCTGCTCGTAG	73	2.03	NM_001123582
Anti-inflammatory cytokines	*il10*	Interleukin 10	CGCTATGGACAGCATCCT AAGTGGTTGTTCTGCGTT	80	2.12	EF165028
	tgfb	Transforming growth factor-beta	AGTTGCCTTGTGATTGTGGGA CTCTTCAGTAGTGGTTTGTCG	191	1.95	EU082211
Resisting oxidative stress	*cat*	Catalase	CCGACCGTCCGTAAATGCTA GCTTTTCAGATAGGCTCTTCATGTAA	140	2.13	BG935638
	sod1	Superoxide dismutase 1	CCACGTCCATGCCTTTGG TCAGCTGCTGCAGTCACGTT	140	1.96	BG936553
	gr	Glutathione reductase	CCAGTGATGGCTTTTTTGAACTT CCGGCCCCCACTATGAC	61	1.99	BG934480
	hif1a	Hypoxia-inducible factor 1A	CCACCTCATGAAGACCCATCA TCTCCACCCACACAAAGCCT	101	1.90	DY708816
Repairing cellular	*hsp70*	Heat shock protein 70	CCCCTGTCCCTGGGTATTG CACCAGGCTGGTTGTCTGAGT	121	1.98	BG933934

	damage	*hsp90*	Heat shock protein 90B	CCACCATGGGCTACATGATG CCTTCACCGCCTTGTCATTC	114	1.90	Q9W6K6

835 E; efficiency of qPCR

836

837 Table 4. Positive and negative ion Multiple Reaction Monitoring parameters for each steroid and
838 isotopically labelled internal standard as analysed on a QTrap 6500+.

Steroid	Q1 Mass (*m/z*)	Q3 Mass (*m/z*)	DP (V)	CE (V)	CXP (V)	Time (min)
Corticosterone (B) 1	347.1	121.1	76	29	8	5.31
Corticosterone (B) 2	347.1	90.9	76	75	12	5.31
D8-corticosterone (d8B)	353.3	125.1	76	29	8	5.16
11-dehydrocorticosterone (A) 1	345.1	121.0	66	31	12	3.55
11-dehydrocorticosterone (A) 2	345.1	91.2	66	83	40	3.55
11-Deoxycorticosterone 1	331.2	97.0	86	29	16	7.45
11-Deoxycorticosterone 2	331.2	109.0	86	31	12	7.45
17-hydroxyprogesterone 1	331.1	109.0	66	29	12	8.05
17-hydroxyprogesterone 2	339.2	100.1	66	31	12	8.05
D8-17-hydroxyprogesterone 1	339.2	96.9	66	29	12	7.97
Pregnenolone (Preg) 1	317.1	281.1	126	17	16	10.36
Pregnenolone (Preg) 2	317.1	159.0	126	29	18	10.36
13C2,D2-Pregnenolone	321.1	285.2	126	17	16	10.34
17-hydroxypregnenolone 1	333.1	297.1	36	13	22	8.31
17-hydroxypregnenolone 2	333.1	132.9	36	27	20	8.31
Cortisol (F) 1	363.1	121.2	76	31	8	3.46
Cortisol (F) 2	363.1	91.1	76	83	10	3.46
D4-cortisol	367.2	121.1	76	31	8	3.42
11-deoxycortisol 1	347.1	97.0	71	27	12	5.69
11-deoxycortisol 2	347.1	109.0	71	33	16	5.69
D5-11-deoxycortisol	352.1	100.1	71	27	12	5.62
Cortisone (E) 1	361.1	163.1	81	31	26	2.94
Cortisone (E) 2	361.1	77.1	81	107	10	2.94
D8-cortisone	369.2	169.0	81	31	26	2.82
11-ketotestosterone (11ketoT) 1	303.2	259.1				3.80
11-ketotestosterone (11ketoT) 2	303.2	121.0				3.80
Testosterone (T) 1	289.1	97.0	101	29	12	7.64
Testosterone (T) 2	289.1	109.2	101	31	6	7.64
$^{13}C_3$-Testosterone ($^{13}C_3$-T)	292.1	100.0	101	29	12	7.64
Androstenedione (A4) 1	287.1	97.0	61	27	14	6.88
Androstenedione (A4) 2	287.1	78.9	61	67	10	6.88
$^{13}C_3$-Androstenedione ($^{13}C_3$-A4)	290.2	100.1	61	27	14	6.88
Dihydrotestosterone (DHT) 1	291.3	255.2	116	21	30	8.96
Dihydrotestosterone (DHT) 2	291.3	91.0	116	55	10	8.96
$^{13}C_3$-Dihydrotestosterone	294.2	258.3	116	21	30	8.96
Aldosterone 1	359.1	188.9	-70	-24	-21	2.62
Aldosterone 2	359.1	331.0	-70	-22	-35	2.62
D8-Aldosterone	367.2	193.9	-70	-48	-29	2.59
Estrone (1)	269.1	144.9	-150	-48	-15	7.20
Estrone (2)	269.1	142.9	-150	-70	-15	7.20
$^{13}C_3$-Estrone ($^{13}C_3$-E1)	272.1	147.8	-150	-52	-21	7.20
Estradiol (1)	271.0	144.9	-110	-52	-21	7.00
Estradiol (2)	271.0	182.9	-110	-52	-19	7.00
$^{13}C_3$-Estradiol ($^{13}C_3$-E2)	274.0	147.9	-110	-52	-21	7.00
Estriol (1)	287.1	171.0	-155	-48	-29	2.54
Estriol (2)	287.1	145.0	-155	-54	-9	2.54
$^{13}C_3$-Estriol ($^{13}C_3$-E3)	290.2	173.9	-110	-48	-29	2.55

839 DP; Declustering Potential, CE; Collision Energy, CXP; Collision Cell Exit Potential, Q1; Quantifier
840 1, Q2; Qualifier 2.

841 Table 5. Steroid levels in the plasma of Atlantic salmon on day 35 and 116 (n=3).

	Low Oxygen				High Oxygen				SE	*P*-value			
	HL-LP LA	LL-LP LB	HL-HP LC	LL-HP LD	HL-LP HA	LL-LP HB	HL-HP HC	LL-HP HD		L	P	O	X
Day 35													
11-dehydrocorticosterone	0.04	0.59	0.33	0.67	0.13	0.05	0.44	0.30	0.08				
11-deoxycorticosterone	0.04	0.04	0.04	0.06	0.03	0.03	0.03	0.04	0.001				
11-deoxycortisol	6.36	10.31	9.21	9.61	3.34	3.14	3.78	1.86	1.04			**	
11-ketoandrostenedione	0.30	0.18	0.40	0.10	1.72	0.65	0.64	0.34	0.16				
11-ketotestosterone	0.04	NA	1.56	0.17	0.63	0.24	0.14	0.13	0.16			**	
11β-hydroxyandrostenedione	2.40	NA	0.53	0.41	1.07	1.87	1.60	0.24	0.39				
17-hydroxypregnenolone	145.7	44.0	406.2	47.9	378.2	47.0	114.2	117.0	57.3				
17-hydroxyprogesterone	10.98	11.01	16.48	13.73	12.73	10.19	10.94	14.19	1.02				
Androstenedione	1.22	0.16	1.64	0.11	1.98	0.96	1.57	0.59	0.21	*			
Corticosterone	0.43	0.57	0.53	0.40	0.41	0.22	0.30	0.29	0.05				
Cortisol	112.9	87.4	63.8	95.0	54.6	36.9	88.2	71.9	12.5				
Cortisone	23.59	34.19	24.53	38.97	15.63	26.37	18.44	21.46	2.38			*	
Dehydroepiandrosterone (DHEA)	40.96	21.03	7.26	11.38	48.41	63.93	92.73	68.58	8.56			**	
Pregnenolone	0.24	0.29	0.17	0.29	0.22	0.36	0.27	0.31	0.03				
Testosterone	0.27	0.09	0.27	0.09	0.46	0.50	0.35	0.30	0.05			*	
Day 116													
11-dehydrocorticosterone	0.57	0.57	0.21	0.30	0.54	0.35	0.48	0.40	0.04		**		Px O
11-ketotestosterone	0.006	0.011	0.005	0.005	0.007	0.007	0.008	0.007	0.001				
17-hydroxypregnenolone	0.03	0.33	0.86	0.12	0.23	0.49	0.16	0.22	0.08				
17-hydroxyprogesterone	0.64	0.36	0.33	0.06	0.06	0.31	0.20	0.30	0.06				Lx P
Aldosterone	1.57	1.45	1.46	0.94	1.74	0.46	0.40	0.96	0.20				
Androstendeione	0.84	0.78	0.67	0.28	0.52	0.68	0.28	0.36	0.07				
Corticosterone	0.06	0.07	0.01	0.03	0.09	0.05	0.05	0.02	0.01		**		
Cortisol	122.3	112.5	45.4	64.3	128.2	76.7	102.0	69.5	8.0		**		
Cortisone	19.6	19.2	12.9	16.3	15.6	16.9	15.2	14.6	0.8				
Dehydroepiandrosterone-S (DHEA)	9.60	8.85	4.01	4.20	7.38	4.41	6.53	4.11	0.61		*		Px O
Dihydrotestosterone	0.077	0.050	0.050	0.050	0.050	0.050	0.050	0.050	0.003				
Estriol	0.020	0.021	0.018	0.017	0.022	0.031	0.016	0.013	0.002				
Pregnenolone	1.44	1.49	0.55	0.81	1.39	0.64	1.03	0.70	0.09		**		Px O
Progesterone	0.006	0.011	0.005	0.005	0.007	0.007	0.008	0.007	0.001				
Testosterone	0.25	0.09	0.05	0.03	0.08	0.07	0.05	0.05	0.02		*		

842 On day 116, 11-deoxycorticosterone, estradiol and estrone were below the lower limit of detection and were removed.
843 Under *P*-value, symbols missing, *, **, *** indicate $P>0.05$, $P<0.05$, $P<0.01$ and $P<0.001$. Letters under "X" indicate a
844 significant interaction ($p<0.05$) where L; lipid, P; pufa and O; oxygen.

845 Table 6. Plasma biochemistry and haematology parameters of Atlantic salmon at day 35 and 116
846 (n=3).

	Low Oxygen				High Oxygen				SE	*P*-value			
	HL-LP LA	LL-LP LB	HL-HP LC	LL-HP LD	HL-LP HA	LL-LP HB	HL-HP HC	LL-HP HD		L	P	O	X
Day 35													
Alanine transaminase (IU/L)	4.0	4.3	10.0	8.0	7.0	12.3	15.0	9.0	1.8				
Amylase (IU/L)	426	469	604	296	496	337	485	294	27	***			Lx P
Aspartate transaminase (IU/L)	40.0	75.0	51.0	46.0	38.7	36.3	63.3	121.7	13.7				
Cholesterol (mmol/L)	5.4	4.7	6.2	4.3	6.5	5.4	6.2	5.2	0.2	**		*	
Creatinine (µmol/L)	26.0	20.7	35.7	24.7	16.3	13.3	18.3	17.0	1.8	*		***	
Glutamate dehydrogenase (IU/L)	21.7	28.0	27.3	24.3	18.0	16.3	25.0	21.3	2.2				
Lipase (µ/L)	27.7	27.7	28.3	32.0	17.0	18.0	19.7	25.7	1.6			***	
Total Protein (g/L)	32.3	33.0	36.7	33.0	34.3	32.7	35.0	32.7	0.6				
Haemoglobin (g/mL)	0.13	0.13	0.14	0.14	0.14	0.13	0.14	0.13	0.00				
Haematocrit (%)	38.0	37.7	39.0	38.3	40.0	39.7	40.7	40.0	0.6				
RBC count (10^6/mL)	1.20	1.13	1.03	0.97	1.07	1.00	0.93	1.03	0.03				
WBC Count (10^3/mL)	25.6	24.1	21.9	16.1	19.7	17.7	18.4	21.6	1.3				
Mean corpuscular volume (MCV; fL/cell)	323	336	381	418	375	402	438	390	12				
Mean corpuscular Hb (MCH; pg/cell)	114	121	134	150	133	130	152	129	4				
Mean corpuscular Hb conc (MCHC; g/dL)	3.53	3.58	3.53	3.57	3.53	3.29	3.47	3.29	0.04			*	
Day 116													
Alanine transaminase (IU/L)	5.0	7.0	2.7	6.5	4.0	5.0	7.5	3.0	0.5				Lx P
Amylase (IU/L)	544	547	637	538	642	622	774	737	32				
Aspartate transaminase (IU/L)	873	967	688	502	921	1169	1133	578	76				
Cholesterol (mmol/L)	7.5	6.8	7.6	6.3	7.4	7.0	8.1	7.2	0.2	**			
Creatinine (µmol/L)	20.0	21.0	19.0	25.0	14.3	16.0	15.3	15.7	0.8			**	
Glutamate dehydrogenase (IU/L)	21.0	29.0	41.0	21.5	15.7	16.7	19.7	25.0	3.3				
Lipase (µ/L)	21.3	18.5	16.7	24.3	16.7	18.0	18.3	17.3	1.0				
Total Protein (g/L)	42.0	42.0	42.3	39.7	43.3	46.7	48.3	45.3	0.9			*	
Haemoglobin (Hb; g/mL)	0.14	0.14	0.17	0.14	0.15	0.13	0.16	0.14	0.01				
Haematocrit (%)	38.0	39.0	36.3	39.3	37.7	40.3	40.7	38.3	0.5				
RBC count (10^6/mL)	1.20	1.27	1.23	1.23	1.30	1.27	1.33	1.17	0.03				
WBC Count (10^3/mL)	19.1	16.2	14.7	16.2	17.3	14.5	20.1	17.4	1.0				
Mean corpuscular volume (MCV; fL/cell)	317	312	299	324	290	319	308	329	6				
Mean corpuscular Hb (MCH; pg/cell)	113	113	138	114	118	105	121	122	4				
Mean corpuscular Hb conc (MCHC; g/dL)	3.6	3.6	4.7	3.5	4.1	3.3	3.9	3.7	0.1				

Under *P*-value, symbols missing, *, **, *** indicate $P>0.05$, $P<0.05$, $P<0.01$ and $P<0.001$. Letters under "X" indicate a significant interaction ($p<0.05$) where L; lipid, P; pufa and O; oxygen.

Table 7. Lipid classes and fatty acids (% of total fatty acids) in the liver.

	Low Oxygen				High Oxygen					*P*-value			
	HL-LP LA	LL-LP LB	HL-HP LC	LL-HP LD	HL-LP HA	LL-LP HB	HL-HP HC	LL-HP HD	SD	L	P	O	X
Total Lipid (% of tissue)	4.5	4.5	4.9	4.3	4.8	4.4	5.2	4.5	0.4	*			
Lipid Class													
Wax/Sterol esters	4.0	2.5	3.8	2.6	5.8	4.7	5.3	3.9	1.5	*		**	
Triacylglycerols	7.3	7.2	7.7	6.2	10.9	6.4	9.2	6.6	3.5				
Free fatty acids	20.2	20.5	20.6	19.2	19.9	18.5	20.6	19.1	1.6				
Cholesterol/sterols	13.0	12.9	12.6	13.3	11.0	12.2	10.9	11.7	1.2			**	
Diacylglycerol	3.0	3.2	2.9	2.8	3.4	2.2	3.5	2.5	0.9				
Total neutral lipids	47.5	46.3	47.5	44.2	51.0	44.0	49.5	43.6	4.2	*			
Unknown glycolipid	1.9	1.7	2.4	2.6	1.3	1.6	1.8	2.1	0.7				
Unkown Polar lipid	1.8	3.3	2.6	2.8	2.3	1.8	2.3	2.3	0.8				
Phosphatidylethanolamine	11.7	12.0	10.5	11.1	10.2	12.3	9.8	9.9	1.2		*		
Phosphatidic acid/tidylglycerol	2.8	3.4	2.8	3.0	3.1	3.6	3.0	4.0	0.7				
Phosphatidylinositol	6.2	5.3	5.2	5.6	5.4	6.6	5.5	5.8	1.2				
Phosphatidylserine	3.2	3.3	3.4	3.5	3.4	3.9	3.4	4.7	0.8				
Phosphatidylcholine	18.3	18.6	18.5	20.6	17.2	20.0	17.4	20.2	2.2				
Sphingomyelin	2.0	1.8	1.8	1.7	2.1	2.0	2.0	2.2	0.3				
Lysophosphatidylcholine	1.5	1.4	2.0	2.0	1.1	1.2	1.9	2.0	0.4		***		
Pigmented material	3.1	2.9	3.3	2.8	2.9	2.9	3.3	3.4	0.6				
Total polar lipids	52.5	53.7	52.5	55.8	49.0	56.0	50.5	56.4	4.2	*			
Fatty acids													
14:0	0.6	0.7	0.8	0.8	0.6	0.7	0.8	0.9	0.1	*	***		
16:0	14.3	14.9	14.3	15.3	13.8	17.1	14.2	16.5	1.3	***			LxP
18:0	5.5	6.5	5.7	6.2	5.6	5.8	5.0	5.5	0.5	**		**	
Total saturated	20.8	22.5	21.1	22.7	20.3	23.9	20.2	23.2	1.6	***			
16:1n-9	1.1	1.1	0.9	0.7	1.1	0.8	0.8	0.7	0.2	**	***	*	
16:1n-7	1.0	1.2	1.4	1.2	1.2	1.2	1.5	1.4	0.2		*		
18:1n-9	37.8	35.8	35.3	28.9	39.3	30.4	36.3	28.4	4.5	***	**		

18:1n-7	1.9	2.0	2.0	2.0	1.9	1.7	2.0	1.9	0.1		*		LxO
20:1n-9	4.2	3.5	3.8	3.3	3.3	2.9	3.7	3.1	0.5	***		**	PxO
24:1n-9	0.7	0.6	0.7	0.7	0.7	0.7	0.7	0.7	0.1				
Total monounsaturated	47.4	44.7	44.7	37.5	48.1	38.2	45.6	36.7	5.0	***	*		
18:2n-6	4.9	4.9	4.5	5.8	4.7	5.8	5.1	5.1	0.6	**			LxPxO
20:2n-6	1.2	1.2	1.2	1.5	1.0	1.3	1.2	1.3	0.2	***	*		LxPxO
20:3n-6	4.3	4.2	2.5	2.9	4.1	5.2	2.3	3.1	1.0	***	***		LxO
20:4n-6 (ARA)	3.8	4.5	2.2	2.7	4.0	4.6	2.0	2.7	1.1	*	***		
22:5n-6	0.4	0.6	0.3	0.4	0.4	0.7	0.3	0.3	0.1	***	***		
Total n-6 PUFA	15.1	16.1	10.9	13.5	14.7	18.2	11.0	12.8	2.5	***	***		LxPxO
18:3n-3	0.3	0.2	0.5	0.6	0.2	0.3	0.6	0.5	0.2		***		LxPxO
20:4n-3	0.2	0.2	0.4	0.4	0.2	0.2	0.4	0.4	0.1		***		
20:5n-3 (EPA)	1.8	1.4	3.4	3.6	2.2	2.0	3.9	4.0	1.0		***	***	LxP
22:5n-3	0.9	1.0	1.4	1.6	1.1	1.3	1.5	1.7	0.3		***	*	
22:6n-3 (DHA)	13.5	13.8	17.3	19.7	12.9	15.8	16.5	20.4	3.0	**	***		
Total n-3 PUFA	16.7	16.7	23.2	26.3	16.9	19.6	23.2	27.3	4.4	*	***		
Total PUFA	31.9	32.8	34.1	39.8	31.6	37.8	34.2	40.1	4.0	**	**		
n-3/n-6	1.1	1.0	2.1	1.9	1.1	1.1	2.1	2.1	0.5	*	***		

Lipid classes and fatty acids <0.2 were removed

Under *P*-value, symbols missing, *, **, *** indicate $P>0.05$, $P<0.05$, $P<0.01$ and $P<0.001$. Letters under "X" indicate a significant interaction ($p<0.05$) where L; lipid, P; pufa and O; oxygen.

854 Table 8. Lipid classes and fatty acids (% of total fatty acids) in the head kidney.

	Low Oxygen				High Oxygen					*P*-value			
	HL-LP LA	LL-LP LB	HL-HP LC	LL-HP LD	HL-LP HA	LL-LP HB	HL-HP HC	LL-HP HD	SD	L	P	O	X
Total lipid (% of tissue)	4.9	3.6	4.4	3.6	5.8	4.3	4.9	4.6	1.0	**		*	
Lipid class													
Wax/Sterol esters	4.1	2.5	4.0	3.0	3.8	4.8	3.7	3.9	1.3				
Triacylglycerols	32.0	24.6	32.3	25.9	40.4	30.5	35.3	36.7	7.8			*	
Free fatty acids	4.8	5.4	5.4	5.8	3.6	4.7	4.7	4.4	1.2				
Cholesterol/sterols	14.3	16.0	14.2	15.6	11.5	12.5	12.5	11.5	2.0			** *	
Diacylglycerol	1.3	1.4	1.4	1.3	1.5	1.5	1.4	1.4	0.2				
Total neutral lipids	56.4	49.9	57.3	51.6	60.8	54.0	57.6	57.9	5.1	*			
Unknown glycolipid	3.2	3.7	3.7	4.2	2.9	2.5	2.7	3.5	0.8			*	
Phosphatidylethanolamine	11.3	13.0	11.4	12.3	9.5	11.0	10.4	9.6	1.7			**	
Phosphatidic acid/tidylglycerol	2.8	3.1	2.1	2.8	3.2	4.4	3.1	3.6	0.9			*	
Phosphatidylinositol	3.5	4.4	3.1	3.8	3.5	4.4	4.1	3.8	0.8				
Phosphatidylserine	4.1	4.7	3.0	4.4	3.5	4.4	4.0	3.7	0.8	*			
Phosphatidylcholine	13.9	16.2	14.5	15.4	12.0	14.3	13.4	12.8	1.8			**	
Sphingomyelin	2.6	2.8	2.4	3.0	2.2	2.7	2.3	2.3	0.5				
Pigmented material	2.1	2.3	2.4	2.4	2.1	2.1	2.2	2.6	0.4				
Total polar lipids	43.6	50.1	42.7	48.4	39.2	46.0	42.4	42.1	5.1	*			
Fatty acid													
14:0	1.0	1.0	1.4	1.5	1.2	1.0	1.4	1.7	0.3		** *		Lx P
16:0	14.4	15.7	15.3	16.2	13.6	15.8	15.0	15.1	1.2	*			
18:0	4.4	5.0	4.7	4.8	4.1	4.6	4.3	4.4	0.4	*		*	
20:0	0.3	0.2	0.3	0.2	0.3	0.2	0.2	0.5	0.2				
Total saturated	20.4	22.4	21.9	23.1	19.6	22.0	21.3	22.1	1.5	**			
16:1n-9	0.6	0.6	0.5	0.4	0.6	0.6	0.4	0.4	0.1		** *		
16:1n-7	1.4	1.4	1.8	1.7	1.7	1.4	1.8	2.1	0.3		**		
18:1n-9	41.8	35.4	37.8	32.1	43.9	36.0	37.8	35.0	4.7	**	*		
18:1n-7	2.4	2.5	2.5	2.5	2.5	2.4	2.4	2.6	0.1		*		
20:1n-9	3.0	2.7	2.7	2.4	3.1	2.7	2.6	2.8	0.4				

22:1n-11	1.1	1.0	1.0	1.0	1.2	0.9	1.0	1.3	0.2			
22:1n-9	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.0			
24:1n-9	0.7	0.7	0.7	0.8	0.6	0.8	0.7	0.7	0.1			
Total monounsaturated	51.5	44.8	47.4	41.7	54.4	45.3	47.4	45.6	5.3	**		
18:2n-6	6.9	7.4	6.7	7.6	7.4	7.5	6.9	8.4	0.8	*		
20:2n-6	0.8	1.1	0.8	1.0	0.8	1.0	0.8	1.0	0.1	** *	*	
20:3n-6	1.9	2.4	1.0	1.3	1.6	2.4	0.9	1.2	0.6	**	** *	
20:4n-6 (ARA)	2.4	3.2	1.4	1.8	2.0	3.3	1.6	1.5	0.9		** *	
22:5n-6	0.2	0.3	0.2	0.2	0.2	0.3	0.2	0.2	0.1	** *	** *	Lx P
Total n-6 PUFA	12.7	14.8	10.4	12.1	12.3	14.9	10.6	12.4	1.7	** *	** *	
18:3n-3	0.9	0.9	1.5	1.6	1.2	0.9	1.6	1.9	0.4		** *	
18:4n-3	0.3	0.2	0.3	0.3	0.4	0.3	0.4	0.4	0.1		**	
20:4n-3	0.3	0.3	0.5	0.5	0.4	0.3	0.5	0.5	0.1		** *	
20:5n-3 (EPA)	2.4	2.4	3.9	4.1	2.3	2.6	4.5	3.5	1.0		** *	
22:5n-3	0.8	1.0	1.2	1.3	0.8	0.9	1.2	1.3	0.2	**	** *	
22:6n-3 (DHA)	9.4	11.8	11.4	13.5	7.9	11.5	11.2	10.6	2.5			
Total n-3 PUFA	14.3	16.8	19.1	21.6	13.2	16.7	19.6	18.5	3.5		**	
Total PUFA	27.1	31.7	29.5	33.8	25.5	31.6	30.2	31.0	3.5	**		
n-3/n-6	1.1	1.1	1.8	1.8	1.1	1.1	1.9	1.5	0.4		** *	

Lipid classes and fatty acids <0.2 were removed

Under *P*-value, symbols missing, *, **, *** indicate $P>0.05$, $P<0.05$, $P<0.01$ and $P<0.001$. Letters under "X" indicate a significant interaction ($p<0.05$) where L; lipid, P; pufa and O; oxygen.

Figures

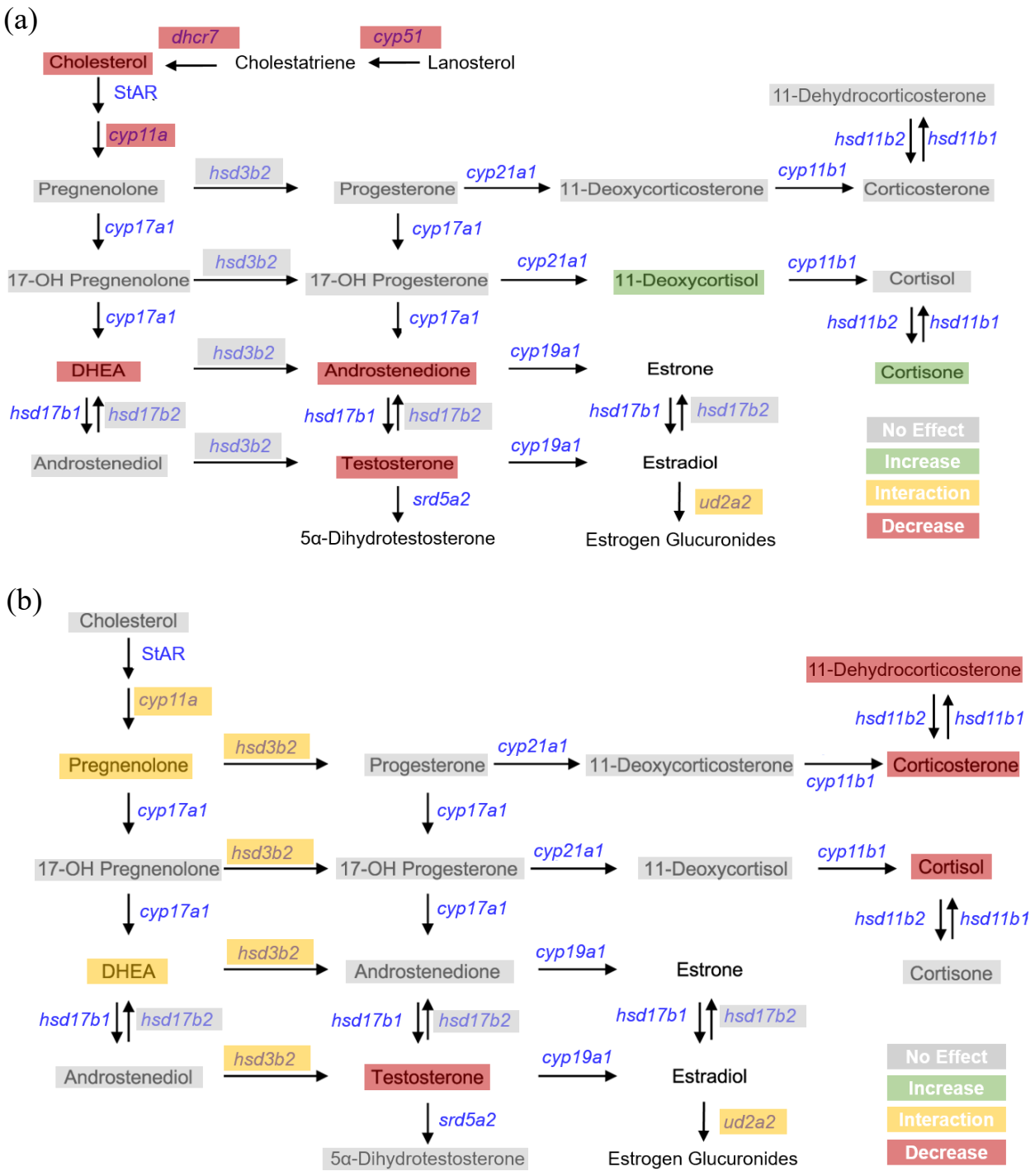

Figure 1. Effect of dietary n-3 LC-PUFA levels on steroidogenesis in the plasma of Atlantic salmon on day 116. Highlighted are those steroids (black) and genes (blue) that were subject to changes in concentration or relative gene expression levels. Steroids/genes not measured are those without an overlay. In terms of results, a) shows hypoxia increased levels of the cortisol intermediates of 11-deoxycortisol and cortisone while downregulating androgenic steroid pathways, and b) showed that

high levels of n-3 LC-PUFA decreased the concentrations of cortisol and corticosterone with several interactions between n-3 LC-PUFA x hypoxia on pregnenolone and DHEA.

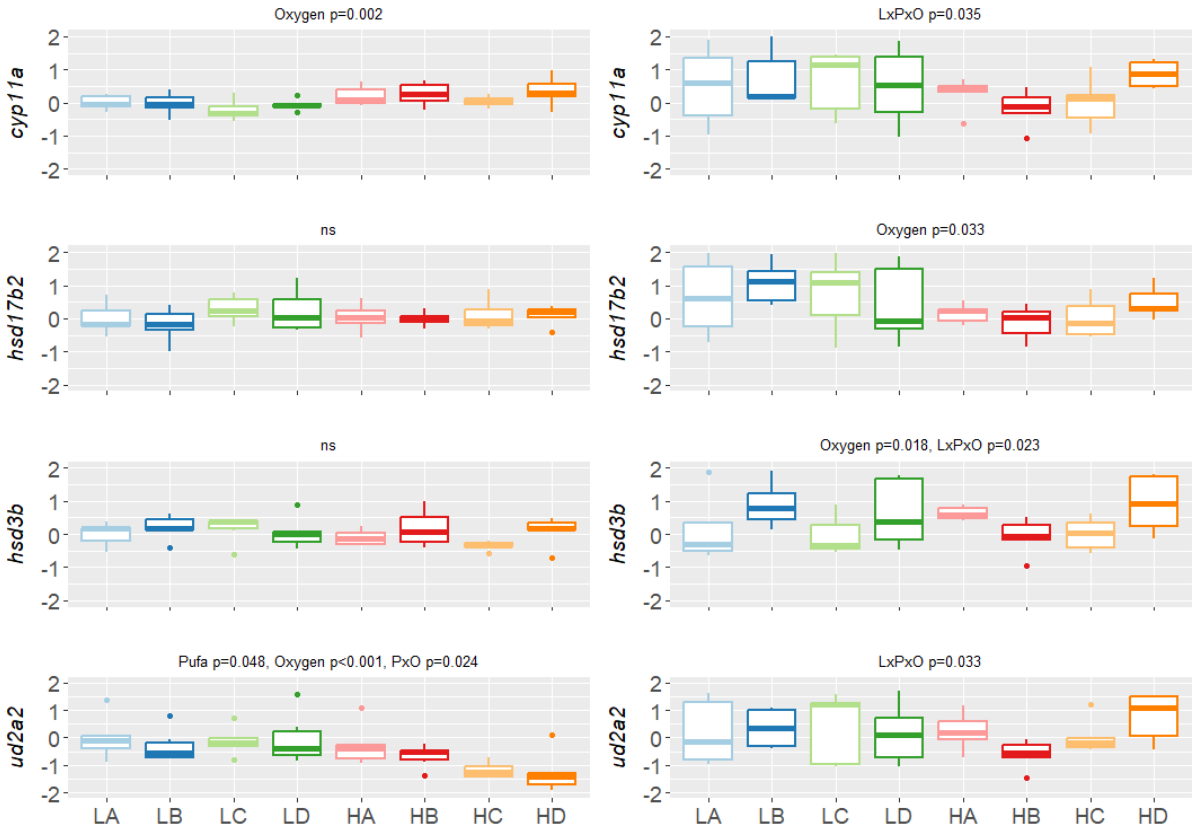

Figure 2. Differential expression (log10 transformed) of steroid genes in the liver relative to three reference genes and the control group (HC) for expression. Day 35 (left) and day 116 (right). Treatments labelled 'L' and 'H' indicate low and high oxygen, respectively. Fish were either fed high lipid (A and C) or low lipid (B and D) with either low PUFA (A and B) or high PUFA (C and D). *P*-values of each factor or interaction are above each plot, 'ns' indicates not significant ($P>0.05$).

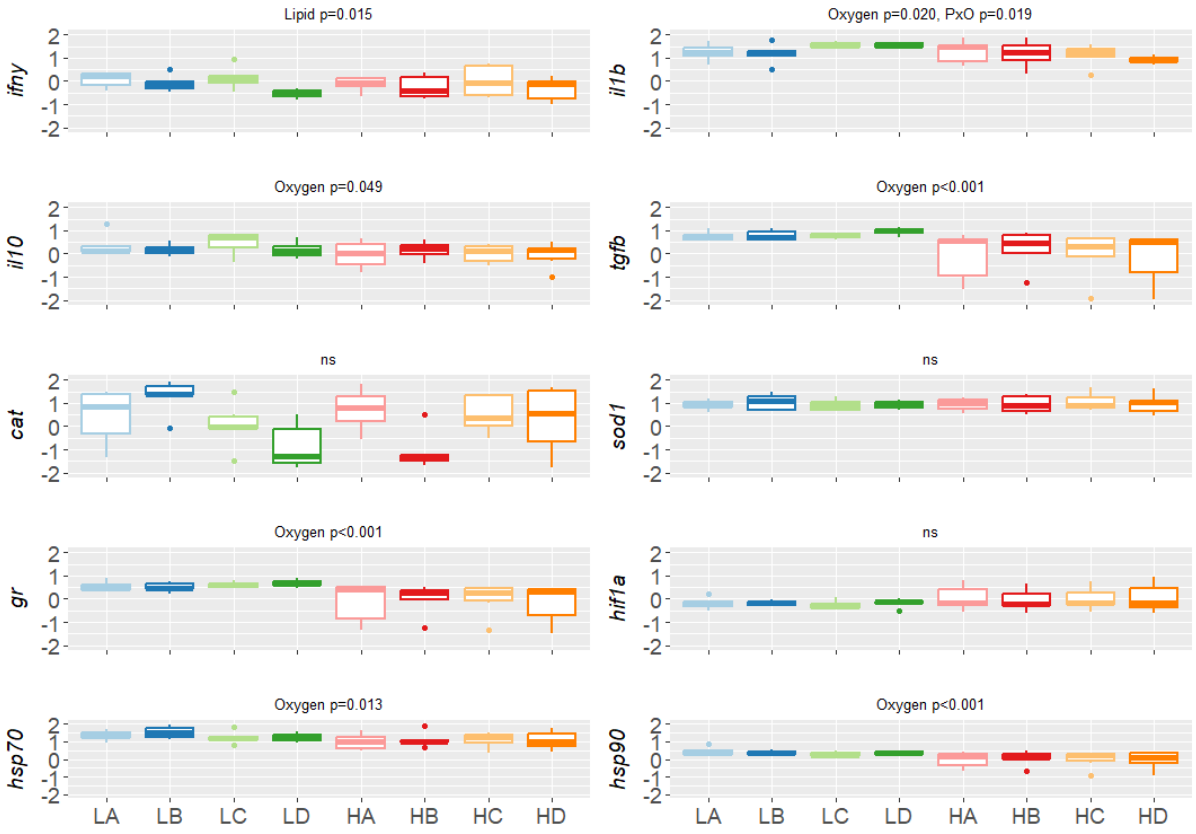

Figure 3. Differential expression (log10 transformed) of immune and oxidative stress genes in the head kidney relative to three reference genes and the control group (HC) for expression. Day 35 (left) and day 116 (right). Treatments labelled 'L' and 'H' indicate low and high oxygen, respectively. Fish were either fed high lipid (A and C) or low lipid (B and D) with either low PUFA (A and B) or high PUFA (C and D). *P*-values of each factor or interaction are above each plot, 'ns' indicates not significant ($P>0.05$).

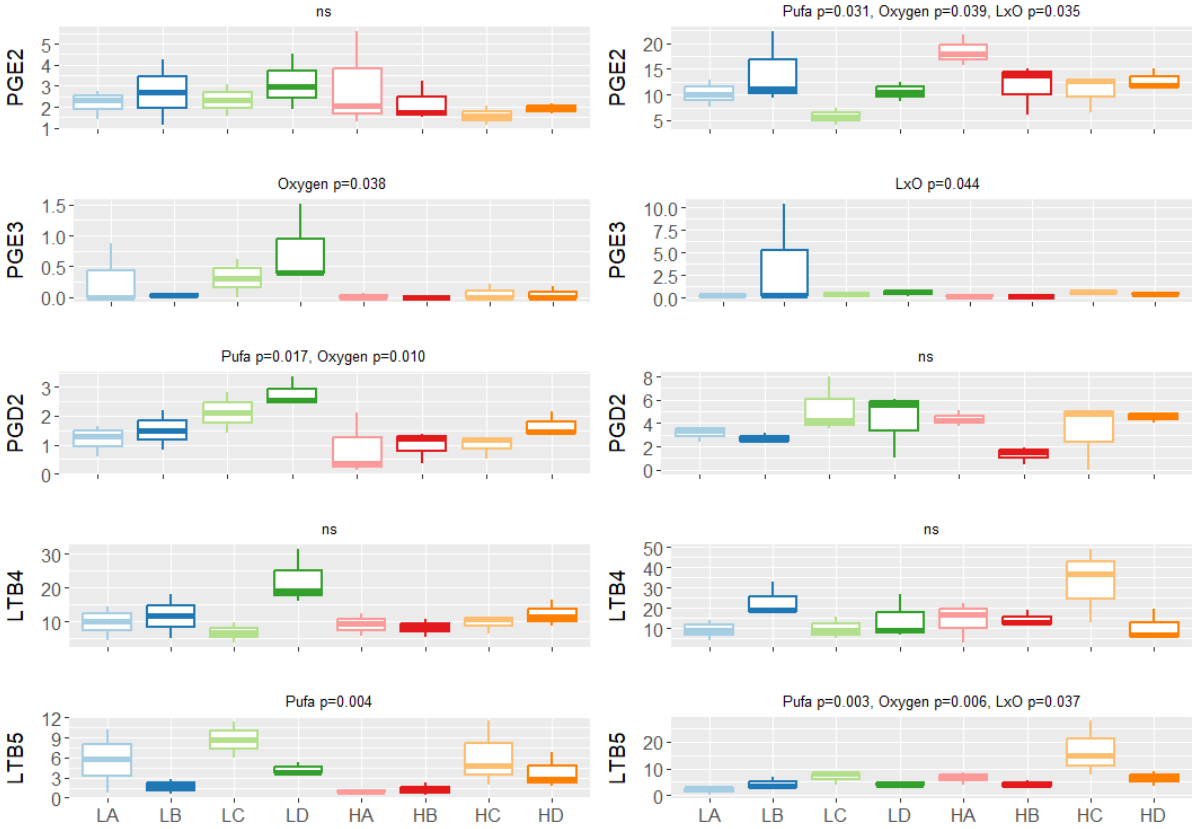

Figure 4. Eicosanoid production in the liver (left) and head kidney (right) at day 116. Treatments labelled 'L' and 'H' indicate low and high oxygen, respectively. Fish were either fed high lipid (A and C) or low lipid (B and D) with either low PUFA (A and B) or high PUFA (C and D). *P*-values of each factor or interaction are above each plot, 'ns' indicates not significant ($P>0.05$).