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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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 VOIs 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...
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 smolitification 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-interrenal (HPA/I) axis, on several levels. Ganga et al. (2006) demonstrated that ARA and EPA stimulated cortisol release in an in-situ study of gilthead seabream. Effects which are central to the regulation of the HPA/I axis (Winberg and Nilsson, 1993), and thus it is central to stress coping processes in both fish and mammals (Hundal 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, Brænden 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 E2 (PGE2) and prostaglandin D2 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 PGE2 and prostaglandin D2 (PGD2) 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., 2016).

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

Diet composition 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.

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.

| Proximate composition (g/100 g) | Diet 1 | Diet 2 | Diet 6 | Diet 1H |
|--------------------------------|--------|--------|--------|---------|
| 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    |
| 16:1-7                         | 3.8    | 3.7    | 1.5    | 5.1     |
| 18:1-7                         | 2.2    | 2.1    | 1.1    | 2       |
| 18:1-9                         | 37.1   | 30.1   | 23.1   | 18.2    |
| 20:1-9                         | 6.4    | 6.4    | 0.7    | 7       |
| 22:1-11                        | 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) 22:5n-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 (Holland 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 Pharama, 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 spin speed 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 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 at each 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 BF3 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 UFMC0001010501,5 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 eDNA plate. A serial dilution curve with five point sets (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 Mastermix (Roche Applied Sciences, Basel, Switzerland) and using the following temperature program: 5 min denaturation and activation at 95 °C, 45 cycles of 10 s denaturation 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 qPCR primer sequences are marked in bold text.

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\textsubscript{2} (99% purity), prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) (98%), PGE\textsubscript{2}-d\textsubscript{4} (99%), leukotriene B\textsubscript{4} (LTB\textsubscript{4}) (97%), leukotriene B\textsubscript{5} (LTB\textsubscript{5}) (98%), and PGE\textsubscript{2}-d\textsubscript{15} (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 subsequent aliquots of 500 µL of acetonitrile (ACN, containing 30 ng/mL PGE\textsubscript{2}-d\textsubscript{4} and 15 ng/mL LTB\textsubscript{4}-d\textsubscript{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 LC/MS/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 RHD Eclipse Plus C18, 95 Å, 2.1 × 50 mm, 1.8 µm chromatographic column.

### Table 2

| Target gene | Forward | Reverse | GenBank accession number | Efficiency |
|-------------|---------|---------|--------------------------|------------|
| Liver       |         |         |                          |            |
| β-actin     | CCAAGCCCAACAGGGGAAAGAAGGGAACACATG GCTGGCGCTCAGGCGAATGATT | BG932897 | 104%                     |
| ARP         | GAAATACCTCAATGCTGGATGTCCTT GCAGGGCAATGTTGCGAT | AY255639 | 85%                      |
| EF1ab       | TGCCCTCCAGAGTTCGCTACG GAGGACCCAGGATGACG | AF321863 | 113%                     |
| 5-Lox       | ACTAATGCTGCTGGGCGGATCATCAAG GCAGGGCAATGTTGCGAT | NM_001139321 | 115%                   |
| Catalase    | CTGGTCTCCAGGCGCTCGTAAAG GCAGGGCAATGTTGCGAT | DT719412 | 107%                     |
| Hsp27       | CACCCGGCTCAGGCTGTATG GCTGGCGCTCAGGCGAATGATT | CV428988 | 107%                     |
| GHR         | TGGACCCAGCTTGGATGTCCTT GCAGGGCAATGTTGCGAT | AF403599 | 113%                     |
| LPL         | TGCTGTAGGAGGAAAAGACTC GCTGGCGCTCAGGCGAATGATT | BI460876 | 104%                     |
| IGF-1       | TCACTCCGGGACCAAGAAGGCA AGGGAACACATG GCTGGCGCTCAGGCGAATGATT | MB1904 | 111%                     |
| TNF-α       | GTGAGCTGCGACTGCGGATG GAGGACCCAGGATGACG | NM_001123617 | 112%                   |
| MCHC        | GGCGAGGGGGGCTACCCGCAGG GAGGACCCAGGATGACG | AP04012-25 | 112%                     |
| MCHII       | GTGAGCCACTACGCTGGCTACG GAGGACCCAGGATGACG | X70165 | 104%                     |
| IL-1β       | GCTGGGGGACACGGGATG GAGGACCCAGGATGACG | XY617117 | 127%                     |
| IL-4 13a    | CCACCAAGGAGGCCCGAAGGATG GAGGACCCAGGATGACG | NM_001204895 | 100%                   |
| PGE\textsubscript{2} (EP4) | CGTGGAGCTGCG ACTGAGGAGGATG GAGGACCCAGGATGACG | Scottish fish immunology center, unpublished | 101%             |
| IFN-γ       | GATTGGTGGGATGACGGTGGATG GAGGACCCAGGATGACG | AV195503 | 100%                     |
| S20         | CGACAGGCTTCAATGCGGATG GAAGGACACATG GCTGGCGCTCAGGCGAATGATT | NM_001140843 | 93.7%                   |
| BDNF        | ATGTGGGCTTCAATGCGGATG GAGGACACATG GCTGGCGCTCAGGCGAATGATT | GU1085761 | 95.0%                   |
| 5-HT1A       | ATGTGGGCTTCAATGCGGATG GAGGACACATG GCTGGCGCTCAGGCGAATGATT | AOX000103617 | 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; MCHC – major histocompatibility complex; IL-1β – interleukin 1β; IL-4 13a – interleukin 4-13a; PGE\textsubscript{2} – prostaglandin E\textsubscript{2}; IFN-γ – interferon γ; BDNF – brain derived neurotrophic factor; 5-HT1A – serotonin 1A 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 PGE2 and PGD2; m/z 349 → 331, 313, 269 for PGE3; m/z 355 → 337, 319, 275 for PGE2-d4; m/z 335 → 317, 195, 129 for LTB4; m/z 333 → 315, 271, 195, 129, 59 for LTB5; and m/z 339 → 321, 197, 130 for LTB4-d4. 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 (PGE2-d4 and LTB4-d4) 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-hydroxytryptophan were analysed using two-way ANOVA with diet and repeated stress as predictors. When tank effects were found to be present, 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. 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, visceral somatic 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, unstrained 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).
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 (Supplementary 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 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.

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.
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 ($\rho = 0.007$). Further analyses revealed a two-way interaction between diet and repeated stress 1 h after exposure to the acute stressor ($\rho = 0.038$). 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 ($\rho = 0.027$, 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 ($\rho = 0.005$), and was also higher than fish fed diet 1 after 1 h ($\rho = 0.002$) (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 ($\rho = 0.005$) and 1 h after ($\rho < 0.001$) 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 ($\rho = 0.036$), 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 ($\rho = 0.006$) (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 4-13α, 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 1 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 Supplementary 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 given 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
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

| FA        | diet 1 | diet 2 | diet 3 | diet 4 |
|-----------|--------|--------|--------|--------|
| n-6/n-3   | 0.28± 0.04 | 0.30±0.04 | 0.28±0.04 | 0.30±0.04 |
| SFA       | 32.7±1.1 | 32.7±1.1 | 32.7±1.1 | 32.7±1.1 |
| EPA       | 20.5±1.1 | 20.5±1.1 | 20.5±1.1 | 20.5±1.1 |
| ARA       | 20.5±1.1 | 20.5±1.1 | 20.5±1.1 | 20.5±1.1 |
| DHA       | 20.5±1.1 | 20.5±1.1 | 20.5±1.1 | 20.5±1.1 |

Table 7

| FA        | diet 1 | diet 2 | diet 3 | diet 4 |
|-----------|--------|--------|--------|--------|
| n-6/n-3   | 0.28± 0.04 | 0.30±0.04 | 0.28±0.04 | 0.30±0.04 |
| SFA       | 32.7±1.1 | 32.7±1.1 | 32.7±1.1 | 32.7±1.1 |
| EPA       | 20.5±1.1 | 20.5±1.1 | 20.5±1.1 | 20.5±1.1 |
| ARA       | 20.5±1.1 | 20.5±1.1 | 20.5±1.1 | 20.5±1.1 |
| DHA       | 20.5±1.1 | 20.5±1.1 | 20.5±1.1 | 20.5±1.1 |

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 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 Högland 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, as shown in line. However, dietary differences in fish fed diet 1-4 could give 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 PGE2 and PGD2 in the cell medium of rat hepatocytes inhibited very low density lipoprotein (VLDL) secretion, causing an accumulation of fat in the cells (Perecz 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 fatter 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 it has been pointed out before, the eicosanoid system is highly complex (Ararajo 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 suppress the expression of the pro-inflammatory cytokine IL-1\(\beta\), 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-1\(\beta\) and TNF\(\alpha\) 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 LT\(_{B4}\) (Alves Martins et al., 2012, Gjøsen 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 LT\(_{B4}\) in the liver (Sissener et al., 2020). These results match those found in the current study, as prior to the acute stressor, more LT\(_{B4}\) was found in fish fed the lower n-6 FA diet while after the acute stressor, the situation was opposite. Although the function of LT\(_{B4}\) in fish is not clear, Holen et al. (2015) suggested that LT\(_{B4}\) is a pro-inflammatory mediator in salmon based on their previous trials (Holen et al., 2014, Holen et al., 2015). LT\(_{B4}\) was secreted by head kidney cells stimulated with LPS and PPI, whereas secretion of LTB\(_4\) over PGE\(_2\) and PGD\(_2\). Mammalian literature also suggests that leukotrienes, and particularly LT\(_{B4}\), 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 LT\(_{B4}\) 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 LT\(_{B4}\) production in response to acute stress seen in the current trial. Thus, also for LT\(_{B4}\) there are indications of an altered acute stress response with different dietary n-6/n-3 ratios.

In the current trial, LT\(_{B4}\) was induced to a greater degree than LT\(_{B4}\) by acute stress. There is little information on the function of LT\(_{B4}\) 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 LT\(_{B4}\) 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\(_3\), TXA\(_3\), and 15-HETET (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-HETET (DHGLA derived eicosanoid) has been shown to inhibit the production of LT\(_{B4}\) (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 VO\(_{s}\) 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

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