# Increasing the dietary n-6/n-3 ratio alters the hepatic eicosanoid production after acute stress in Atlantic salmon *(Salmo salar)*

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## ARTICLE INFO

## ABSTRACT

Earlier studies have indicated that a high inclusion of n-6 fatty acids (FA) in feeds for Atlantic salmon can affect the stress response. To test this hypothesis, Atlantic salmon *(Salmo salar)* were fed diets containing varying dietary n-6/n-3 FA ratios and different absolute levels of n-6 and n-3 FAs. The fish were divided into two different stress challenge groups, where one group was exposed to three weekly hypoxia challenges for 4 weeks (repeated stress), while one group was left undisturbed. At the end of the experiment, both groups were exposed to an acute stressor (lowering of water level). Thus, effects of the diets on acute stress, repeated stress and the combined effect of these could be investigated. In general, there were few effects of the repeated stress, while fish in all diet groups responded strongly to the acute stress based on several stress markers. Dietary n-6/n-3 ratio did not affect growth, all fish appeared phenotypically healthy, and all groups were able to mount an acute stress response. However, there was an interaction between diet and repeated stress on cortisol response after acute stress, possibly indicating altered hypothalamic-pituitary adrenal axis reactivity in fish fed high n-6/n-3 FA ratio. Hepatic levels of prostaglandin $D_2$ ($PGD_2$) and leukotriene $B_4$ responded differently to acute stress depending on the dietary n-6/n3 FA ratio, indicating an altered acute stress response. Additionally, increasing the dietary n-6/n-3 FA content led to higher levels of $PGD_2$ and $PGE_2$ as well as higher liver triacylglycerol. In summary, the results suggest that increasing the dietary n-6/n-3 FA ratio in salmon feeds can affect the way they respond to stressors in an aquaculture setting, possibly affecting the fish robustness.

## 1. Introduction

The limited availability of fish oil (FO) for use in fish feeds has contributed to its replacement by vegetable oils (VO), mainly rapeseed oil in Norwegian fish feeds (Aas et al., 2019). Most VOs are rich in the n-6 fatty acid (FA) 18:2n-6 (linoleic acid, LA), which salmon readily can convert into 20:4n-6 (arachidonic acid, ARA). Thus, tissue levels of ARA in Atlantic salmon are highly dependent on dietary LA and will increase with increasing dietary LA content (Sissener et al., 2020). Hence, the changes in oil composition in typical salmon feeds have resulted in a higher n-6 FA and lower n-3 FA content in both feed and fillet of

Norwegian salmon, compared to when fed a more marine based diet (Sissener et al., 2016a). Studies on the impacts of feeds rich in n-6 FAs on the health and welfare of Atlantic salmon show somewhat contradictory results. Some trials show seemingly no adverse effects of high dietary n-6 FAs (Grisdale-Helland et al., 2002, Sissener et al., 2017, Menoyo et al., 2007, Emam et al., 2020), while in others 30% mortality was experienced following transport (Bell et al., 1991a) and 28% following light sedation and weighing (Sissener et al., unpublished data).

The n-6 and n-3 FAs are the precursors of eicosanoids, which are highly potent lipid signalling molecules. Moreover, they compete for the same enzymes for eicosanoid production, and their final products can

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have opposing effects (Schmitz and Ecker, 2008). Despite a higher prevalence of 20:5n-3 (eicosapentaenoic acid, EPA) compared to ARA in fish tissues, ARA is still the preferred eicosanoid precursor in fish (Bell et al., 1994). An increase in dietary LA is also shown to cause a higher production of ARA derived eicosanoids in fish (Sissener et al., 2020, Alves Martins et al., 2012, Bell et al., 1998). Eicosanoids are constitutively produced, but exposure to stress, trauma or disease can trigger an increased biosynthesis (Arts and Kohler, 2009). Hence, changes to the dietary n-6 and n-3 FA and the relationship between them could potentially modify the stress response of fish through altered eicosanoid production.

In accordance with the above studies, which suggest that the dietary n-6 and n-3 FA composition can modify the stress response via an altered eicosanoid production, there are also several studies showing that VO in the feed can affect plasma cortisol levels in fish. For instance, Jutfelt et al. (2007) reported that feeding Atlantic salmon a diet with sunflower oil (high in LA) resulted in elevated cortisol levels during smoltification compared to when feeding a diet with FO. Moreover, a slower increase in plasma cortisol after crowding was observed in gilthead seabream *(Sparus aurata)* fed a soy oil diet (high in LA) compared to a FO diet (Ganga et al. 2011). Furthermore, in the latter study, fish fed the soy oil diet took longer time to recuperate and had not regained basal cortisol levels after one week. The type of stressor used can also determine which impact of dietary n-6 FA is seen. This is supported in a study by Koven et al. (2003), who reported reduced mortality when sea bream larvae was fed a diet high in ARA when exposed to an acute handling stressor. However, when exposed to a chronic stressor (repetitive salinity changes) increased mortality was observed in fish fed a high ARA diet.

The observed dietary effects on cortisol levels may be due to the FA composition being able to interact with the stress axis, the hypothalamic-pituitary-adrenal/interrenal (HPA/I; in mammals/in fish) axis, on several levels. Ganga et al. (2006) demonstrated that ARA and EPA stimulated cortisol release in an in-situ study of gilthead seabream interrenal cells. Furthermore, there are a several mammalian studies demonstrating that dietary fatty acids interact with the stress axis on the central level (for references see Maes et al., 2009). Effects which are related to changes in neurotransmission of serotonin (5-HT) and the metabolic faith of its intermediate precursor tryptophan (TRP). Since 5-HT is important in stress coping mechanisms, it plays a central role in the regulation of the HPA/I axis (Winberg and Nilsson, 1993), and thus it is important in stress coping processes in both fish and mammals (Höglund et al., 2020). However, if the FA composition affects the stress coping ability through effects on brain 5-HT signalling in fish is to our best knowledge unknown.

Feeding diets high in LA and low in EPA and 22:6n-3 (docosahexaenoic acid, DHA) to Atlantic salmon causes an increased liver triacylglycerol (TAG) content (Alvheim et al., 2013, Ruyter et al., 2006, Bransden et al., 2003). Several trials have found that a reduced content of EPA + DHA in salmon feeds can cause increased liver TAG (Bou et al., 2017, Sanden et al., 2016). However, a higher liver TAG is also seen with increasing dietary LA despite equal dietary levels of EPA + DHA (Hundal et al., 2020). Additionally, studies using rat hepatocytes have demonstrated that prostaglandin $E_2$ ($PGE_2$) and prostaglandin $D_2$ can inhibit secretion of very-low-density lipoproteins (VLDL) (Perez et al., 2006, Björnsson et al., 1992). This could suggest that a higher dietary n-6 results in a higher liver TAG through increased $PGE_2$ and prostaglandin $D_2$ ($PGD_2$) levels. Increased liver TAG can be seen as a general sign of dietary imbalance and can potentially be linked to reduced robustness (Sissener et al., 2016a).

Atlantic salmon in aquaculture are exposed to many different stressors such as fluctuations in temperature, salinity and oxygen levels, transportation, delousing, vaccination, etc., which will elicit a stress response. The stress response is a necessary response to challenging situations, but chronic or repeated stress may lead to allostatic overload, a situation where the fish is no longer able to respond properly to additional stressors mammals (Höglund et al., 2020, Madaro et al.,

2015). We need to understand how optimal nutrition can contribute to a healthy, robust fish capable of coping with stressful situations and environmental changes. Hence, this trial was designed both to test the effects of altered dietary n-6/n-3 ratio, and effects of absolute levels of n-6 and n-3 on the response to repeated and acute stress, in addition to any interaction between them.

## 2. Material & methods

### 2.1. Diets and fish trial

Diets and experimental design have been published elsewhere (Hundal et al., 2020). Briefly, the trial included four diets designed for elucidating the effects of total dietary n-3 and n-6 FAs and the ratio between them on fish robustness and stress coping abilities. The first three diets contained equal levels of n-3 FAs with increasing n-6 FAs aiming for n-6/n-3 FA ratios of 1, 2 and 6. Roughly, half of the n-3 FAs were 18:3n-3 and the other half EPA + DHA, while the n-6 FA level was increased by including LA. The fourth diet contained twice as much n-3 FAs with equal n-6 level as the second diet, hence resulting in an n-6/n-3 ratio of 1. From now on the diets will be referred to by their dietary n-6/n-3 ratio (diet 1, 2 and 6). The final diet will be labelled 1H due to its higher absolute n-3 FA content. Analysed dietary n-6/n-3 FA ratios are given in Table 1. Diet formulation has been published elsewhere (Hundal et al., 2020), the only difference between the diets were the oil blends used to achieve the desired FA composition. The analysed proximate and FA composition of the diets are shown in Table 1 (previously published in Hundal et al., 2020).

The trial was performed at Skretting ARC Research station (Lerang, Norway) and conducted according to the guidelines of the Norwegian State Commission for Laboratory Animals. The National food safety authorities approved the protocol (identification number: ID 13576).

**Table 1**
Analysed dietary proximate and fatty acid composition, and TFA of the diets (4 mm), as the mean of two technical replicates analysed. Previously published in Hundal et al., 2020.

|  | Diet 1 | Diet 2 | Diet 6 | Diet 1H |
| --- | --- | --- | --- | --- |
| **Proximate composition (g/100 g)** | | | | |
| Lipid | 29.8 | 30.0 | 28.6 | 28.8 |
| Protein | 44.5 | 44.1 | 44.5 | 44.8 |
| Ash | 5.1 | 5.2 | 5.5 | 5.4 |
| **Fatty acids (% of TFA)** | | | | |
| ΣSFA | 19.7 | 19.7 | 16.6 | 19.8 |
| 12:0 | 1.3 | 1.7 | 0.4 | 0.1 |
| 14:0 | 3.3 | 3.5 | 1.5 | 4.4 |
| 16:0 | 11.2 | 10.4 | 9.4 | 11.5 |
| 18:0 | 2.5 | 2.6 | 3.7 | 2.7 |
| ΣMUFA | 59.4 | 52.2 | 27.3 | 43.5 |
| 16:1n-7 | 3.8 | 3.7 | 1.5 | 5.1 |
| 18:1n-7 | 2.2 | 2 | 1.1 | 2 |
| 18:1n-9 | 37.1 | 30.1 | 23.1 | 18.2 |
| 20:1n-9 | 6.4 | 6.4 | 0.7 | 7 |
| 22:1n-11 | 7.7 | 7.7 | 0.7 | 8.5 |
| Σn-6 | 11.1 | 18.2 | 46.9 | 18.4 |
| (LA) 18:2n-6 | 10.8 | 17.9 | 46.7 | 17.6 |
| (ARA) 20:4n-6 | 0.1 | 0.1 | 0.1 | 0.2 |
| Σn-3 | 7.7 | 7.7 | 7.8 | 15.1 |
| 18:3n-3 | 2.9 | 2.9 | 3.1 | 5.7 |
| (EPA) 20:5n-3 | 2.4 | 2.4 | 2.0 | 4.4 |
| (DHA) 22:6n-3 | 1.5 | 1.5 | 1.8 | 3.1 |
| EPA + DHA | 3.9 | 3.9 | 3.8 | 7.5 |
| ΣPUFA | 20.9 | 28.1 | 56.1 | 36.8 |
| **n-6/n-3** | 1.4 | 2.4 | 6.1 | 1.2 |
| **TFA (mg/g feed)** | 270.7 | 272.7 | 296.5 | 255.3 |

Diet codes are given by dietary n-6/n-3 ratio. Diet 1H has a double absolute n-6 and n-3 FA content compared to diet 1; TFA - total fatty acids; SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; LA – linoleic acid, ARA -arachidonic acid, EPA - eicosapentaenoic acid; DHA - docosahexaenoic acid; PUFA - polyunsaturated fatty acids.

The trial started with a pre-feeding period from 11th of November 2017 to 26th of February 2018 to let tissues stabilise according to the FA composition of the diets. A mixed population of both sexes of Atlantic salmon (SalmoBreed, Erfjord, Stamfisk AS) (~80 g) were distributed randomly to 4 circular tanks (3 m diameter, 7000 L, 735 fish/tank) supplied with running sea water at 8 °C and exposed to 24 h light and fed the experimental diets (3 mm, proximate and FA composition published in Hundal et al., 2020). The 26th of February 2018 the fish (mean weight 259 ± 2 g) were distributed into 24 tanks (1 m diameter, 450 L, 25 fish per tank), 12 assigned for repeated stress and 12 for controls, supplied with flow through sea water at 11.7 ± 0.2 °C and 24 h light photoperiod. Within each section, the experimental diets (4 mm pellets) were fed to triplicate tanks to satiation, in slight excess of expected intake by automatic feeders (Hølland Teknologi AS, Sandnes, Norway). Excess feed was collected by feed collectors to monitor feed intake. Standard husbandry procedures at the station were used.

The first two weeks of the trial the fish were allowed to acclimate to the new tanks, before implementation of repeated stress. Repeated stress was achieved by repeatedly shutting off the water inlet (3 times per week) to create hypoxia/hypercapnia. The fish respired the oxygen saturation down to 35% before the water inlet was turned back on. It took approximately 30 min from the closing of the water inlet (start of stress test) until oxygen levels were back to normal (end of stress test). The repeated stress exposures were performed for 4 consecutive weeks. At the end of the trial, all fish (repeatedly stressed and unstressed controls) were exposed to acute stress by lowering the water level in the tanks for 30 min. During the acute stress, water was lowered till it barely covered the fish in the cone of tank. The water level was kept like this for 30 min before the water was raised to normal level. Oxygen was maintained during the stress by normal water renewal. No mortalities were recorded in the trial, neither during the period of repeated stress, not after the acute stress.

## 2.2. Sampling

Sampling was performed the 10th to 12th of April 2018. Fish were sampled at three different time points relative to the acute stress test; before stress, 1 h after and 24 h after. The postprandial time was 12 h. The fish were sacrificed using an overdose of anaesthetic (Tricaine Pharmaq, 0.3 g/L), and weight and length were measured on all fish before blood was taken from the caudal vein with vacutainers coated with EDTA. Blood was centrifuged for 7 min at 4000*g* to separate plasma and red blood cells (RBC). RBC were washed thrice in physiological saltwater. Before the acute stressor, pooled plasma samples were collected using five fish per tank, and RBC samples were collected from three individual fish per tank. Pooled plasma samples were also collected 1 h and 24 h after the acute stress. Individual plasma samples from five fish were taken for cortisol analysis, both before and 1 h and 24 h after stress. HCT was measured on five individual fish per tank per time point. Individual liver samples were taken from five fish per tank for gene expression analysis at each time point. For eicosanoids, liver samples from five fish were taken before acute stress, and from three fish 1 h and 24 h after acute stress. Pooled liver samples of five fish per tank was taken for lipid class analysis. Brain samples were collected before acute stress from five fish and pooled per tank for FA analysis. Both before and 1 h after acute stress, telencephalon and hypothalamus were quickly dissected out by experienced personnel. Liver and brain samples were flash frozen in liquid nitrogen, then put on dry ice and stored at −80 °C until analysed. RBC and plasma samples were frozen on dry ice and stored at −80 °C until analyses.

## 2.3. Lipid class and fatty acid analysis

Lipids from liver and brain were extracted in a chloroform/methanol 2:1 mixture (Merck, Darmstadt, Germany). For samples to be analysed for lipid classes, 1% BHT (2,6-di-tert-butyl-methylphenol, Sigma-

Aldrich AS, Norway) was added. The chloroform:methanol mixture was added directly at approximately 20 times the sample weight for liver, whereas for brain methanol was added first and shaken for 2 h before adding chloroform to improve the extraction of polar lipids (PL). The samples were frozen overnight at −20 °C. Lipid class analysis was performed using high performance thin layer chromatography as described previously (Torstensen et al., 2004). After lipid extraction as described above, the samples were filtered and the quantification of lipid class composition was carried out by HPTLC as described by Torstensen et al. (2011a). Lipid classes were quantified by scanning densitometry using a CAMAG TLC Scanner 3 and calculated using an integrator (WinCATS-Planar Chromatography, version 1.2.0; CAMAG, Berlin, Germany). Quantitative determination (mg lipid class/g tissue) of lipid classes was performed by establishing standard equations for each lipid class within a linear area, in addition to including a standard mixture of all the lipid classes at each high-performance TLC plate for corrections of between plate variations. Neutral lipids (NL) and PL were separated in brain samples using solid-phase extraction, as described in Sissener et al. (2016b). Nonadecanoic acid (19:0) was added as internal standard to the extracts for the quantitative determination of fatty acyl methyl esters. FA analysis was then performed on the NL and PL fraction following the method used in Torstensen et al. (2011b). In short, the extracts were filtered, evaporated and then saponified and methylated using BF$_3$ in methanol. The separation of FA was performed on an AutoGC (Autosystem XL, Perkin Elmer Inc., Waltham, MA, USA) equipped with a flame ionization detector. The software Chromeleon® version 7.2 (Thermo Scientific, Waltham, MA, USA) was used for integration. RBC were analysed for FA composition using ultra-fast GC (described in Sissener et al. (2016b)), which is a faster method but slightly limited because it does not separate monounsaturated FA according to the double bond position. The system used for FA detection in the red blood cells was a Trace GC Ultra (Thermo Corporation) with SSL-injector, flame Ionization Detector, and the column was a Wax column (P/N UFMC00001010501,5 m long, 0.1 mm. Id., 0.1 μm film thickness). Chromeleon was the integrator used.

Feed FA composition was analysed by acid catalysed methylation and extraction in hexane before separation in a GC, as described in Sissener et al. (2016c). Feed FA composition was performed by Skretting ARC.

## 2.4. RNA isolation and quantitative real time PCR

For analysis of gene expression in liver, samples from the fish fed the lowest and the highest n-6/n-3 FA ratio (diet 1 and diet 6) were used. RNA was extracted from the liver using EZ1 universal tissue kit (Qiagen, Crawley, UK) according to the manufacturers instruction using the biorobot EZ1 (Qiagen) with 10 μL DNase. Quantitative and qualitative assessments of the RNA were performed with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), respectively. When assessing the RNA integrity, the RNA 6000 Nano LabChip® kit (Agilent Technologies) was used. The absorbance ratio A260/280 was 2.1 ± 0.0, A260/230 was 2.2 ± 0.1 and the RIN-value was higher than 8.3 for all samples, indicating RNA samples suitable for RT-qPCR. A two-step method was applied to measure levels of target gene mRNA in the samples. First, a reverse transcription reaction was run on a 96-well cDNA plate. A serial dilution curve with five set points (3.1 to 100 ng/μL) of total RNA (mix of all RNA) was set up in triplicate for PCR efficiency calculations. Samples were set up in duplicate, and non-amplification control and non-template control were included as negative controls. Aliquots of 10 μL of sample (50 ng/μL ± 5%) or standard were diluted to 50 μL using the TaqMan Reverse Transcription Reagent containing Multiscribe RT (50 U/μL) and oligo (dT) primers (kit N808–0234; Applied Biosystems, Foster City, Ca, USA). The reverse transcription reaction was carried out on the GeneAmp PCR 9700 (Applied Biosystems), with the following temperature program:

incubation at 10 min at 25 °C, RT reaction for 60 min at 48 °C in 50 μL total volume and then inactivation for 5 min at 95 °C. qPCR was run on a LightCycler® 480 Real-Time PCR System with the SYBR Green Master-mix (Roche Applied Sciences, Basel, Switzerland) and using the following temperature program: 5 min denaturisation and activation at 95 °C, 45 cycles of 10 s denaturisation at 95 °C, 10 s annealing at 60 °C and 10 s synthesis at 72 °C. A melting point analysis was performed before cooling to 4 °C. The stability of the reference genes (β-actin, ARP and EF1ab) was calculated using CFX Maestro software (Bio-Rad CFX Maestro version 1.1, Bio-Rad Laboratories, Hercules, CA, USA), which performs a stability analysis based on the GeNorm algorithm. Normalisation was performed using the CFX Maestro. The PCR primer sequences used are provided in Table 2.

Gene expression in brain was performed by NIVA on the fish fed the two most different n-6/n-3 FA ratios (diet 1 and diet 6) for the samples taken before the acute stressor and 1 h after. RNA was extracted from telencephalon using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The extracted RNA was treated for contaminating genomic DNA with TURBO DNA-free kit by Invitrogen™ (Carlsbad, CA, USA). Quantitative and qualitative assessments of the RNA were performed with a NanoDrop 1000 spectrophotometer and an Agilent 2100 Bioanalyzer, respectively. The RIN values were $9.3 \pm 0.06$ (mean $\pm$ SEM) proving excellent RNA quality. cDNA was synthesized from 1.0 μg total RNA by using iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) following the manufacturers protocol. The qPCR reaction contained 5 μL Power SYBR™ Green PCR Master Mix, 1 μl 10 μM forward and reverse primer, and 5 μl cDNA (diluted 1:10). Primer sequences given in Table 2. Real time PCR was carried out using a Roche 96 LightCycler (Roche Diagnostics, Penzberg, Germany) with 10 min preincubation at 95 °C, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a melting curve. The reference genes tested were S20, hprt1 and ppia. The stability of the three reference genes ef1αa, s20 and hprt1 was evaluated (following the protocol by Silver et al. (2006)), after which s20 was selected as the most stable reference gene and used as the internal control gene for calculations. All genes were run together with a standard curve to assess primer efficiency.

### 2.5. Analysis of blood and plasma

Whole blood haematocrit was measured immediately after sampling in a Thermo Scientific Pico 17 haematocrit centrifuge. Analyses of the plasma glucose and chloride were performed on a Maxmat Biomedical Analyzer (SM1167, Maxmat S.A., Montpellier, France), using Maxmat reagents and the appropriate calibrators and controls for the different methods.

Cortisol in plasma was analysed using a commercially available DetectX® cortisol enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI, USA) following the manufacturers protocol. The absorbance of the prepared ELISA plate was read in a plate reader at 450 nm and the concentrations were calculated using the four-parameter logistics curve.

### 2.6. Eicosanoid analysis

Liver samples from the diet 1 and diet 6 were analysed for eicosanoids. Eicosanoid standards used in the analyses were $PGE_2$ (99% purity), prostaglandin $E_3$ ($PGE_3$) (98%), $PGE_2$-$d_4$ (99%), leukotriene $B_4$ ($LTB_4$) (97%), leukotriene $B_5$ ($LTB_5$) (98%), $LTB_4$-$d_4$ (99%), all purchased from Cayman Chemical (ANN Arbor, MI, USA). The samples were kept frozen on dry ice, crushed to a fine powder and homogenised prior to analysis. A mortar was cooled with liquid nitrogen, then the liver samples were crushed with a pestle while submerged in liquid nitrogen. The samples were put back into tubes and stored at −80 °C until further analysis. The samples were weighed (approx. 300 mg), and successive aliquots of 500 μL of acetonitrile (ACN, containing 30 ng/mL $PGE_2$-$d_4$ and 15 ng/mL $LTB_4$-$d_4$) and pure chloroform were added before vortex mixing for 30 s. The extract was collected and transferred through a filtration system to a new tube. The extraction was repeated a second time, but with pure CAN only. The resulting 2 mL extract was then evaporated at room temperature using a RapidVap (Labconco, Kansas City, MO, USA). The dried samples were diluted in 50 μL methanol before injection into an LCMS/MS system (Agilent 6495 QQQ triple quadrupole, Agilent Technologies, Waldbronn, Germany) with an electrospray ionization (ESI) interface and iFunnel ionization to quantify the eicosanoids. The UHPLC system was equipped with a Zorbax RRHD Eclipse Plus C18, 95 Å, 2.1 × 50 mm, 1.8 μm chromatographic column.

**Table 2**

qPCR primer sequences, their accession numbers and efficiency for target and reference genes (marked in bold text).

| Target gene | Forward | Reverse | GenBank accession number | Efficiency |
|---|---|---|---|---|
| Liver | | | | |
| **β-actin** | CCAAAGCCAACAGGGAGAA | AGGGACAACACTGCCTGGAT | BG933897 | 104% |
| **ARP** | GAAAATCATCCAATTGCTGGATG | CTTCCCACGCAAGGACAGA | AY255630 | 85% |
| **EF1ab** | TGCCCCTCCAGGATGTCTAC | CACGGCCCACAGGTACTG | AF321836 | 113% |
| 5-Lox | ACTAAGTTTGCTGCTTCGG | CTGACTCCAGACCTCGTG | NM_0011398321 | 115% |
| Catalase | CCAGATGTGGGCCGCTAACAA | TCTGGCGCTCCTCCTCATTC | Est04a09 | 107% |
| SOD | GTTTCTCTCCAGCCTGCTCTAAG | CCGCTCTCCTTGTCGAAGC | DY718412 | 107% |
| Hsp27 | CCAGCTGCCTGAGGATGTG | CCTCGGTGCCCAATGATG | CV428908 | 109% |
| GHR | TGGACACCCAGTGCTTGATG | TCCCTGAAGCCAATGGTGAT | AF403539 | 113% |
| LPL | TGCTGGTAGCGGAGAAAGACAT | CTGACCACCAGGAAGACACCAT | BI468076 | 104% |
| IGF-1 | TGACTTCGGCGGCAACA | GCCATAGCCCGTTGGTTTACT | M81904 | 111% |
| TNF-α | GTGTATGTGGGAGCAGTGTT | GAAGCCTGTTCTCTGTGACT | NM_001123617 | 112% |
| MHCI | GCGACAGGTTTCTACCCCAGT | TGTCAGGTGGGAGCTTTTCTG | AF504013–25 | 112% |
| MHCII | GTGGAGCACATCAGCCTCACT | GACGCACCGATGGCTATCTTA | X70165 | 104% |
| IL-1β | GCTGGAGAGTGCTGTGGAAGAAC | CGTAGACAGGTTCAAATGCACTTTGTG | AY617117 | 127% |
| IL4-13a | CCACCACAAAATGCAAGGAGTTCT | CCTGGTTGTCTTGGCTCTTCAC | NM_001204895 | 103% |
| PGE₂ (EP4) | CTGATTATGATGCACAAGCGGTTCA | GTTTACAAAAATCCGCAGCACCAAAG | Scottish fish immunology center, unpublished | 101% |
| IFNγ | GATGGGCTGGATGACTTTAGGATG | CCTCCGCTCACTGTCCTCAAA | AY795563 | 103% |
| Brain | | | | |
| **S20** | GCAGACCTTATCCGTGGAGCTA | TGGTGATGCGCAGAGTCTTG | NM_001140843.1 | 93.7% |
| BDNF | ATGTCTGGGCAGACCGTTAC | GTTGTCCTGCATTGGGAGTT | GU108576.1 | 95.0% |
| 5-HT1Aα | ATGCTGGTCCTCTACGGGCG | CGTGGTTCACCGCGCCGTTT | AGKD01067361.1 : 7182–7844 | 104.2% |

ARP – acidic ribosomal protein; EF1ab – elongation factor 1ab; 5-Lox – 5-lipoxygenase; SOD – superoxide dismutase; Hsp27 – heat shock protein 27; GHR – growth hormone receptor; LPL – lipoprotein lipase; IGF-1 – insulin-like growth factor 1; TNF-α – tumor necrosis factor α; MHCI – major histocompatibility complex; IL-1β – interleukin 1β; IL4-13a – interleukin 4-13a; PGE₂ EP4 – prostaglandin E₂ (EP4); IFNγ – interferon γ; BDNF – brain derived neurotrophic factor; 5-HT1Aα – serotonin 1Aα receptor.

The mobile phase delivered at 0.4 mL/min in gradient mode consisted of ultra-pure water with 0.1% formic acid (solution A) and an equal volume mixture of acetonitrile and methanol with 0.1% formic acid (solution B). The solvent gradient was as follows: solution A was reduced from 60 to 5% from 0.00 to 4.00 min, kept at 5% between 4.00 and 5.50 min, increased to 60% between 5.50 and 5.51 min and kept at 60% between 5.51 and 10.00 min. Mass spectrometric detection was performed by multiple reactions monitoring (MRM) in negative mode. The monitored transitions in ion counts per second (icps) were: $m/z$ 351 → 333, 315, 271 for $PGE_2$ and $PGD_2$; $m/z$ 349 → 331, 313, 269 for $PGE_3$; $m/z$ 355 → 337, 319, 275 for $PGE_2$-$d_4$; $m/z$ 335 → 317, 195, 129 for $LTB_4$; $m/z$ 333 → 315, 271, 195, 129, 59 for $LTB_5$; and $m/z$ 339 → 321, 197, 130 for $LTB_4$-$d_4$. The ESI parameters were gas temperature (120 °C), gas flow rate (19 L/min), nebulizer pressure (20 psi), sheath gas temperature (300 °C), sheath gas flow (10 L/min), capillary voltage (3500 V) and nozzle voltage (2000V). The integration of the chromatograms was performed using the MassHunter Qualitative Navigator software (version 8.0). The levels of eicosanoids were estimated by means of the internal standards ($PGE_2$-$d_4$ and $LTB_4$-$d_4$) and expressed as pg eicosanoid/g liver.

### 2.7. Analysis of brain serotonergic neurochemistry

Serotonergic activity was analysed by NIVA. The frozen brain samples (telencephalon and hypothalamus) were homogenised in 4% ($w/v$) ice-cold perchloric acid containing 0.2% EDTA and 94.2 ng/mL of 3,4-dihydroxybenzyl amine hydrobromide deoxyepinephrine (the internal standard), using an MSE 100 W ultrasonic disintegrator (Henderson Biomedical, United Kingdom). Prior to analysis, the samples were thawed on ice, and centrifuged at 17,000 rpm for 5 min. The supernatant was then removed and 5-HT, and its principal catabolite 5-hydroxtrindolaceticacid (5-HIAA) were quantified using high-performance liquid chromatography (HPLC) with electrochemical detection. Generally, the 5-HIAA/5-HT ratio is used as a reliable proxy for determining monoamine activity/signalling (Höglund et al., 2019). In the present study, the aforementioned ratio was used for quantifying 5-HT activity.

The HPLC system consisted of a solvent-delivery system (Shimadzu, LC-10 CE), equipped with an auto injector (Famos, Spark), a reverse phase column (4.6 × 100 mm, Hichrom, C18, 3.5 µm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes at −40 and + 320 mV. A conditioning electrode (ESA 5020) with a potential of +400 mV was employed before the analytical electrodes, to oxidize possible contaminants. The mobile phase consisted of 86.25 mM/L sodium phosphate, 1.4 mM/L sodium octyl sulfate and 12.26 µM/L EDTA in deionized (resistance 18.2 MW) water containing 7% ACN brought to a pH of 3.1 with phosphoric acid. The samples were quantified by comparison with standard solutions of known concentrations and corrected for recovery of the internal standard using HPLC software (CSW, DataApex Ltd., Czech Republic).

### 2.8. Statistics

Statistical analyses were performed using the free software environment R (R Development Core Team, 2011). Differences between the groups for performance data, feed intake, FA composition and lipid class were analysed using two-way ANOVA with diet and repeated stress as predictors (only sampled prior to acute stress). Plasma markers, eicosanoids in liver and gene expression were analysed for differences between groups using a three-way ANOVA, with diet, repeated stress and acute stress as predictors. When tank effects were found to be present, nested ANOVA was used. Three-way interactions were further investigated by maintaining one predictor constant and analysing the remaining response variable. Tukey's HSD post hoc test was used when significant effects were found. Homogeneity of variances and normality were checked using Levene's test and Shapiro Wilk's test, respectively. Graphical evaluation was also performed with residuals vs fitted plot for

homogeneity of variances and QQ-plot for normality. A $p$-value of <0.05 was considered statistically significant. Results are expressed as mean and standard deviation (mean ± SD).

## 3. Results

### 3.1. Performance summary

Only diet effects (no effects of repeated stress) were found on growth parameters. The highest final weight and length was recorded in fish fed diet 1H, and it was significantly higher than in fish given diet 2 and 6. No effects were found on specific growth rate, feed conversion ratio, condition factor, viscerasomatic index or hepatosomatic index (Table 3). Repeatedly stressed fish had a significantly higher feed conversion ratio than control fish (0.82 vs 0.78, respectively, $p$ = 0.024).

### 3.2. Feed intake

The first two weeks (acclimation period), the fish given diet 2 had a significantly lower feed intake than fish fed diets 1 and 1H. After the first hypoxia stress test (feed intake and all hypoxia exposures are marked in Fig. 1) feed intake was reduced significantly for the exposed fish. However, unstressed control fish fed diet 6 ate as little as fish exposed to the repeated stressor. The next two weeks the control fish ate more than those exposed to hypoxia (except fish fed diet 6). However, after this initial 2-week period no differences in the feed intake was found. At the end of the trial, fish fed diet 1H had eaten significantly more than fish fed diet 2 and 6.

### 3.3. The highest dietary n-6/n-3 ratio caused higher liver TAG

Only diet effects were found on the liver lipid class composition. No differences were found in the polar lipid classes. However, there were significant differences in the total neutral lipids. TAG was the cause of these differences, with fish given diet 6 having significantly higher liver TAG than fish given diet 2 and 1H (Table 4).

### 3.4. The FA composition of the red blood cells was more influenced by n-6/n-3 FA ratio than their dietary absolute levels. EPA was significantly reduced by higher dietary n-6/n-3 FA ratio

All n-3 FA analysed, except DHA, decreased significantly with higher dietary n-6/n-3 FA ratio despite similar dietary n-3 FA (diet 1, 2 and 6,

**Table 3**
Performance summary of Atlantic salmon fed diets with varying dietary n-6/n-3 ratios and absolute levels of n-6 and n-3 FA. All data grouped by diet, but not repeated stress. Weight, length and CF were measured on all fish ($n$ = 150 per diet). HSI and VSI were measured on 20 fish per tank ($n$ = 120 per diet). Different superscript letters denote significant statistical difference ($p$ < 0.05 two-way ANOVA with Tukey HSD post hoc). Numbers are mean with standard deviation.

|  | Diet 1 | Diet 2 | Diet 6 | Diet 1H |
|---|---|---|---|---|
| Final weight (g) | 399.3 ± 52.9 [ab] | 383.7 ± 53.6 [b] | 385.2 ± 54.8 [b] | 407.0 ± 56.3 [a] |
| Final length (cm) | 30.7 ± 1.6 [ab] | 30.3 ± 1.4 [b] | 30.3 ± 1.6 [b] | 30.9 ± 1.5 [a] |
| SGR, % day $^{-1}$ | 1.0 ± 0.0 | 0.9 ± 0.1 | 0.9 ± 0.1 | 1.0 ± 0.1 |
| FCR | 0.8 ± 0.0 | 0.8 ± 0.1 | 0.8 ± 0.1 | 0.8 ± 0.1 |
| CF | 1.4 ± 0.1 | 1.4 ± 0.1 | 1.4 ± 0.1 | 1.4 ± 0.1 |
| VSI, % | 12.0 ± 2.3 | 12.1 ± 1.8 | 12.2 ± 2.6 | 11.6 ± 1.8 |
| HSI, % | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.2 |

Diet codes are given by dietary n-6/n-3 ratio. Diet 1H has a double absolute n-6 and n-3 FA content compared to diet 1. SGR, FCR, CF, VSI and HSI were calculated according to standard formulae. SGR – specific growth rate; FCR – feed conversion ratio; CF – condition factor; VSI – viscerasomatic index; HSI – hepatosomatic index.
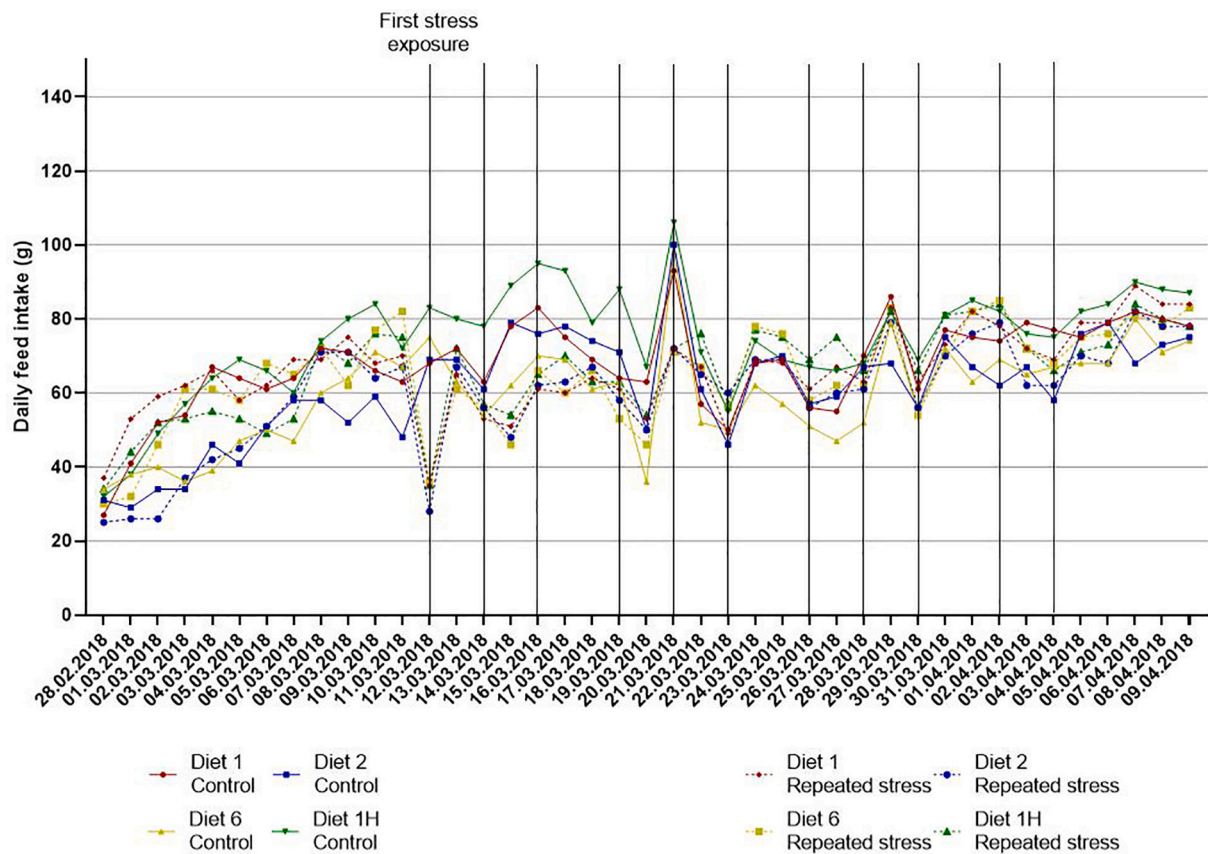
**Fig. 1.** Daily feed intake (g) of Atlantic salmon fed diets with varying dietary n-6/n-3 FA ratios and absolute levels of n-6 and n-3 FA. The fish were either exposed to a repeated stressor (hypoxia 3 times per week, 4 weeks, marked with black lines) or left undisturbed. Diet codes are given by dietary n-6/n-3 ratio. Diet 1H has a double absolute n-6 and n-3 content compared to diet 1.

**Table 4**

Liver lipid classes (mg/g) of Atlantic salmon fed diets with varying dietary n-6/n-3 ratios and absolute levels of n-6 and n-3 FA. Data grouped by diet, and pooled for repeated stress and unstressed controls. Pooled samples of five fish per tank were used ($n = 6$ tanks per diet). Different superscript letters denote significant statistical difference ($p < 0.05$, two-way ANOVA with Tukey HSD post hoc). Numbers are mean with standard deviation.

|  | Diet 1 | Diet 2 | Diet 6 | Diet 1H |
|---|---|---|---|---|
| LPC | 0.05 ± 0.12 | ND | ND | ND |
| SM | 2.38 ± 0.56 | 2.10 ± 0.70 | 2.58 ± 0.44 | 2.05 ± 0.29 |
| PC | 26.63 ± 1.65 | 26.93 ± 2.20 | 28.47 ± 0.99 | 27.17 ± 0.75 |
| PS | 3.02 ± 0.44 | 2.87 ± 0.76 | 3.12 ± 0.51 | 2.70 ± 0.32 |
| PI | 2.70 ± 0.27 | 2.55 ± 0.23 | 2.60 ± 0.55 | 3.10 ± 0.40 |
| CL | 0.90 ± 0.15 | 0.78 ± 0.26 | 0.92 ± 0.15 | 0.75 ± 0.10 |
| PE | 6.88 ± 0.92 | 6.98 ± 0.82 | 6.28 ± 0.81 | 6.52 ± 1.19 |
| **Sum polar** | **42.57 ± 3.04** | **42.23 ± 4.26** | **43.93 ± 2.47** | **42.33 ± 1.77** |
| DAG | 0.25 ± 0.14 | 0.15 ± 0.08 | 0.27 ± 0.16 | 0.15 ± 0.05 |
| CHOL | 3.37 ± 0.23 | 3.17 ± 0.47 | 3.58 ± 0.19 | 3.30 ± 0.43 |
| FFA | ND | ND | ND | ND |
| TAG | 8.40 ± 3.44 [ab] | 7.62 ± 2.03 [b] | 13.53 ± 5.08 [a] | 6.07 ± 0.85 [b] |
| CE | ND | ND | ND | ND |
| **Sum neutral** | **12.03 ± 3.66** [ab] | **10.87 ± 2.27** [b] | **17.37 ± 5.28** [a] | **9.53 ± 0.90** [b] |
| **Sum lipids** | **54.57 ± 6.00** | **53.10 ± 6.37** | **61.30 ± 7.49** | **51.82 ± 1.91** |

Diet codes are given by dietary n-6/n-3 FA ratio. Diet 1H has a double absolute n-6 and n-3 content compared to diet 1; ND - not detected; LPC - lyso-phoshocholine; SM - sphingomyelin; PC - phosphatidylcholine; PS - phosphatidylserine; PI - phosphatidylinositol; CL - cardiolipin; PE - phosphatidylethanolamine; DAG - diacylglycerol; CHOL - cholesterol; FFA - free fatty acid; TAG - triacylglycerol; CE - cholesteryl ester.

Supplementry Table 1). A higher dietary n-3 content caused a higher n-3 level, even though the dietary n-6 FA was increased simultaneously (diet 1H). Every n-6 FA analysed reflected the dietary content, with n-6/n-3 FA ratio being the main decisive factor (Supplementry Table 1). A higher absolute content of dietary n-6 did not lead to a higher n-6 FA content in the RBC provided n-6/n-3 was kept low (diet 1H vs diet 2).

*3.5. Stress markers in the blood clearly indicated an effect of the acute stressor*

HCT, plasma chloride, glucose and cortisol all increased significantly 1 h after the acute stressor, and were significantly reduced after 24 h in all dietary groups. While HCT and plasma cortisol levels were back to baseline levels after 24 h, plasma glucose and chloride had still not

**Table 5**

Stress markers in blood of Atlantic salmon fed diets with varying dietary n-6/n-3 FA ratios and absolute levels of n-6 and n-3 FA, and exposed to repeated stress/control and acute stressors. Data are grouped by acute stress in this table, not diet and repeated stress, as no significant effects were seen for these variables. Pooled samples of five fish per tank were used for plasma chloride and glucose ($n = 24$ per time point). HCT was measured on 5 fish per tank ($n = 120$ per time point). Different superscript letters denote significant statistical difference ($p < 0.05$, three-way ANOVA with Tukey HSD post hoc). Numbers are mean with standard deviation.

|  | Before | 1 h | 24 h |
|---|---|---|---|
| HCT | 39.10 ± 2.47 [a] | 40.70 ± 2.77 [b] | 39.25 ± 2.44 [a] |
| Plasma chloride (mmol/L) | 146.07 ± 10.40 [a] | 165.63 ± 4.11 [c] | 156.33 ± 2.34 [b] |
| Plasma glucose (mmol/L) | 4.74 ± 0.50 [a] | 7.73 ± 0.57 [c] | 5.15 ± 0.29 [b] |

recovered completely (Table 5).

Although clear effects of the acute stressors were seen on all the stress markers, neither dietary nor repeated stress effects were seen on HCT, plasma chloride or glucose (supplementary Table 2).

Cortisol, however, exhibited a three-way interaction between diet, repeated and acute stress ($p = 0.007$). Further analyses revealed a two-way interaction between diet and repeated stress 1 h after exposure to the acute stressor ($p = 0.038$). The fish not exposed to repeated stress had similar cortisol response regardless of diet, while repeatedly stressed fish given diet 6 had suppressed cortisol response compared to fish given diet 1 ($p = 0.027$, Fig. 2).

### 3.6. Eicosanoids in the liver and their response to acute stress were affected by diet

#### 3.6.1. Prostaglandins

Hepatic levels of the ARA derived eicosanoid $PGD_2$ increased with higher dietary n-6 FA. $PGD_2$ also responded clearly to acute stress, being significantly reduced 1 h after the acute stressor for diet 1, with a similar trend for diet 6. However, a diet × acute stress interaction was also found. While fish given diet 1 had started recovering towards basal levels after 24 h, the liver $PGD_2$ of fish given diet 6 was still declining. Furthermore, fish given diet 6 had higher pre-acute stress levels than fish given diet 1 ($p = 0.005$), and was also higher than fish fed diet 1 after 1 h ($p = 0.002$)(Fig. 3a).

The ARA derived eicosanoid $PGE_2$ was significantly higher in liver of fish fed diet 6 compared to fish fed diet 1 (Fig. 3b). Analysing each time point separately, fish given diet 6 had significantly higher levels both before the acute stressor ($p = 0.005$) and 1 h after ($p \leq 0.001$) than fish given diet 1.

The EPA derived eicosanoid $PGE_3$ in liver was stable regardless which variable was looked at; neither diet, repeated or acute stress had an effect, nor was any interaction found Fig. 3c.

#### 3.6.2. Leukotrienes

Content of the ARA metabolite $LTB_4$ in liver showed a significant interaction between diet and acute stress (Fig. 3d). The two dietary groups responded oppositely to the acute stressor. $LTB_4$ in the liver of fish given diet 1 decreased significantly 1 h after stress and increased again after 24 h. For fish given diet 6, the levels $LTB_4$ had increased significantly 1 h after acute stress. After 24 h, $LTB_4$ had started decreasing again. Interestingly, fish given diet 1 had significantly higher

levels of $LTB_4$ prior to the acute stressor ($p = 0.036$), despite lower dietary contents of n-6 FA. Contrarily, 1 h after stress fish given diet 6 had significantly higher $LTB_4$ levels than fish given diet 1 ($p = 0.006$) (Fig. 3d). It is also worth noting that, of the analysed eicosanoids, $LTB_4$ was the eicosanoid with the highest concentration prior to the acute stressor.

The EPA derived metabolite $LTB_5$ showed a marked response to acute stress (Fig. 3e). Its levels increased significantly 1 h after stress and were significantly reduced again after 24 h, although they had not returned to basal levels. No other variables showed any effect.

### 3.7. Gene expression in liver and brain was little affected

In liver, catalase, 5-lipoxygenase, lipoprotein lipase (LPL), growth hormone receptor, heat shock protein 27, interferon γ, interleukin 4-13a, insulin-like growth factor 1 (IGF-1), superoxide dismutase and tumor necrosis factor α were all significantly affected by acute stress. Most were not back to starting levels 24 h after acute stress. LPL had a significant three-way interaction effect, caused by a significantly higher transcription after 24 h in control fish (not repeatedly stressed) given diet 1. IGF-1 was significantly lower in fish given diet 6, and the diet-×acute stress interaction had a $p$-value of 0.078. Fish fed diet 6 had a weak reduction in the expression of IGF-1 after acute stress and fish fed diet 1 had a significant reduction after acute stress. Interleukin 1β, major histocompatibility complex I and II and $PGE_2$ EP4 receptor did not exhibit any significant effects of the variables in the trial. Gene expression of brain derived neurotrophic factor and 5-HT 1A (serotonin receptor) was not affected by diet, acute stress or repeated stress (gene expression results reported in Supplemetnary Table 2).

### 3.8. Brain fatty acid composition

In the brain PL fraction, all measured n-6 FAs were significantly higher in fish given diet 6 compared to fish fed the other three diets (Table 6). The n-6 FA also reflected dietary n-6/n-3 FA ratio rather than absolute contents (meaning samples from diet group 1H were similar to diet group 1 rather than 2). Brain PL content of LA was greatly reduced as compared to diets. Mostly, the n-3 FA reflected dietary differences with fish fed diet 1H having higher levels. However, despite similar dietary content, EPA was significantly reduced by the highest dietary n-6/n-3 FA ratio. DHA remained stable regardless of dietary n-6/n-3 FA ratio or dietary n-3 FA content. Interestingly, 18:1n-9 was also
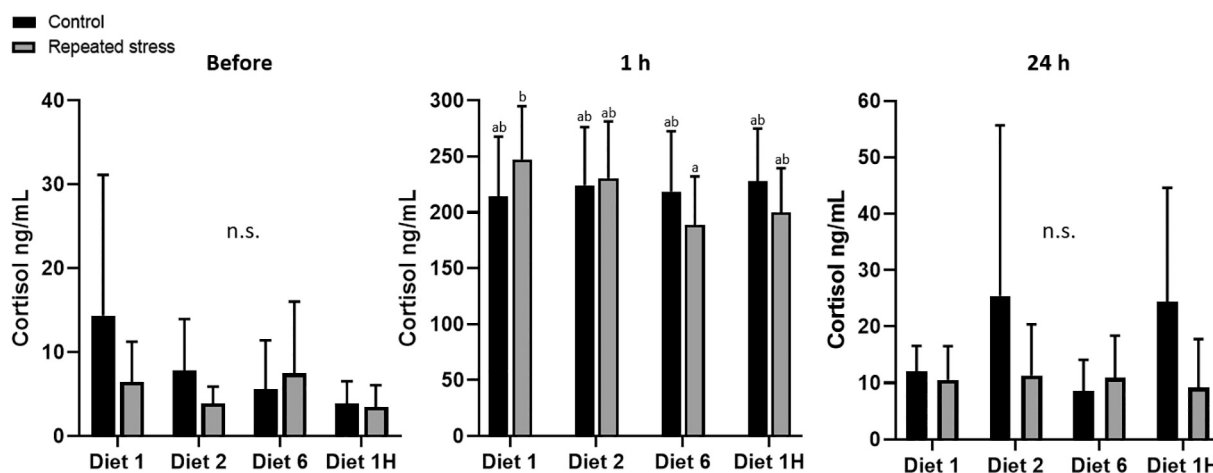


**Fig. 2.** Plasma cortisol (ng/mL) of Atlantic salmon before, 1 h after and 24 h after an acute stressor ($n = 15$ per diet/repeated stress/acute stress, hence per bar in figure). The fish had been fed diets with varying dietary n-6/n-3 ratios and absolute levels of n-6 and n-3 FA. The fish were either exposed to a repeated stressor (hypoxia 3 times per week, 4 weeks) or left undisturbed. Different letters denote significant statistical difference (p < 0.05, three-way ANOVA with Tukey HSD post hoc). Numbers are mean with bars representing standard deviation. Diet codes are given by dietary n-6/n-3 ratio. Diet 1H has a double absolute n-6 and n-3 FA content compared to diet 1.
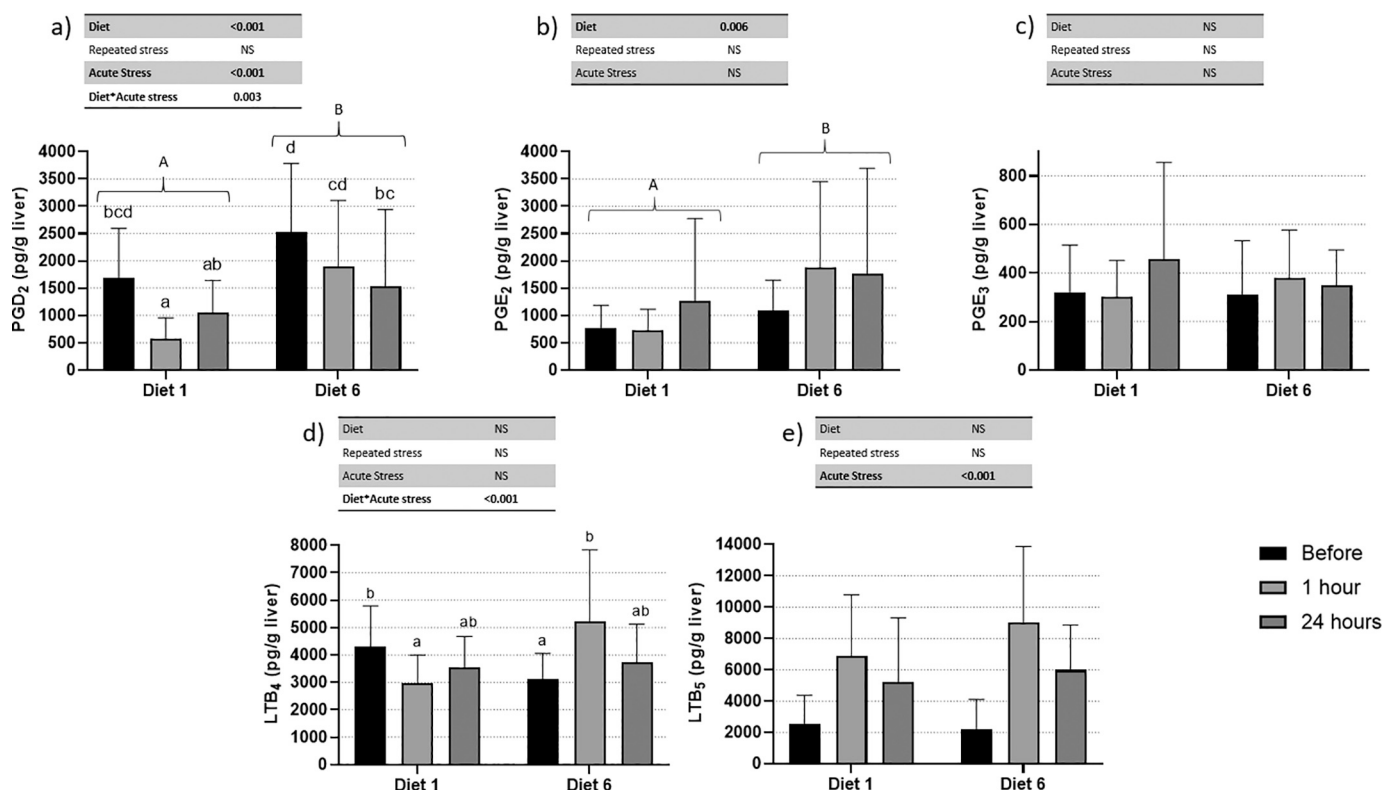
**Fig. 3.** Level of prostaglandins (PGD2, PGE2, PGE3, a, b and c respectively) and leukotrienes (LTB4, LTB5, d and e, respectively) in liver (pg/g) of Atlantic salmon fed varying dietary n-6/n-3 FA ratios and total n-6 and n-3 FA, and exposed to repeated and/or acute stressors. Repeatedly stressed fish and controls are pooled (per diet and time after acute stressor) in the plots. Five fish from each tank were sampled before stress (n = 30), three fish per tank after 1 and 24 h (n = 18). Numbers are mean and bars standard deviation. Different capital letters denote significant main diet effect, small letters are the result of post hoc after significant interaction (three-way ANOVA with Tukey HSD post hoc). Interaction effects only shown when significant. PGD2: prostaglandin $D_2$, PGE2: prostaglandin $E_2$, PGE3: prostaglandin $E_3$, LTB4: leukotriene $B_4$, LTB5: leukotriene $B_5$, diet 1 and diet 6: diet names by dietary n-6/n-3 FA ratio.

remarkably stable (~20.5%) despite large dietary variability. The lower n-6 and higher n-3 FA in brain PL compared to the diets, resulted in brain PL n-6/n-3 FA ratios being much lower than the diets (range 0.1–0.2), although they did reflect dietary differences.

In the brain NL fraction, sum n-6 FA reflected the absolute level of LA in the feed, as did LA, 20:2n-6 and ARA (Table 6). For these FAs, the tissue levels reflected dietary n-6 FA content rather than the n-6/n-3 FA ratio, as diet group 1H was more similar to diet group 2 than 1. The main difference found in the various n-3 FA was that there was more in the group fed a higher n-3 level. The NL n-6/n-3 FA ratio (range 1.0–3.5) was reduced compared to the diets, but much higher than in the brain PL.

### 3.9. Levels of brain serotonin and of its metabolite were only affected by acute stress

The different diets had no effects on the response in 5-HT, 5-HIAA or 5-HIAA/5-HT to either acute or repetitive stress, nor were any effects of the repetitive stress itself seen. However, the brain 5-HT was significantly decreased and 5-HIAA and 5-HIAA/5-HT significantly increased in response to acute stress (Table 7).

### 4. Discussion

As we exchange the FO in Atlantic salmon feed for VO, we particularly reduce the content of the essential LC-PUFAs EPA and DHA and increase the content of LA. We hypothesized that dietary FA composition, particularly the n-6/n-3 FA ratio, would affect fish stress response and change its robustness. Hence, the fish in the current trial were exposed to both repeated and acute stress to reveal potential effects of

dietary FAs during challenging conditions. The results showed that the fish seemed to adapt to the repeated hypoxia stressor without major effects on health, while the acute stressor induced a clear stress response in all diet groups. The eicosanoid levels in the liver after acute stress were dependent on the dietary n-6/n-3 FA ratio, indicating an altered acute stress response.

The repeated stress in this trial was induced by combined hypoxia and hypercapnia, where the inlet water was shut until oxygen saturation had reached 35%. After the first hypoxia stress, a significant decrease in feed intake was seen for all exposed tanks, as would be expected for stressed fish (reviewed in Conde-Sieira et al., 2018). However, the fact that feed intake then normalised for the repeatedly stressed tanks, suggests an adaptation to this stressor for all dietary groups. This is also reflected in the baseline cortisol levels (before acute stress), which were generally low and did not differ between treatment groups. In line with our results, Remen et al. (2012) repeatedly exposed Atlantic salmon to hypoxia, but only the first exposure led to elevated plasma cortisol. Furthermore, that we could not detect any differences in final weights between the repeatedly stressed fish and the controls lends support to adaptive responses to the repeated hypoxia stress in the present study. The highest final weight in the diet group with more n-3 FAs (coinciding with differences in feed intake) is in line with previously published results in a growth trial using the same feeds as the current trial (Hundal et al., 2020).

Fish in all diet groups, as well as both the repeatedly stressed and the control fish displayed a pronounced response to acute stress, as evidenced by both gene expression in liver, liver eicosanoids, brain monoamines, plasma glucose, chloride and cortisol. The fact that both repeatedly stressed and control fish were able to mount a cortisol response to the acute stressor further supports that the fish had adapted

**Table 6**

Selected FA (% of TFA) in the brain PL and NL of Atlantic salmon fed varying dietary n-6/n-3 FA ratios and absolute levels of n-6 and n-3 FA and exposed to a repeated stressor or not. Data are pooled over repeatedly stressed and control fish. Pooled samples of five fish per tank ($n = 6$ tanks per diet). Different superscript letters denote significant statistical difference ($p < 0.05$, two-way ANOVA with Tukey HSD post hoc). Numbers are mean with standard deviation.

| | Diet 1 | Diet 2 | Diet 6 | Diet 1H |
|---|---|---|---|---|
| Polar lipids | | | | |
| ΣSFA | 23.9 ± 0.5 | 24.2 ± 0.7 | 24.3 ± 0.6 | 24.0 ± 0.8 |
| ΣMUFA | 31.7 ± 1.0 | 31.3 ± 0.9 | 30.6 ± 1.6 | 31.8 ± 1.7 |
| 18:1n-9 | 20.5 ± 0.7 | 20.4 ± 0.7 | 20.2 ± 0.9 | 20.4 ± 1.2 |
| Σn-6 | 3.2 ± 0.3 [a] | 3.7 ± 0.3 [a] | 6.4 ± 0.6 [b] | 3.2 ± 0.1 [a] |
| 18:2n-6 (LA) | 0.8 ± 0.1 [a] | 1.1 ± 0.2 [a] | 2.5 ± 0.4 [b] | 0.9 ± 0.1 [a] |
| 20:2n.6 | 0.2 ± 0.0 [a] | 0.3 ± 0.0 [a] | 0.6 ± 0.1 [b] | 0.2 ± 0.0 [a] |
| 20:3n-6 | 0.4 ± 0.0 [b] | 0.5 ± 0.1 [c] | 0.8 ± 0.1 [d] | 0.3 ± 0.0 [a] |
| 20:4n-6 (ARA) | 1.2 ± 0.1 [a] | 1.4 ± 0.1 [b] | 1.9 ± 0.1 [c] | 1.3 ± 0.0 [ab] |
| Σn-3 | 34.0 ± 0.7 [ab] | 34.3 ± 0.7 [ab] | 33.0 ± 0.7 [a] | 34.8 ± 1.5 [b] |
| 18:3n-3 | 0.1 ± 0.0 [a] | 0.1 ± 0.0 [a] | 0.1 ± 0.0 [a] | 0.2 ± 0.0 [b] |
| 20:5n-3 (EPA) | 5.2 ± 0.2 [b] | 5.2 ± 0.1 [b] | 4.7 ± 0.1 [a] | 5.6 ± 0.2 [c] |
| 22:5n-3 | 2.0 ± 0.1 | 2.0 ± 0.1 | 1.6 ± 0.1 | 2.1 ± 0.1 |
| 22:6n-3 (DHA) | 26.0 ± 0.8 | 26.3 ± 1.2 | 25.9 ± 0.7 | 26.2 ± 1.5 |
| FA ratios | | | | |
| n-6/n-3 | 0.1 ± 0.0 [a] | 0.1 ± 0.0 [a] | 0.2 ± 0.0 [b] | 0.1 ± 0.0 [a] |
| ARA/EPA | 0.2 ± 0.0 [ab] | 0.3 ± 0.0 [b] | 0.4 ± 0.0 [c] | 0.2 ± 0.0 [a] |
| **Sum FA (mg/g)** | 31.5 ± 2.5 | 30.2 ± 1.7 | 30.2 ± 5.4 | 31.7 ± 1.2 |
| Neutral lipids | | | | |
| ΣSFA | 20.2 ± 0.5 [c] | 19.6 ± 0.5 [c] | 18.2 ± 0.4 [a] | 21.1 ± 0.4 [b] |
| ΣMUFA | 55.5 ± 0. 9 [d] | 52.3 ± 0.7 [c] | 32.7 ± 0.5 [a] | 44.1 ± 0.4 [b] |
| 18:1n-9 | 35.0 ± 0.9 [c] | 31.2 ± 0.7 [b] | 23.8 ± 1.3 [a] | 22.5 ± 0.3 [a] |
| Σn-6 | 11.5 ± 0.4 [a] | 15.7 ± 1.5 [b] | 37.6 ± 1.9 [c] | 17.0 ± 0.4 [b] |
| 18:2n-6 (LA) | 9.5 ± 0.4 [a] | 13.2 ± 1.3 [b] | 32.8 ± 1.8 [c] | 14.7 ± 0.4 [b] |
| 18:3n-6 | 0.3 ± 0.0 [a] | 0.4 ± 0.1 [b] | 0.9 ± 0.1 [c] | 0.3 ± 0.0 [a] |
| 20:2n.6 | 0.6 ± 0.0 [a] | 0.9 ± 0.1 [b] | 1.7 ± 0.1 [c] | 0.9 ± 0.0 [b] |
| 20:3n-6 | 0.5 ± 0.0 [a] | 0.6 ± 0.1 [b] | 1.3 ± 0.0 [c] | 0.4 ± 0.0 [a] |
| 20:4n-6 (ARA) | 0.6 ± 0.1 [a] | 0.6 ± 0.1 [a] | 0.8 ± 0.1 [b] | 0.7 ± 0.0 [b] |
| Σn-3 | 11.4 ± 0.6 [a] | 11.2 ± 0.7 [a] | 10.7 ± 0.5 [a] | 16.6 ± 0.3 [b] |
| 18:3n-3 | 2.0 ± 0.1 [a] | 2.1 ± 0.1 [a] | 2.1 ± 0.0 [a] | 4.0 ± 0.1 [b] |
| 18:4n-3 | 0.71 ± 0.03 [ab] | 0.75 ± 0.05 [b] | 0.65 ± 0.07 [a] | 1.00 ± 0.03 [c] |
| 20:5n-3 (EPA) | 2.0 ± 0.2 [a] | 1.8 ± 0.2 [a] | 1.7 ± 0.2 [a] | 3.0 ± 0.1 [b] |
| 21:5n-3 | 0.14 ± 0.02 [b] | 0.14 ± 0.01 [b] | 0.11 ± 0.01 [a] | 0.21 ± 0.03 [c] |
| 22:5n-3 | 0.7 ± 0.0 [b] | 0.6 ± 0.0 [ab] | 0.6 ± 0.0 [a] | 1.0 ± 0.0 [c] |
| 22:6n-3 (DHA) | 5.0 ± 0.4 [a] | 4.9 ± 0.5 [a] | 4.8 ± 0.4 [a] | 6.2 ± 0.3 [b] |
| FA ratios | | | | |
| n-6/n-3 | 1.0 ± 0.1 [a] | 1.4 ± 0.2 [b] | 3.5 ± 0.3 [c] | 1.0 ± 0.0 [a] |
| ARA/EPA | 0.28 ± 0.01 [b] | 0.32 ± 0.01 [c] | 0.46 ± 0.02 [d] | 0.24 ± 0.01 [a] |
| **Sum FA (mg/g)** | 18.4 ± 5.1 | 24.4 ± 11.7 | 31.4 ± 18.1 | 20.3 ± 4.4 |

SFA - saturated fatty acid; MUFA - monounsaturated fatty acid; LA - linoleic acid; ARA - arachidonic acid; EPA - eicosapentaenoic acid; DHA - docosahexaenoic acid; FA - fatty acid; Diet codes are given by dietary n-6/n-3 FA ratio. Diet 1H has a double absolute n-6 and n-3 FA content compared to diet 1.

**Table 7**

Brain content of serotonin (5-HT), its metabolite (5-HIAA) and the ratio between them (measure of serotonergic activity) in Atlantic salmon fed varying dietary n-6/n-3 ratios and total n-6 and n-3 FA, and exposed to repeated and/or acute stressors. Data grouped by acute stress, not diet and repeated stress. Five fish were sampled from each tank before stress and 1 h after, however a few fish were removed from analysis due to error in the lab. Hence, $n = 52$ before acute stress and $n = 54$ 1 h after acute stress. Different letters denote significant differences (three-way ANOVA, using a Tukey HSD post hoc).

| | Before | 1 h |
|---|---|---|
| 5-HT (ng/g) | 284.5 ± 55.1 [a] | 226.8 ± 53.9 [b] |
| 5-HIAA (ng/g) | 88.8 ± 27.2 [a] | 110.7 ± 46.5 [b] |
| 5-HIAA/5-HT | 0.31 ± 0.08 [a] | 0.48 ± 0.10 [b] |

5-HT – serotonin, 5-HIAA – 5-hydroxyindoleacetic acid, 5-HIAA/5-HT – released/produced ratio.

to the repeated stress, as a general down-regulation of the HPI axis and supressed cortisol response would be expected following repeated stress (Moltesen et al., 2016, McKenzie et al., 2012, Barton et al., 2005, Madaro et al., 2015, Höglund et al., 2020, Brodeur et al., 1997, Hontela et al., 1997). However, there was an interaction effect of diet and repeated hypoxia 1 h after exposure to acute stress, showing a slight depression in the cortisol response to acute stress in repeatedly stressed fish fed diet 6 compared to fish fed diet 1. Generally, chronic stress-induced suppression of the HPI axis reactivity has been interpreted as an indication of allostatic overload (Moltesen et al., 2016, Madaro et al., 2015, Höglund et al., 2020). Allostatic overload is a state when the accumulated effect of repeated and/or chronic stress results in the coping mechanism (the stress response) becoming maladaptive. As the hypoxia challenge did not result in blunted cortisol response by itself, our results suggest an additional effect of the dietary FA composition on the HPI reactivity of the fish. However, it must also be noted that the interaction effect seen on plasma cortisol was minor and might also be a random effect. Such an interpretation is supported by the fact that the cortisol in fish fed diet 6 did not differ from fish fed diet 1H, which had the same n-6/n-3 ratio as diet 1. While several previous studies have shown that ARA and EPA can affect cortisol response in fish (Alves Martins et al., 2013, Alves Martins et al., 2011, Jutfelt et al., 2007), no effect of diet by itself was seen in our study.

The brain's 5-HTergic system plays a key role in the integration of behavioural and physiological stress responses in vertebrates (Puglisi-Allegra and Andolina, 2015, Winberg and Nilsson, 1993), and as such is a central mediator of allostatic processes (reviewed by Beauchaine et al., 2011). Specifically, 5-HT modulates the release of glucocorticoids by interacting with the HPA/I axis on the hypothalamic level. Furthermore, n-3 FA deficient feed has been shown to affect central 5-HT signalling in mammals (McNamara et al., 2010), such dietary effects have been associated with inflammatory induced changes in the intermediate precursor of 5-HT tryptophan (TRP) in mammals (for references see review by Höglund et al., 2019). In the present study both 5-HT turnover and cortisol increased in response to acute stress. However, 5-HT turnover did not reflect the interaction effect seen on plasma cortisol 1 h after acute stress. As we were not able to detect dietary effects on inflammatory markers in the liver, other mechanisms than inflammatory induced changes in 5-HT signalling may underlie the combined effect of diet and repeated stress on HPI axis reactivity the present study. Dietary FAs may interact with the HPI axis reactivity on other levels, as mammalian studies show that PGE2 affects ACTH release from the pituitary. While eicosanoids in brain were not analysed in the current study, we found that a higher n-6/n-3 in the diet affected PGE2 in the liver. However, it is important to note that the brain FA composition was relatively little affected by diet compared to other tissues, such as the red blood cells. This is in line with previous studies, showing a highly conserved FA composition of the salmon brain (Sissener et al., 2016b), consequently one might expect less dietary effects on eicosanoids in brain compared to other tissues.

Regarding n-6 FA derived eicosanoids in the liver, this was where we observed both dietary effects in the basal levels and also interaction effects with acute stress, indicating that the dietary FA composition modulated how the fish responded to acute stress. ARA in liver polar lipids increases with higher dietary LA (Sissener et al., 2020, Alves Martins et al., 2012), which probably caused the increase in ARA derived prostaglandins in liver of fish fed higher levels of LA in our study. This is in line with results from different fish species given a higher dietary LA or ARA where they also observed higher levels of ARA derived eicosanoids in various tissues (Sissener et al., 2020, Alves Martins et al., 2012, Li et al., 2012, Bransden et al., 2005, Bell et al., 1998, Bell et al., 1995, Bell et al., 1991b). An increase in the levels of hepatic PGE$_2$ and PGD$_2$ in the cell medium of rat hepatocytes inhibited very low density lipoprotein (VLDL) secretion, causing an accumulation of fat in the cells (Perez et al., 2006, Björnsson et al., 1992). In the current trial, fish given the diet highest in LA had numerically higher

content of liver TAG than fish given diets lower in this FA. Correspondingly, these fish also had a significantly higher production of $PGE_2$ and $PGD_2$ than fish provided with less dietary LA. In several trials, Atlantic salmon fed diets high in LA has been reported to get a fattier liver (Alvheim et al., 2013, Ruyter et al., 2006, Bransden et al., 2003). A higher $PGE_2$ and $PGD_2$ level due to increased dietary LA could be one explanatory factor in the mechanisms behind this.

A major concern when increasing the dietary LA in fish feeds has been the possibility of an increased production of n-6 FA derived eicosanoids. Indeed, in the current trial a higher production of the n-6 FA derived $PGE_2$ and $PGD_2$ was discovered when feeding a diet with a higher n-6/n-3 ratio. The concern over n-6 FA derived eicosanoids is due to the dogma of n-6 FA derived eicosanoids being pro-inflammatory, as opposed to the n-3 FA derived ones being anti-inflammatory. However, as has been pointed out before, the eicosanoid system is highly complex (Araujo et al., 2019, Holen et al., 2015) and it is not so straightforward. $PGE_2$ possesses anti-inflammatory properties and can supress the production of pro-inflammatory cytokines and mediate hepatoprotective properties in immune-mediated liver injury in mammals (Yin et al., 2007). $PGE_2$ will also in fish models supress the expression of the pro-inflammatory cytokine IL-1β, as seen in Atlantic salmon SHK cells (Fast et al., 2005) and cod head kidney cells (Furne et al., 2013). There has been performed very little work of the function of $PGD_2$ in fish, although it has been detected in both Atlantic salmon liver (Sissener et al., 2020) and intestine (Oxley et al., 2010), and in gilthead seabream acidophilic granulocytes (Gómez-Abellán et al., 2015). In Atlantic salmon intestine, $PGD_2$ was found to be reduced after stress (Oxley et al., 2010), which is in line with current results in liver. Gómez-Abellán et al. (2015) demonstrated that $PGD_2$ and its derivatives likely have an important role in the resolution of inflammation in gilthead seabream, and the effects were particularly clear in a pro-inflammatory environment. A higher dietary n-6/n-3 ratio in the current study did cause elevated levels of $PGE_2$ and $PGD_2$, but no differences were seen in expression of either pro- or anti-inflammatory cytokines. Additionally, the cytokine mRNA expression was expressed at very low levels. This corresponds to results from LPS stimulated leukocytes from Atlantic salmon fed diets with large differences in n-6/n-3 ratios where no differences in expression of IL-1β and TNFα were found (Seierstad et al., 2009). Prostaglandins are molecules with many different targets, and it is possible that there were other downstream effects not discovered in the current trial. Although we did not discover any indication of ongoing inflammation in the liver, fish given the lowest dietary n-6/n-3 ratio had started recovering to pre-stress levels of $PGD_2$ after 24 h whereas the fish given the highest n-6/n-3 ratio had not. This indicates an altered stress response caused by the increased dietary n-6/n-3 ratio.

Studies have shown that fish (or cells of fish) given higher dietary n-6 FA have a higher production of $LTB_4$ (Alves Martins et al., 2012, Gjøen et al., 2004, Bell et al., 1996). However, all these trials have in common that some sort of stimuli was applied prior to the eicosanoid measurement. Contrarily, unchallenged Atlantic salmon fed soy oil diets (high in LA) compared to fish fed palm oil or rapeseed oil diets (lower LA) had less LTB4 in the liver (Sissener et al., 2020). These results match those found in the current study, as prior to the acute stressor, more $LTB_4$ was found in fish fed the lower n-6 FA diet while after the acute stressor, the situation was opposite. Although the function of $LTB_4$ in fish is not clear, Holen et al. (2015) suggested that LTB4 is a pro-inflammatory mediator in salmon based on their previous trials (Holen et al., 2014, Holen et al., 2012), where head kidney cells stimulated with LPS and PIC preferably secreted $LTB_4$ over $PGE_2$ and $PGE_3$. Mammalian literature also suggests that leukotrienes, and particularly $LTB_4$, are the main mediators of liver injury (Tolman 2000). Data from the current study suggests that leukotrienes are more highly induced than prostaglandins also in the acute stress response, as leukotriene concentrations after acute stress were higher and responded stronger to stress than the prostaglandins. The fact that the two investigated dietary groups had opposite $LTB_4$ responses to the acute stressor might be related to the ARA/EPA ratio in the liver PL.

In a previously published trial using the same diets (Hundal et al., 2020), feeding diet 1 resulted in an ARA/EPA ratio of 0.4 in liver PL, but feeding diet 6 resulted in an ARA/EPA ratio at 1.9. A higher availability of ARA compared to EPA could have caused this shift in $LTB_4$ production in response to acute stress seen in the current trial. Thus, also for $LTB_4$ there are indications of an altered acute stress response with different dietary n-6/n-3 ratios.

In the current trial, $LTB_5$ was induced to a greater degree than $LTB_4$ by acute stress. There is little information on the function of $LTB_5$ in fish, but the current data indicate that it has an important role in the acute stress response. This needs to be further investigated. $PGE_3$, another EPA derived eicosanoid, showed no responses to either acute or repeated stress, suggesting that $PGE_3$ does not take part in the stress response of Atlantic salmon. It is noteworthy that no effects of diet were seen for $LTB_5$ or $PGE_3$, even though increasing dietary n-6/n-3 FA ratio from 1 to 6 causes a reduction in liver PL EPA content (from 7.6 to 3.3% of TFA) (Hundal et al., 2020), meaning that less EPA was available as a precursor in diet group 6 than in diet group 1.

Dihomo-gamma-linoleic acid (DHGLA, 20:3n-6) is an important eicosanoid substrate for both cyclooxygenase and lipoxygenase enzymes producing eicosanoids such as $PGE_1$, $TxA_1$ and 15-HETrE (Kapoor and Huang, 2006), and thus competes with ARA (and EPA) for these enzymes. In the current study, DHGLA increased with increasing dietary n-6/n-3 ratio in brain PL and RBC, and we have previously demonstrated increases in several other polar lipids (Hundal et al., 2020). However, those data also demonstrate that DHGLA is replacing n-3 FA in PL to a greater degree than other n-6 FA. Hence DHGLA could be a way for the fish to counteract effects of ARA derived eicosanoids, and particularly when there is little EPA present. Indeed, in mammals 15-HETrE (DHGLA derived eicosanoid) has been shown to inhibit the production of $LTB_4$ (Kapoor and Huang, 2006). Unfortunately, we did not analyse eicosanoids produced from DHGLA.

In summary, Atlantic salmon seemed able to adapt to a repeated hypoxia stressor and the response was hardly influenced by the dietary n-6 FA, n-3 FA or their ratio. However, the eicosanoid levels after acute stress in Atlantic salmon liver fed a high n-6/n-3 diet was altered compared to fish fed a low n-6/n-3 diet, indicating an altered acute stress response. Hence, producers of aquaculture feeds should be mindful when increasing the inclusion of VOs rich in n-6 FAs as this can affect the stress response and thus possibly the robustness of the fish.

## Author statement

The data have not been presented in a paper before, except the feed data as the diets were used for several trials. The manuscript has not been submitted for publication elsewhere. All authors have approved of the final manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.736272.

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