Optimisation of dietary n-3 and n-6 fatty acids for a robust Atlantic salmon (*Salmo salar*)

Bjørg Kristine Hundal

Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

This PhD was completed at the Institute of Marine Research in Bergen, Norway. During these years, I have been part of the research programme Feed and Nutrition. My PhD thesis has been supervised on a daily basis by my main supervisor Dr. Nini H. Sissener and co-supervisor Dr. Nina S. Liland. Prof. Erik-Jan Lock and Prof. Rune Waagbø have also been co-supervisors on my PhD thesis. My work has been a part of the OptiHealth project (FHF grant no. 901282) led by Bente Ruyter (Nofima). The project group at the IMR consisted of Dr. Nini H. Sissener, Dr. Nina S. Liland, PhD student Chandrasekar Selvam and myself. Pedro Araujo at IMR, Grethe Rosenlund and Ingunn Stubhaug at Skretting ARC, Marta Bou and Esmail Lutfi at Nofima, Trygve Sigholt at Biomar, Erik Höglund at NIVA and Brett Glencross at Institute of Aquaculture at the University of Stirling (currently working at IFFO – the marine ingredients organisation) have all been involved in the research project.

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- Bjørg Kristine -

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Summary

An optimal diet for Atlantic salmon (*Salmo salar)* should promote a healthy fish that is robust to changes in its environmental conditions and can withstand the handling it will encounter under farming conditions, all while promoting good and rapid growth. The plant ingredients commonly used in aquafeeds do not have an ideal FA composition for salmon. In particular, they are lacking the n-3 fatty acids (FA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are essential nutrients for salmon. Further, they are rich in the n-s6 FA linoleic acid (LA) and the n-9 FA oleic acid (OA), which are not common in the natural diet of salmon. The exact requirement of EPA and DHA for Atlantic salmon is, however, still not known. There are also indications that a higher inclusion of dietary n-6 FA can increase the requirement for EPA and DHA. Many previous trials investigating these nutrients have been short-term, land-based trials where the fish have been shielded from difficult situations. Though such trials can define minimum requirements, the practical requirements need to be determined in a challenging environment. The focus of this thesis has been to investigate the effect of dietary EPA, DHA and n-6 FA on the robustness of Atlantic salmon exposed to challenging environmental conditions.

It has been suspected that dietary n-6 can affect the requirement of EPA and DHA, and that it may have an effect on the response to chronic and acute stress. To investigate this, a feeding experiment was conducted with three diets containing equal absolute amounts of n-3 FA and increasing n-6 FA (n-6/n-3 ratios of 1, 2 and 6), as well as a final diet with double absolute n-3 FA content and an n-6/n-3 ratio of 1. This allowed for a separation between effects of ratio and absolute amounts on tissue FA levels. These diets were used in a 13-week growth trial and a 4-week stress trial. In the stress trial, half the fish were exposed to a repeated stressor (hypoxia) three times weekly, while the other half were undisturbed controls. At the end of the experiment, all fish were exposed to a confinement stressor. These trials confirmed that dietary n-6 FA increases the requirement for EPA, but not DHA. Despite equal level of dietary EPA + DHA, an increasing level of dietary n-6 FA (a higher n-6/n-3 ratio) resulted in lower levels of EPA in polar lipids (liver polar lipids, red blood cells, skin phospholipids, brain polar

lipids). A higher feed n-6/n-3 ratio increased the level of all n-6 FA (including the longer-chain n-6 FA). However, maintaining a low n-6/n-3 ratio inhibited the increased incorporation of n-6 into polar lipids despite higher dietary absolute levels of n-6 FA. Generally, polar lipids reflected the relative n-6 and n-3 level of the feed, while in neutral lipids the FA composition was more related to the absolute contents. These results indicate a competition between n-3 and n-6 FA for incorporation into polar lipids.

When investigating the effect of these diets on the stress response, it was found that all fish appeared phenotypically healthy, and were able to mount an acute stress response. There were hardly any significant effects of the repeated hypoxia stressor, possibly indicating an adaptation. Hepatic production of prostaglandin D2 (PGD_2) and leukotriene B4 (LTB_4) responded differently to acute stress depending on the feed n-6/n-3 FA ratio, which suggests a dietary impact on the acute stress response. Based on still declining rather than recovering PGD_2 levels 24 hours after exposure to the acute stressor, fish fed an n-6/n-3 ratio of 6 recovered more slowly from the stress compared to fish fed a ratio of 1. Furthermore, the n-6/n-3 ratio of 6 resulted in rising levels of LTB_4 in the fish liver one hour after acute stress compared to a decline seen in fish fed a ratio of 1. A general increase of the arachidonic acid (ARA) derived prostaglandins PGE_2 and PGD_2 was further seen in fish fed the high n-6/n-3 ratio. Eicosanoids are highly potent molecules, particularly the ones derived from ARA. Upsetting the balance between their n-3 and n-6 FA precursors, and hence their production, might lead to overly strong responses when salmon are exposed to stressors. A low dietary n-6/n-3 ratio will therefore likely be beneficial for the stress coping ability of salmon.

The final study examined how graded levels of EPA and DHA from 10 to 35 g/kg feed affected liver lipid metabolism in salmon kept in open sea cages during a full production cycle. Changes to the hepatic energy metabolism when reducing dietary EPA + DHA were discovered using a metabolomics approach. An inhibition of hepatic β-oxidation likely occurred in the fish fed the lowest levels of EPA + DHA (10 g/kg), as evidenced by less tricarboxylic acid cycle intermediates originating from β-oxidation. Additionally, in the low EPA + DHA groups, other pathways providing metabolic

energy, such as the pentose phosphate pathway, branched chain amino acid catabolism and creatine metabolism were activated in the liver. Increases in various acylcarnitines in the liver of the same fish, in particular accumulation of 3-hydroxyacrnintines (intermediates in mitochondrial β-oxidation), supported this and indicated disturbances in the hepatic lipid metabolism. Elevated liver lipids were furthermore observed in fish fed lower levels of EPA and DHA, aligning well with the metabolite data. The study showed that diets containing 10 and 13 g/kg EPA and DHA were insufficient for maintaining good liver metabolic health in Atlantic salmon. However, 35 g/kg dietary EPA and DHA was also significantly better than 16 g/kg, indicating that 16 g/kg might be suboptimal as well.

List of publications

I. Hundal BK, Liland NS, Rosenlund G, Bou M, Stubhaug I & Sissener NH. (2021a) Increasing dietary n-6 fatty acids while keeping n-3 fatty acids stable decreases EPA in polar lipids of farmed Atlantic salmon *(Salmo salar).* British Journal of Nutrition, vol. 125, issue 1, pp. 10-25. doi: https://doi.org/10.1017/S0007114520002494

II. Hundal BK, Liland NS, Rosenlund G, Höglund E, Araujo P, Stubhaug I & Sissener NH. (2021b). Increasing the dietary n-6/n-3 ratio alters the hepatic eicosanoid production after acute stress in Atlantic salmon *(Salmo salar).* Aquaculture, vol. 534, article number 736272. doi: https://doi.org/10.1016/j.aquaculture.2020.736272

III. Hundal BK, Lutfi E, Sigholt T, Rosenlund G, Liland NS, Glencross B, Sissener NH. (2022). A Piece of the Puzzle—Possible mechanisms for why low dietary EPA and DHA cause hepatic lipid accumulation in Atlantic salmon (*Salmo salar*). Metabolites, 12, issue 2, article number 159. doi: https://doi.org/10.3390/metabo12020159

Abbreviations

ACN acetonitrile
AGD amoebic gill disease
ALA alpha linolenic acid
ALAT alanine aminotransferase
ARA arachidonic acid
CACT carnitine acylcarnitine translocase
CMS cardiomyopathy syndrome
COX cyclooxygenase
CRH corticotropin releasing hormone
CPT1 carnitine palmitoyl transferase 1
CPT2 carnitine palmitoyl transferase 2
EPA eicosapentaenoic acid
DHA docosahexaenoic acid
FA fatty acid
FFA free fatty acid
FAS fatty acid synthetase
HDL high-density lipoprotein
FO fish oil
HPI axis hypothalamus-pituitary-interrenal axis
HSP heat shock protein
LA linoleic acid
LDL low-density lipoprotein
LOX lipoxygenase
LPC lysophosphatidylcholine
LTB_4 leukotriene B4
LTB_5 leukotriene B5
MUFA monounsaturated fatty acid
OA oleic acid
PC phosphatidylcholine
PE phosphatidylethanolamine
PGD_2 prostaglandin D2

PGE_2 prostaglandin E2

PGE_3 prostaglandin E3

PUFA polyunsaturat fatty acid

ROS reactive oxygen species

SFA saturated fatty acid

SREBP1 sterol regulatory element-binding protein 1

TCA tricarboxylic acid cycle

TFA total fatty acids

TAG triacylglycerol

VLDL very-low-density lipoproteins

VO vegetable oil

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1 BACKGROUND

The current commercial diet of Atlantic salmon is considerably different from just a few decades ago. There is a stable, but limited, amount of the marine feed ingredients fishmeal and fish oil (FO) worldwide (FAO, 2020). Concurrently, the aquaculture industry continues its yearly growth (FAO, 2020), causing increased demand for these resources. Additionally, there is resistance towards using wild caught fish ingredients, as it can compete directly with human consumption. For these reasons, there has been a gradual decline in the use if marine ingredients in aquafeeds, mainly in favour of terrestrial vegetable alternatives (Aas et al., 2022, Tacon and Metian, 2015). Although this change has allowed for growth in the aquaculture industry, increases in plant ingredients could cause negative effects on fish health and robustness due to an imbalanced nutrient composition and elevated levels of fibres and anti-nutritional factors (Turchini et al., 2009, Gatlin et al., 2007). However, provided good knowledge on nutrient requirements, it is possible to balance different raw materials to achieve a good nutrient composition (Turchini et al., 2019).

Current Norwegian salmon grow out diets are high in lipids, starting at approximately 28 % fat at the start of the sea water phase and increasing gradually up to around 38 % fat towards the end of the production cycle (pers.comm. Trygve Sigholt). Which lipid ingredients are used will determine the final fatty acid (FA) composition. FA are not only used for energy and as structural components in the fish, but also have important bioactive roles. Hence, an altered FA composition could affect the health and robustness of the fish. The focus of this thesis was to investigate dietary levels of the essential n-3 FA eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6-3, DHA), dietary n-6 FA, the relationship between them, and how they can affect fish robustness.

1.1 From marine to terrestrial ingredients

As we reduce the inclusion of marine oils, it needs replacement by an alternative lipid source. Norwegian aquaculture for instance, has since the early nineties drastically reduced the inclusion of marine ingredients. The levels have been reduced from 90 % of the feed being of marine origin to an inclusion of less than 23 % in 2020. In the place of marine feed resources, terrestrial plant ingredients have been included (Aas et al.,

2022). However, ingredients of animal origin can also be used, and new marine ingredients such as microalgae are emerging as viable options. A main difference between FO and vegetable oils (VO) is the complete absence of the nutritionally important long chain n-3 FAs EPA and DHA, which are found almost exclusively in marine oils. Instead, VOs typically contain the shorter chain n-3 FA, α-linolenic acid (18:3n-3, ALA). They are also generally rich in the n-6 FA linoleic acid (18:2n-6, LA) and the monounsaturated FA (MUFA) oleic acid (18:1n-9, OA) (Table 1). Furthermore, FOs generally contain an even distribution of FAs, while VOs are usually dominated by one or a few FAs (Table 1).

Consequently, exchanging FO for VO in salmon feeds alters the FA composition of the feed to a large extent. The declining dietary EPA + DHA and rising dietary ALA, LA and OA has been reported to cause similar changes to farmed salmon composition in Scotland, Norway and Tasmania (Aas et al., 2019, Sprague et al., 2016, Nichols et al., 2014). As fish growth, health, welfare and tissue composition are all affected by dietary FA to some degree, the alternative oils used is of great importance.

The Norwegian monitoring program for fish feed (Sele et al., 2021) found an average of 24.2 g EPA + DHA per kg feed (average n-6/n-3 ratio of 0.63) for samples of salmon feed collected in 2020 (Table *2*). The maximum level of dietary EPA + DHA found in these samples was 4-fold higher than the minimum value (Table *2*). This indicates a high variation in the dietary EPA + DHA in commercial feeds, and that some feeds might be well below the averaged 24.2 g/kg EPA + DHA. However, these data are based on samples from feed for all life stages. Levels of EPA + DHA in feeds used at the end of the sea water phase is at approximately 24 g/kg at 38 % lipids (pers. Comm. Trygve Sigholt). The FA composition of samples of Norwegian salmon feed collected in 2020 is provided in Table 2, with average, minimum and maximum values.

Table 1: Fatty acid composition (weight %) of typical vegetable oils used in feeds for Atlantic salmon, in addition to three types of fish oil and a krill oil.

	Capelin[1]	Sardine[1]	Anchovy[1]	Krill[1]	Soy[2]	Olive[2]	Sunflower[2]	Linseed[2]	Palm[2]	Coconut[2]	Rapeseed[2]
12:0	-	-	-	-	-	-	0.5	-	0.4	48.2	-
14:0	6.3	7.2	7.8	16.3	0.1	-	0.1	-	1.1	18.5	0.1
16:0	11.0	16.2	18.2	19.0	10.8	12.1	6.4	6.1	43.8	8.7	5.1
18:0	0.9	2.9	4.2	1.4	3.9	2.6	4.5	3.4	4.4	2.7	1.7
Sum SFA	**18.9**	**27.3**	**30.2**	**38.6**	**15.7**	**15.3**	**12.8**	**10.0**	**50.4**	**92.6**	**8.0**
18:1n-7	1.9	3.1	-	7.8	-	-	-	-	-	-	-
18:1n-9 (OA)	6.7	9.3	16.0	15.8	23.9	72.5	22.1	18.4	39.1	6.0	60.1
20:1n-9	15.9	1.5	1.0	1.4	0.1	0.3	0.2	-	0.1	0.1	1.4
22:1n-11	20.2	0.9	1.6	0.3	-	-	-	-	-	-	-
Sum MUFA	**56.5**	**25.4**	**29.6**	**38.1**	**24.2**	**73.8**	**22.4**	**18.4**	**39.4**	**6.1**	**62.4**
18:2n-6 (LA)	1.3	1.1	2.8	1.6	52.1	9.4	65.6	16.8	10.2	1.8	21.5
20:4n-6 (ARA)	-	0.8	0.1	0.1	-	-	-	-	-	-	-
Sum n-6	**1.3**	**1.9**	**2.9**	**2.1**	**52.1**	**9.4**	**65.6**	**16.8**	**10.2**	**1.8**	**21.5**
18:3n-3 (ALA)	0.7	0.6	1.8	0.8	7.8	0.6	0.5	55.0	0.3	0.1	9.9
18:4n-3	4.8	3.2	2.3	3.6	-	-	-	-	-	-	-
20:5n-3 (EPA)	7.5	18.4	14.8	6.4	-	-	-	-	-	-	-
22:5n-3 (DPA)	0.6	2.2	1.8	0.2	-	-	-	-	-	-	-

22:6n-3 (DHA)	5.7	12.3	10.9	2.3	-	-	-	-	-	-	-
Sum n-3	**19.8**	**37.7**	**29.3**	**14.6**	**7.8**	**0.6**	**0.5**	**55.0**	**0.3**	**0.1**	**9.9**
n-6/n-3	**0.07**	**0.05**	**0.10**	**0.14**	**6.7**	**16**	**131**	**0.3**	**34**	**18**	**2.2**

[1] From Sissener et al. (2016a), [2] From Dubois et al. (2007)

Table 2: Fatty acid composition of Norwegian Atlantic salmon feeds (g/kg). Samples collected from feeds for all life stages. Provided are the average, the minimum and maximum levels. Data from Sele et al. (2021).

Fatty acid	Average	Minimum	Maximum
14:0	8.6	5.4	19.2
16:0	27.7	20.0	48.1
18:0	9.6	2.7	13.5
Sum SFA	**51.3**	**32.7**	**82.9**
18:1n-9	97.7	11.9	142.0
22:1n-9	2.0	1.0	4.3
Sum MUFA	**133.0**	**57.9**	**185.0**
18:2n-6	38.8	7.6	64.5
20:4n-6	1.1	0.7	2.5
Sum n-6	**40.5**	**9.0**	**65.5**
18:3n-3	23.0	2.4	31.7
20:5n-3	13.0	9.2	32.7
22:6n-3	11.2	7.0	28.2
Sum EPA + DHA	**24.2**	**16.6**	**60.9**
Sum n-3	53.2	36.8	81.7
Sum PUFA	**94.7**	**47.9**	**125.0**
Sum FA	**283.5**	**142.0**	**354.0**
n-3/n-6	1.6	0.9	5.1
Sum EPA + DHA (% of TFA)	8.9	5.5	21.2

1.2 Lipid metabolism in Atlantic salmon

The major pathways of lipid metabolism in fish are generally the same as in mammals, and constitute of digestion and absorption, lipogenesis, oxidation and transport, and they have been the topic of multiple reviews (e.g. Tocher and Glencross, 2015, Tocher et al., 2008, Leaver et al., 2008). An imbalance between these processes can lead to an accumulation or depletion of fat. The liver is a key organ for lipid metabolic processes and is also an important hub for the distribution of lipids to other tissues. An overview of the major pathways involved in hepatic FA metabolism can be found in Figure 1.

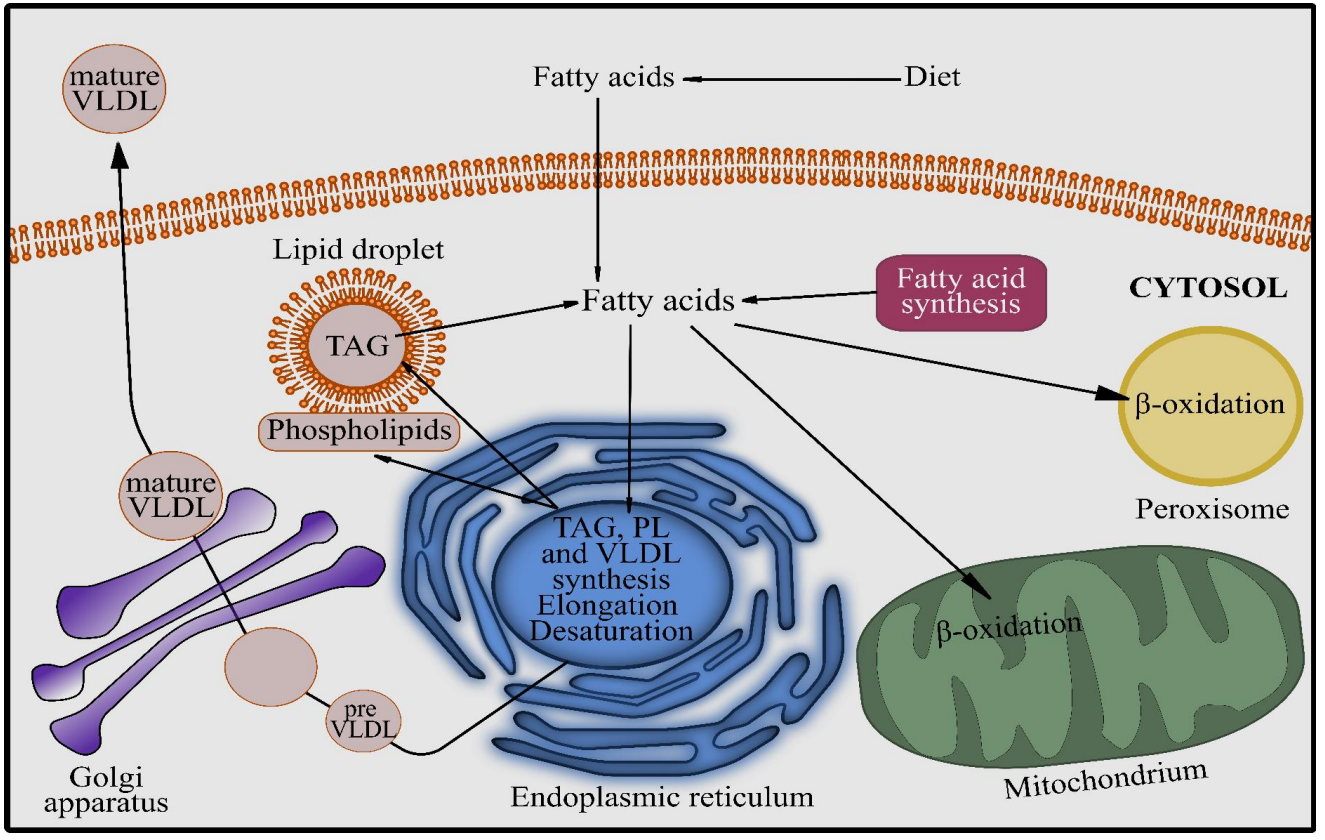

Figure 1: Overview of the main pathways of hepatic lipid metabolism. Depending on energy status of the fish (i.e. time since last feeding), dietary or endogenously synthesised fatty acids can be used for TAG and phospholipid synthesis, production of lipid droplets and VLDL or β-oxidation in either mitochondria or peroxisomes. Fatty acids from both the diet or endogenous synthesis can also be further modified (elongated and desaturated). TAG – triacylglycerol, VLDL - very-low-density lipoproteins. Figure created by Bjørg Kristine Hundal.

Digestion, absorption and transport

Most of the lipid digestion of Atlantic salmon happens in the proximal intestine and pyloric caeca. Various lipases catalyse the degradation of lipids to free fatty acids (FFA), partial acylglycerols, lysophospholipids, cholesterol and fatty alcohols (Tocher and Glencross, 2015). In humans, triacylglycerol (TAG) is degraded into FFA and monoacylglycerol mainly by pancreatic lipase, which is dependent on its coenzyme colipase. Fish lack the enzyme colipase and thus a functioning pancreatic lipase (Sæle et al., 2010), hence all three FA are released from the glycerol backbone due to nonspecific lipases. TAG being completely broken down into FFA and glycerol has also been demonstrated in salmon (Bogevik et al., 2008). Due to the hydrophobic properties of lipids, they must be emulsified with bile salts to be taken up by the enterocytes. Once inside the enterocytes, the FA are re-esterified to form TAG and phospholipids. Combined with apolipoproteins the TAG and phospholipids form chylomicrons, which are transported from the intestine to the liver and releasing FFA to the tissues along the way. Fish lack the enzymatic capability to edit the ApoB100 mRNA (Turchini et al., 2022, Damsteegt et al., 2018, Conticello et al., 2005), and therefore cannot make the truncated ApoB48 used to produce chylomicrons in mammals. Chylomicron-like particles have been detected in fish (Azarm et al., 2013, Fremont et al., 1981), but being smaller in size and composed of the same apolipoproteins as in very-low-density lipoproteins (VLDL), resulting in less distinction between the two lipoprotein types. For transport out of the liver, lipids combine with apolipoproteins, like ApoB100, to form VLDL. VLDL circulates to the peripheral tissues, where lipids are released. This increases the density of the particles, forming low-density lipoprotein (LDL) and thereafter high-density lipoprotein (HDL) (Turchini et al., 2022, Tocher and Glencross, 2015). The main function of LDL is to provide cholesterol for peripheral tissues, while HDL returns lipids and cholesterol from the tissues to the liver (Torstensen and Tocher, 2010).

Lipogenesis

Fish have the biosynthetic capability to produce 16:0 and 18:0 from acetyl-CoA. The first committed step forms malonyl-CoA and is catalysed by acetyl-CoA carboxylase in the cytosol. The multienzyme fatty acid synthase (FAS) complex then catalyses the production of these FA by condensation of malonyl-CoA to acetyl-CoA, and then adding one malonyl-CoA at a time to the growing acyl-chain using NADPH for reducing power. Dietary n-3 long-chain polyunsaturated FA (PUFA) can inhibit lipogenesis in salmon (Betancor et al., 2014). Fish can form the MUFA OA (18:1n-9) and 16:1n-7 but lack the ability to further desaturate them and form PUFA. Therefore, fish must acquire the PUFA LA (18:2n-6) and ALA (18:3n-3) through the diet. These dietary 18-carbon PUFA can be further elongated and desaturated into longer chain PUFA such as EPA, DHA and ARA by fish using fatty acyl desaturases and fatty acyl elongases. The diet of salmon is naturally rich in EPA and DHA, and DHA inhibits desaturation and elongation (Betancor et al., 2014, Thomassen et al., 2012). Further, although salmon possesses the capability to elongate and desaturate 18-carbon PUFA, this capacity is limited and not sufficient to reach similar tissue levels of EPA and DHA as when fed high dietary levels (Østbye et al., 2021, Bou et al., 2017b, Bou et al., 2017c, Rosenlund et al., 2016).

Fatty acid oxidation

While lipogenesis is performed in the cytosol, FA oxidation in fish occurs within specialised organelles named peroxisomes and mitochondria, as is also the case in mammals (Tocher and Glencross, 2015, Leaver et al., 2008). The separation of these processes in separate cell compartments secures that a futile cycle of anabolic and catabolic reactions is avoided. In addition, malonyl-CoA (first intermediate of lipogenesis) inhibits mitochondrial β-oxidation by inhibition of carnitine palmitoyl transferase 1 (CPT1) and thus transport of FA into the mitochondria (Foster, 2004, Frøyland et al., 1998). The capacity of fish for FA catabolism is dependent on life stage, fish size and season (Sanden et al., 2016, Stubhaug et al., 2007, Stubhaug et al., 2006, Nordgarden et al., 2003). FA longer than 12 carbon cannot directly cross the

mitochondrial membrane and need active transport to pass over. FA and CoA are first converted into acyl-CoA. CPT1 then catalyses the formation of acylcarnitines from acyl-CoA, which are then capable of crossing the mitochondrial membrane in exchange for a free carnitine from within the mitochondrial matrix using the carnitine acylcarnitine translocase (CACT). Inside the mitochondria, CPT2 catalyses the reformation of acyl-CoA from acylcarnitine (Longo et al., 2016). The β-oxidation is a four-step reaction leading to consecutive removal of the two-carbon unit acetyl-CoA and production of NADH. Much of this acetyl-CoA is shuttled into the tricarboxylic acid (TCA) cycle for further production of NADH and of $FADH_2$, to be used in the production of ATP through oxidative phosphorylation (Tocher and Glencross, 2015). Peroxisomal β-oxidation utilises a different set of enzymes than its mitochondrial counterpart, and much of the energy produced dissipates as heat (Lodhi and Semenkovich, 2014). Otherwise, it is similar to the mitochondrial β-oxidation (Leaver et al., 2008). The salmon liver has a high capacity for peroxisomal β-oxidation (Stubhaug et al., 2007, Frøyland et al., 1998), and during parr-smolt transformation it contributes with up to 100 % of the β-oxidation capacity (Stubhaug et al., 2007). Acyl-CoA oxidase is the rate limiting enzyme in peroxisomal β-oxidation (Aoyama et al., 1994).

The relative rates of oxidation for different types of FA classes in salmon have been determined to be MUFA > saturated FA (SFA) > n-6 PUFA > n-3 PUFA (Norambuena et al., 2015), with shorter chain favoured over longer chain (Tocher, 2015, Tocher and Glencross, 2015). On the other hand, dietary FA present in excess generally have lower deposition in salmon, indicative of preferential oxidation of dietary FA present in excess (Norambuena et al., 2015, Sanden et al., 2011, Stubhaug et al., 2005a, Torstensen and Stubhaug, 2004, Torstensen et al., 2004). Not even the essential FA EPA and arachidonic acid (20:4n-6, ARA) are exempted when present in sufficient amounts (Norambuena et al., 2015). DHA is apparently spared this catabolic fate, being selectively deposited (Katan et al., 2019, Betancor et al., 2014, Glencross et al., 2014), but even retention of DHA decreases slightly when sufficiently high amounts are included in the diet (Glencross et al., 2014, Stubhaug et al., 2007). That dietary EPA

increases hepatic β-oxidation in mammals is well documented (Shang et al., 2017, Ukropec et al., 2003, Halvorsen et al., 2001, Madsen et al., 1999). Though there is evidence of an increased capacity for β-oxidation in salmon liver with more dietary EPA and DHA *in vivo* (Stubhaug et al., 2007, Stubhaug et al., 2006) and *in vitro* (Kjær et al., 2008b, Vegusdal et al., 2005), there are also studies where no effects of dietary EPA and DHA on β-oxidation capacity were seen (Sanden et al., 2011, Kjær et al., 2008a, Stubhaug et al., 2005a). Kjær et al. (2008a) also investigated excessive dietary EPA + DHA (> 50 % of total FA (TFA)), in which case detrimental effects on β-oxidation were seen due to oxidative damage to the mitochondria. There is very little work done on EPA alone in fish. However, one study investigating feeds with transgenic camelina oil (high EPA), found that compared to wild type camelina (low EPA + DHA but not deficient) it resulted in significantly lower liver lipids (Betancor et al., 2015). When the high EPA diet was compared to an FO diet, however, the fish fed the high EPA diet had significantly higher liver lipids (Betancor et al., 2015). There were higher levels of all n-3 FA in the liver of fish fed the high EPA diet, except DHA, which was significantly lower. The authors therefore suggested that lack of DHA might play an important role in the increased adiposity of the liver (Betancor et al., 2015). Another hypothesis about the reason for the hypolipidemic effects of EPA and DHA, is that rather than stimulating the β-oxidation system itself EPA and DHA can increase β-oxidation by promoting uptake of FA into the cells (Torstensen and Stubhaug, 2004).

1.3 Importance of dietary n-3 and n-6 fatty acids for salmon

1.3.1 The minimum and practical requirements of EPA and DHA

Early studies on salmon fry reported a minimum dietary EPA + DHA requirement between 5 and 10 g/kg EPA + DHA to achieve optimal growth (Ruyter et al., 2000a, Ruyter et al., 2000b). As little as 2 g/kg EPA + DHA was reported to be sufficient to support good growth in juvenile salmon (Emery et al., 2016). However, these were relatively short-term, tank-based trials performed on smaller fish. Additionally, in the latter trial fish were fed commercial diets high in EPA and DHA prior to the experiment. Owing to the short duration of the trial (14 weeks), these diets might have supplied sufficient n-3 PUFA to sustain the fish for the length of the trial. Two long-term feeding

trials (fish kept either 38, 51 or 59 weeks) in the sea water phase, albeit in a controlled environment in land-based tanks, demonstrated that 10 g/kg EPA + DHA was sufficient to maintain growth and health (Rosenlund et al., 2016). Despite reduced growth, fish in the same trial but fed 5 g/kg EPA + DHA had a high survival (99 %), although some negative health effects were seen (higher plasma cortisol, increased liver fat, reductions in brain polar lipid DHA) (Rosenlund et al., 2016, Sissener et al., 2016a, Sissener et al., 2016b). These studies suggest a minimum requirement for growth and health of Atlantic salmon kept in a stable and controlled environment at 10 g EPA + DHA per kg feed during long-term feeding. On the other hand, feeding salmon EPA + DHA at ≤10 g/kg during a full production cycle was proven insufficient to maintain a robust salmon in more realistic commercial-like conditions in open net-pens (Bou et al., 2017a). This study recorded higher mortalities in fish fed EPA + DHA at ≤10 g/kg compared to 17 g/kg after delousing procedures, with these differences in mortality being particularly prominent during the summer months when water temperatures were relatively high (Bou et al., 2017a). Using dietary concentrations of EPA + DHA at 26 g/kg compared to 16 g/kg did not lead to additional health benefits in a full-scale sea cage trial where the fish were exposed to both delousing and disease outbreaks (Sissener et al., 2016c). These studies indicate that the practical requirement of EPA + DHA for Atlantic salmon in the sea water phase lies between 10 and 16 g/kg.

1.3.2 Effects of dietary n-6 fatty acids on n-3 fatty acid requirement

There is no doubt that the level of EPA + DHA in the feed is important for the incorporation of these two FA into cellular membranes (Bou et al., 2017b, Sissener et al., 2016b). However, there are indications that the dietary n-6 FA and the relationship between n-6 and n-3 FA in the feed might be equally important (reviewed in Sissener, 2018). Maintaining constant dietary EPA + DHA and ARA while adding more LA to feeds for salmon resulted in reductions in EPA in polar lipids, with concurrent increases in polar lipid ARA (Sissener et al., 2020). This was suggested to be related to higher ARA production owing to higher precursor levels (LA), together with reduced synthesis of EPA because of competition over shared enzymes, or the dislocation of EPA from polar lipids in favour of the more available ARA. This trial clearly demonstrated that

dietary LA affects deposition and metabolism of EPA and ARA. Similarly, diets formulated with constant dietary EPA + DHA and varying LA and ALA (different n-6/n-3), gave reduced EPA in liver lipid when dietary LA increased in salmon (Katan et al., 2019). In line with the aforementioned trials, feeding gilthead sea bream (*Sparus aurata*) larva increasing ARA resulted in lower full body EPA levels (Alves Martins et al., 2012). Excessive dietary LA similarly caused an apparent deficiency in ALA for the freshwater fish Eurasian Perch *(Perca fluviatilis* Linnaeus 1758*)* (Blanchard et al., 2008). Conversely, decreasing the dietary level of EPA and DHA (constant n-6) led to increases in polar lipid 20:3n-6 and ARA in salmon, indicating an increase in the n-6 biosynthetic pathway when lacking in n-3 FA (Bou et al., 2017c). DHA is the only n-3 FA that remains relatively constant despite dietary alterations in LA or ARA (Sissener et al., 2020, Alves Martins et al., 2012). Two subsequential trials with the freshwater fish Murray cod (*Maccullochellapeelii, Mitchell 1838*) demonstrated that both the absolute (Francis et al., 2009) and relative (Senadheera et al., 2010) dietary n-6 and n-3 FA content influenced the final FA make-up of the tissues. All these trials demonstrate that both the relative and absolute dietary levels of n-6 and n-3 FA are important for the final tissue FA composition, but they do not reveal which of the two matter more. Further, it is likely that the required dietary EPA + DHA to ensure a robust salmon is higher when the dietary n-6 FA level is increased.

1.3.3 Homeoviscous adaptation

A main feature of the biological membrane is the lipid bilayer which possess several properties counting phase behaviour, fluidity/viscosity, thickness rigidity and curvature which in part is determined by its composition of lipids (Holthuis and Menon, 2014). The membrane content of saturated vs. unsaturated FA is vital for determining how the acyl chains are packed in the membrane, hence also its viscosity and permeability (Ernst et al., 2016). While SFA generally leads to tighter packing making the membrane more rigid, unsaturated FA results in lower density and thus higher fluidity (Ernst et al., 2016). To modify the degree of membrane saturation, phospholipid molecular species and head group and sterol incorporation in response to changing environmental temperature is termed homeoviscous adaptation (Ernst et al., 2016). Salmon increase the content of PUFA and reduce SFA in its membranes at decreasing temperatures (Liu

et al., 2020a). An increase in membrane unsaturation supports normal membrane functionality in fish faced with reduced temperatures (Liu et al., 2020a, Tiku et al., 1996). FA typical of VO are shorter and less saturated than their marine counterparts (Table 1), resulting in thinner membranes (Holthuis and Menon, 2014). The relative proportion of EPA and DHA in the cell membrane is important for the regulation of fluidity during temperature fluctuations. As they have a much lower melting point than more saturated and shorter chain FA, they help keep the correct fluidity even at low temperature, thus increasing their requirement at lower temperatures (reviewed in Arts and Kohler, 2009). Sterols increase the thickness of cellular membranes (Holthuis and Menon, 2014). By replacing FO with VO in fish feed, the dietary cholesterol is reduced whereas the level of phytosterols increases (Liland et al., 2013a). Phytosterols increase the bilayer thickness, which is a measure of their ability to condense the membrane lipids. However, their condensing effect is less efficient than that of cholesterol (Kamal and Raghunathan, 2012). It therefore seems possible that dietary lipids can affect the physical properties of the membrane.

1.3.4 Eicosanoids

EPA and ARA are two of the precursors for a group of biologically active signalling molecules called eicosanoids. They are important in the initiation and resolution of inflammation, and also have functions in immunity (reviewed in: Calder, 2017, Arts and Kohler, 2009). EPA is also the precursor for specialised pro-resolving mediators called resolvins (Calder, 2020a). Eicosanoids are constitutively produced, but exposure to stress, trauma or disease can trigger an increased biosynthesis (reviewed in Arts and Kohler, 2009). As dietary n-3 and n-6 FA can modulate membrane content of these FA and the downstream production of eicosanoids, changes to the dietary absolute and relative contents have the potential to modify the potency of stress and immune responses through altered eicosanoid production (further discussed in section 1.5.2).

The n-6 and n-3 derived eicosanoids are not interconvertible and can have opposing physiological effects. Many n-6 and n-3 FA are identical with the exception of the n-3 double bond, which results in different spatial structure. This structural difference generally leads to n-3 derived eicosanoids in many cases being less potent inflammatory

agents than the n-6 FA derived (reviewed in Calder, 2015). For example, Wada et al. (2007) discovered that EPA derived prostanoids had much lower affinity for eicosanoid receptors than the ARA derived ones. Further, prostaglandin E_3 (PGE_3, derived from EPA) is significantly less efficient at inducing inflammatory effects than its ARA counterpart, PGE_2 (Bagga et al., 2003). This suggests that the EPA derived eicosanoids are less metabolically active than the ARA derived eicosanoids, which would lead to weaker inflammatory responses. However, the EPA and ARA derived eicosanoids also have separate biological functions, often directly opposing (reviewed in Schmitz and Ecker, 2008). For instance, PGI_2 and PGE_2 (from ARA) are pro-arrythmic, whereas PGI_3 and PGE_3 (from EPA) are anti-arrythmic (Li et al., 1997).

While in mammalian cell membranes, arachidonic acid (20:4n-6, ARA) is more prevalent than EPA (Calder, 2017), the opposite is the case in salmon polar lipids (Sissener et al., 2017c, Martinez-Rubio et al., 2013). Still, ARA seems to be the preferred eicosanoid precursor in also fish (Furne et al., 2013, Olsen et al., 2012, Liu et al., 2006, Bell et al., 1994). For production of eicosanoids, EPA and ARA must first be released from the cell membrane. EPA and ARA released from the membrane can then be acted upon by COX, lipoxygenases (LOX) and cytochrome p450 enzymes to yield metabolites in the eicosanoid family. As shown in Figure 2, there is competition between ARA and EPA over eicosanoid producing enzymes. There is discrimination in favour of utilising ARA for both COX (Furne et al., 2013, Olsen et al., 2012, Liu et al., 2006, Bell et al., 1994) and LOX (Tocher and Sargent, 1987) pathways in fish. The LOX pathway seems to be preferred over the COX pathway in salmon (Sissener et al., 2020, Holen et al., 2018, Holen et al., 2015).

Dietary and tissue LA can be converted to ARA in salmon (Katan et al., 2019). Trials where salmon were fed increased dietary LA found increased production of ARA derived eicosanoids in gills and leukocytes (Bell et al., 1992, Bell et al., 1991b). Sissener et al. (2020) found similar results in plasma, though somewhat unexpectedly the opposite results were found in liver where increased dietary LA resulted in significantly less ARA derived eicosanoids. However, the hepatic relationship between

LTB_4 and LTB_5 matched the polar lipid ARA:EPA ratio, though no such relationship was seen between PGE_2 and PGE_3. Feeding salmon diets high in ALA compared to a diet high in LA resulted in increased EPA in the membrane phospholipids (heart, leukocytes), and a concurrent reduction in production of ARA derived eicosanoids (plasma, stimulated blood, stimulated cardiac myocytes) (Bell et al., 1993a). Hence, the balance between the n-6 and n-3 FA in the cell membranes can contribute to determining which eicosanoids are produced.

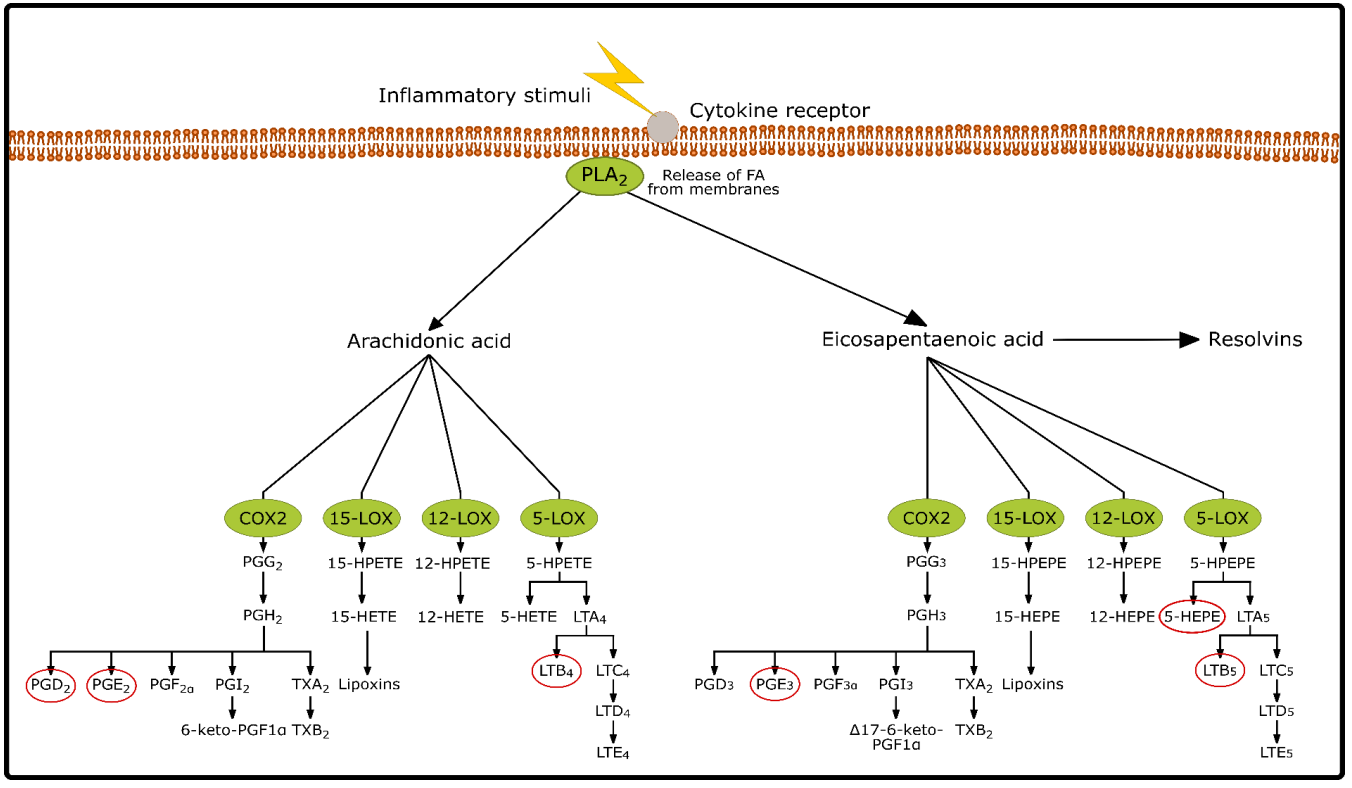

Figure 2: Eicosanoid production from n-3 and n-6 PUFA. n-3 and n-6 fatty acids are released from membranes by phospholipases (such as PLA_2) in response to inflammatory stimuli. These free fatty acids are then acted upon by different COX and LOX enzymes to form eicosanoids which will give rise to the inflammatory response. Some ARA and EPA derived eicosanoids are depicted here. EPA can also form inflammation resolving mediators called resolvins. The eicosanoids marked by a red circle have been analysed in the present thesis. COX – cyclooxygenase, LOX – lipoxygenase, PLA_2 – phospholipase A_2, PUFA – polyunsaturated fatty acid. Figure created by Bjørg Kristine Hundal. Based on data from figure 1 in (Araujo et al., 2019)

1.4 Fatty acids implicated in the accumulation of liver lipids in Atlantic salmon

This paragraph will discuss some FA which, either in excess or deficit, are associated with accumulation of lipids in the liver of Atlantic salmon. A major drawback when investigating the effect of reducing or increasing a certain feed ingredient, such as a FA, is that in its place something else must be equally increased or reduced. Consequently, the observed effects could be due to the modification of the target nutrient, of the accompanying altered nutrient, or a combination of the changes, thus complicating interpretation of results.

In salmon, a high level of liver fat is considered a general sign of dietary imbalance (Sissener et al., 2016a), and a high content of liver lipids is associated with increased mortality (Dessen et al., 2021, Bou et al., 2017a, Mørkøre et al., 2013). This does not automatically imply a causal link between more liver fat and the observed increases in mortality, but it could mean that it is a symptom of a less robust fish.

Early studies on rainbow trout investigating the effects of essential FA deficiency (feeding PUFA free diets) demonstrated that the fish developed a swollen liver with high fat content as part of the pathology (Castell et al., 1972). Indeed, increased liver fat content is a recurring issue when salmon diets are low in EPA and DHA (≤10 g/kg) (Bou et al., 2017a, Sanden et al., 2016), and Tocher et al. (2003) found a significant negative correlation between higher dietary EPA + DHA and liver lipid content. Increasing dietary EPA + DHA from 16 to 43 g/kg also reduced liver lipid contents, although not significantly (Betancor et al., 2018). However, these fish were kept at 13°C. When salmon were fed low n-3 PUFA (regression design: 5 – 17 g/kg EPA + DHA) they accumulated liver lipids at 6°C, but not at 12°C (Sissener et al., 2017c). As discussed in section 1.3.3, low temperature could increase the requirement for EPA and DHA. This suggests that the effect of dietary EPA + DHA on liver lipids will be more apparent at low water temperatures.

Several mechanisms have been implicated in the hypolipidemic effects of EPA and DHA. They are reported to inhibit lipogenesis in salmon through inhibition of sterol regulatory element-binding protein 1 (SREBP1) and FAS (Xue et al., 2020, Katan et

al., 2019), thus reduced dietary EPA and DHA content could promote hepatic lipogenesis (Leaver et al., 2008). Some studies have also indicated that too little dietary EPA + DHA will decrease the production of VLDL (Jordal et al., 2007), thus hindering lipids from being secreted. This can lead to a lipid accumulation in the liver. Results regarding the effect of EPA and DHA on β-oxidation in salmon are varying, both demonstrating increased (Kjær et al., 2008b, Stubhaug et al., 2007, Stubhaug et al., 2006, Vegusdal et al., 2005, Torstensen and Stubhaug, 2004) or unchanged (Kjær et al., 2008a, Stubhaug et al., 2005a) β-oxidation capacity with more EPA + DHA provided in the diet. Salmon fed diets containing a high-DHA/low-EPA algal oil had higher occurrence of pale livers and higher total lipids in the liver than fish fed FO diets with a more balanced DHA/EPA ratio. The authors hypothesised this to be due to mild chronic stress in the liver caused by lack of EPA (Kousoulaki et al., 2020). Some also suggest that effects of EPA and DHA on FA oxidation are not related to changes in FA oxidation itself. Torstensen and Stubhaug (2004) found that differences in β-oxidation between hepatocytes stimulated with different FA[1] disappeared when the amount of β-oxidation was assessed relative to FA uptake. They therefore proposed that β-oxidation is regulated at the level of FA uptake. Ultimately, it is clear that an accumulation of hepatic lipid occurs with too low dietary EPA and DHA, but there is likely not one single explanation for this.

Furthermore, reducing the dietary EPA and DHA causes an increase in other FA, some of which also are believed to cause lipid accumulation in the liver. VO are usually dominated by one FA or a few, while FO has a more varied FA composition with smaller amount of several different FA (Section 1.1, Table 1). LA is a FA common to VO, and fish fed a higher dietary level of this FA usually also has an increase in liver fat (Alvheim et al., 2013, Ruyter et al., 2006, Bransden et al., 2003, Tocher et al., 2003). Alvheim et al. (2013) found elevated hepatic levels of ARA derived endocannabinoids (subgroup of eicosanoids) in salmon fed a soy oil based diet (high LA) compared to a FO diet. These endocannabinoids can increase the hepatic SREBP1c activity, and therefore also its target enzymes FAS and acetyl-CoA oxidase in mice (Osei-Hyiaman

[1] 16:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:5n-3, 22:6n-3

et al., 2005). Alvheim et al. (2013) therefore hypothesised that the increased liver TAG in soy oil fed salmon could partly derive from increased lipogenesis induced by elevated ARA derived endocannabinoids. Another possible explanation for increased hepatic lipid content with higher dietary n-6 FA is through decreased VLDL clearance from the liver, as the n-6 derived PGE_2 and PGD_2 has been demonstrated to inhibit secretion of VLDL from rat hepatocytes (Perez et al., 2006, Björnsson et al., 1992).

A third FA implicated in the accumulation of fat in the liver is OA, as seen in trials feeding salmon with increased OA levels (Liland et al., 2013a, Torstensen et al., 2011) and in salmon hepatocytes stimulated with OA (Espe et al., 2019). It has been hypothesised that OA is metabolised slower than other FA in fish (Caballero et al., 2002). Moreover Kjær et al. (2008b) showed that OA can stimulate TAG synthesis in salmon hepatocytes.

Changes in the liver lipid content of salmon can thus be induced by several different factors. Since reducing one nutrient inevitably means the increase of others, this adds to the difficulty of pinpointing the exact cause. As the liver is responsible for transport of lipids to other tissues through the production of VLDL, changes to the hepatic metabolism might affect the whole organism. However, what significance the liver lipid level has on the health and robustness of salmon is still not completely clear, though associations with reduced robustness have been found.

1.5 Stress response in Atlantic salmon

1.5.1 Stress response in fish

Stress can be defined as the cascade of physiological responses that happen when an organism attempts to re-establish homeostasis or resist death when faced with a stressor (Schreck and Tort, 2016). Stress can be acute (brief), such as when fish are netted or chased, in which case the stress response is an appropriate and necessary response. In this setting, it enables the fish to respond to its environmental changes or threats and restore homeostasis. However, stress can also be chronic (lasting over time), such as overcrowding or repeated temperature changes. Bottom-line, the stress response is the provision of energy for overcoming the stressor, while suppressing processes

unimportant for immediate survival such as immune responses and growth. Consequently, during chronic stress the coping mechanism itself (the stress response) can become detrimental.

Physiological stress response

There are three levels to the physiological stress response (see Figure 3 for overview). The primary response, the alarm phase, is a neuroendocrine response involving a rapid release of stress hormones (catecholamines and corticosteroids). Catecholamines are stored within chromaffin cells in the head kidney and can be released within seconds via the simpatico-chromaffin axis (Iwama et al., 2005, Reid et al., 1998). The release of cortisol is dependent on the hypothalamus-pituitary-interrenal axis (HPI axis). First, the stressor triggers production of corticotropin releasing hormone (CRH) in the hypothalamus. Then CRH stimulates production of adrenocorticotropic hormone in the pituitary, which again induces production and release of cortisol from the interrenal tissue (Iwama et al., 2005). This process takes a few minutes, meaning that cortisol is less affected by sampling effects (Ellis et al., 2012). Hence, plasma cortisol is a widely-used indicator for stress. Clearance of catecholamines is usually seconds to minutes, while cortisol normally leaves circulation after a few hours (Faught et al., 2016).

The secondary stress response is the effects of the hormones released during the alarm phase, and entails making energy available for fight, flight or coping mechanisms. Catecholamines trigger, amongst others, cardiovascular and respiratory responses and increased glucose levels in plasma (Rodnick and Planas, 2016). The main effect of cortisol in stress is also the mobilisation of energy substrates; it increases the breakdown of muscle, the plasma glucose and plasma FFA (Sadoul and Vijayan, 2016). Cortisol also affects the hydromineral balance (Takei and Hwang, 2016) and suppresses the immune system (Schreck and Tort, 2016).

The tertiary stress response can be observed only after longer periods of stress, when changes are visible in fish performance or on a population level. The fish can adapt to the stressor, as fish repeatedly exposed to a stressor can cope better with it than when exposed to it a single time (Remen et al., 2012, Jentoft et al., 2005). However, tertiary

stress responses can also be maladaptive if severe or continued for too long, with effects such as immunosuppression and growth retardation.

When investigating the stress response, a time-series sampling is necessary to capture pre-stress, rise and recovery by measuring suitable indicators of stress (Sopinka et al., 2016). As mentioned, catecholamines are elevated instantaneously (within seconds) and cortisol minutes after exposure to acute stress. An hour after exposure to an acute stressor, it is expected to find elevated levels of plasma cortisol as a measure of the primary response (Fast et al., 2008). Markers of the secondary response, such as higher plasma glucose (Fast et al., 2008, Cai, 2014), alterations in gene expression (Olsen et al., 2012, Cai, 2014) or changed eicosanoid levels (Olsen et al., 2012) are also anticipated to be found at this time point. Twenty-four hours after acute stress, clearance of primary stress indicators is normally expected (Faught et al., 2016, Fast et al., 2008). Furthermore, restoration of pre-stress levels is expected after 24 hours. If not completely returned to pre-stress levels, recovery is at least expected to have been initiated (Fast et al., 2008, Cai, 2014). However, the duration and severity of the stress response will depend on the type of stressor and the state of the stress-coping mechanisms in the fish, and a completely accurate prediction of responses is difficult.

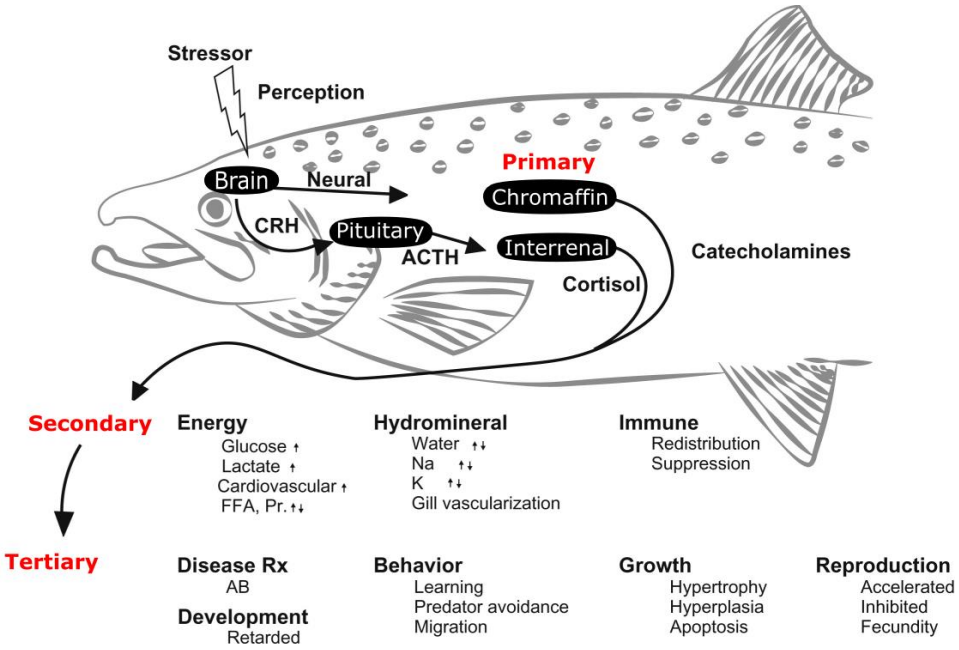

Figure 3: Schematic overview of the physiological stress response in fish. The stressor is first perceived, resulting in activation of the primary stress response (within drawing of fish). The secondary response is the physiological changes in energy availability, hydromineral balance and immune responses. The tertiary response can be seen at the whole animal level. Source: Schreck and Tort (2016)

Cellular stress response

Heat shock proteins (HSP) are a family of highly conserved proteins that function in the cellular stress response and aid the cell in maintaining the structural integrity of proteins and uphold a normal cell function (Roberts et al., 2010, Iwama et al., 1998). Although they are part of the cellular stress response, they also have constitutive chaperone functions for the proper folding of proteins in an unstressed situation. The low molecular weight HSP are, however, are function specific and don't have constitutive functions in an unstressed cell (Roberts et al., 2010). While it is clear that HSP aid the cell in maintaining homeostasis during the cellular stress response (Roberts et al., 2010), there are inconsistencies in the literature regarding their suitability as general indicators of stress. The HSP response to stress seemingly varies substantially in regards to tissue, family of HSP, species, developmental stage and type of (Iwama et al., 2004). HSP70 mRNA has been found to be a good indicator for handling stress (Olsvik et al., 2011),

the protein level of HSP70 an indicator for thermal stress (Gallant et al., 2017), HSP90a mRNA for thermal stress and combined thermal and hypoxia stress (Beemelmanns et al., 2021) and HSP27 for a lowered water level (confinement stress) in Atlantic salmon (Cai, 2014). Meanwhile, investigations by Zarate and Bradley (2003) indicated that HSP (HSP90, HSP70 and HSP30) were not sensitive markers for hatchery stress (physical stress caused by feed deprivation, anaesthesia, capture- and crowding stress, hyperoxia and hypoxia) in Atlantic salmon. The use of HSP as indicators of the stress therefore probably needs to be done in both a stressor- and species specific manner.

Oxidative stress is also part of the cellular stress response, and occurs when there is an imbalance between the produced reactive oxygen species (ROS) from metabolic processes and the cellular capacity to remove them (Hoseinifar et al., 2020). An accumulation of ROS can cause damage to DNA and proteins as well as causing lipid peroxidation in unsaturated membrane-bound FA. The anti-oxidant defence consists of a complex redox network, with both enzymatic (e.g. superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic (e.g. vitamin C, vitamin E and glutathione) systems (Hamre et al., 2022, Martínez-Álvarez et al., 2005).

1.5.2 Effect of dietary lipids on the stress response

Plasma cortisol is an extensively used indicator for stress (as described in section 1.5.1) and is shown to be affected by the dietary FA composition. Smoltifying salmon fed sunflower oil diets (high in LA) had significantly higher plasma cortisol levels than fish fed FO diets (Jutfelt et al., 2007). A delayed cortisol response was observed in gilthead seabream *(Sparus aurata)* fed a soy oil diet (high in LA) compared to a FO diet (Ganga et al., 2011). In a more recent trial however, there were no significant differences in plasma cortisol after a confinement stressor for salmon fed a soy oil diet compared to fish fed diets with FO, olive oil or rapeseed oil (N.H. Sissener, unpublished results).The cortisol response to acute stress in Senegalese sole (*Solea senegalensis*) post-larvae increased with dietary supply of ARA (up to 1.7% of feed) (Alves Martins et al., 2013, Alves Martins et al., 2011). However, different types of stressors are not affected in the same manner by dietary ARA. While sea bream larvae fed high ARA exposed to an (acute stress) handling stressor had reduced mortality, repetitive salinity changes

(chronic stress) caused increased mortality (Koven et al., 2003). Salmon fed EPA + DHA at 5 g/kg had elevated basal and post-stress (lowering of water stand) plasma cortisol levels compared to fish fed higher levels of EPA + DHA (from 9 to 17 g/kg) (Sissener et al., 2016a). Dietary EPA + DHA at 5 g/kg was also not sufficient to maintain growth or DHA content in red blood cells. To support growth and red blood cell DHA level, 9 g EPA + DHA kg feed was required. This indicates that the dietary EPA + DHA must be at a very low level to affect plasma cortisol. Hence, dietary EPA + DHA levels well below minimum requirement results in chronically elevated plasma cortisol in salmon. The effects of dietary FA on cortisol production might stem from their interaction with the HPI axis, as ARA and EPA has been demonstrated to induce cortisol release in interrenal cells of gilthead seabream (Ganga et al., 2006). When the COX pathway was inhibited, the increasing effects of EPA and ARA on cortisol release was removed. The effect was therefore likely mediated by the COX products of ARA and EPA. Dietary n-6 and n-3 PUFA affect the eicosanoid production in fish (section 1.3.4) and the production of many eicosanoids have been demonstrated to be significantly affected (most reduced, and some increased) in salmon intestine after stress (Olsen et al., 2012). Thus, there is likely merit to the hypothesis that dietary FA can affect the stress response in salmon.

2 AIMS

The overall goal of the present doctoral thesis was to investigate the effect of dietary EPA, DHA and n-6 FA on the robustness of Atlantic salmon exposed to challenging environmental conditions. Four specific sub-aims were defined to achieve the main goal.

I: Determine whether the requirement of EPA and DHA is affected by dietary n-6 FA level (**Paper I**).

II: Investigate which is more important of the absolute and relative level of dietary n-3 and n-6 FA in determining the tissue FA composition (**Paper I, Paper II**).

III: Elucidate the impact of dietary n-3 FA, n-6 FA and their ratio on fish health and proper stress coping ability (**Paper II**).

IV: Understand how dietary EPA and DHA ranging from 10 to 35 g/kg feed will affect liver lipid metabolism in salmon kept in open sea cages during a full production cycle (**Paper III**).

3 METHODOLOGICAL CONSIDERATIONS

3.1 Experimental design

The greater part of the lifespan of an aquaculture salmon takes place in the sea water phase, during which most of the losses also occur. Around 15% of all farmed salmon died during the seawater phase in Norway in 2020 (Oliveira et al., 2021), which is both of major welfare and economic concerns. Salmon farming comprises of many stressors from environment and management, among others temperature, salinity and oxygen fluctuations, transportation, delousing and diseases, which all contribute to mortalities (Oliveira et al., 2021). Being able to respond appropriately to such challenges is essential for the fish. However, should these events become chronic or repeated they could lead to allostatic overload, meaning that fish will no longer cope suitably with additional stressors (Höglund et al., 2020, Madaro et al., 2015). Recent trials have indicated that suboptimal lipid nutrition can contribute to a less robust fish (section 1.3.1, 1.3.2 and 1.5.2). Many studies on FA requirement have been performed in indoor tanks, as short-term, and/or under controlled conditions (i.e. Sanden et al., 2016, Liland et al., 2013a, Ruyter et al., 2006, Tocher et al., 2003). However, the real aquaculture setting is in open sea cages, long-term and the fish will likely face many challenges during production. Such short-term, land based, controlled trials cannot give the full answer to what the practical requirements are (reviewed in Sissener et al., 2016a). There is therefore a need for knowledge on the practical requirements of EPA + DHA during long-term feeding under challenging conditions, and how optimal lipid nutrition can contribute to a healthy, robust salmon capable of handling the challenges it will be faced with. Therefore, the trials in the current thesis aimed to test how differences in dietary n-3 and n-6 FA would affect salmon stress response and robustness under more challenging conditions.

The diets used in **Paper I and II** were designed to elucidate the effect of dietary n-6 FA on the requirement of EPA + DHA, and whether the absolute or relative levels of n-3 and n-6 FA would be more important. Increasing the dietary n-6 FA leads to increased n-6 FA tissue levels (Sissener et al., 2017b, Bransden et al., 2003), and decreasing dietary n-3 FA decreases tissue n-s FA levels (Rosenlund et al., 2016, Sissener et al.,

2016c). As many earlier trials have simply exchanged FO for VO, the dietary n-6 level has been increased concomitantly with reduced dietary n-3 FA, making it challenging to separate the effects of relative and absolute dietary contents. For **Paper I and II**, three diets with equal EPA + DHA + ALA were formulated. However, they had increasing dietary LA, which resulted in dietary n-6/n-3 ratios of 1, 2 and 6. This design allowed for investigation of the effect of dietary n-6/n-3 ratio on the tissue levels of EPA and DHA. While an n-6/n-3 ratio of 1 and 2 could realistically be used by the industry, a ratio of 6 is more extreme but was included to provoke possible effects. The dietary EPA + DHA in these three diets was 10.8, 10.8 and 11.5 g/kg, respectively. Dietary EPA + DHA were chosen to be close to minimum requirement to ensure effects of increasing n-6 were not masked by high dietary levels of these FA. A final diet formulated with twice as much EPA + DHA + ALA was also included (EPA + DHA at 19.8 g/kg), but with an n-6/n-3 ratio of 1 like the first diet (See Figure 4 for a schematic overview of diet design). This permitted us to separate the effects of relative and absolute dietary n-6 and n-3 FA levels. In **Paper I**, fish fed these diets were kept under controlled, stable conditions. Meanwhile, half of the fish in **Paper II** were exposed to a repetitive stressor (oxygen reduction) over four weeks, and the other half were used as controls. At the end of the trial, all fish (stressed and unstressed controls) were subjected to an acute stressor (lowering of water stand). This permitted investigation of the impact of dietary FA composition on the stress response.

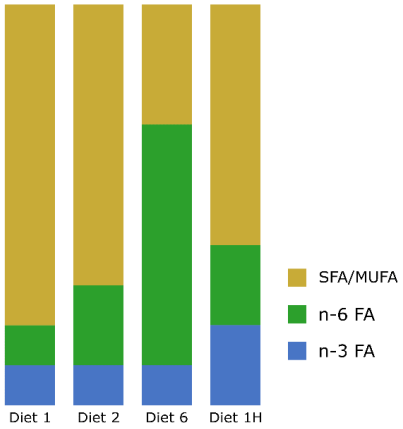

Figure 4: Schematic overview of the dietary design in **Paper I** and **II**

Based on earlier studies, the current practical requirements of EPA + DHA for salmon in the sea water phase are thought to lie between 10 and 16 g/kg feed (section 1.3.1). The diets used in **Paper III** were formulated to contain 10, 13 and 16 g/kg EPA + DHA in the total feed to determine the practical requirement of EPA + DHA. The fish were fed these diets for 14 months in open sea cages, where they were exposed to variations in environmental conditions and management procedures such as delousing and a natural outbreak of cardiomyopathy syndrome (CMS). This aided in establishing the practical level of requirement, as opposed to minimum requirements determined under strictly experimental conditions. A diet with 35 g/kg EPA + DHA was included to investigate whether beneficial effects of further increasing the dietary level of these FA could be seen. The practical requirement of EPA + DHA can vary with life stages and environmental conditions. The requirement can also be dependent on other nutrients in the feed, such as n-6 FA (section 1.3.2). Hence, the trial in **Paper III** gave the opportunity to study the effects of long term feeding of different dietary levels of EPA + DHA in a more realistic representation of aquaculture. Results on growth, welfare and fillet quality from this trial have been published in Lutfi et al. (2022), while effects of these diets on the stress response are presented in Selvam et al. (2022). Work performed in the present thesis investigated the effects of these diets on the hepatic lipid

metabolism **(Paper III)**, as dietary EPA + DHA is known to be intricately entangled with a well-functioning lipid metabolism in salmon.

3.2 Method for eicosanoid analysis in salmon liver tissue

Commonly used methods for investigation of eicosanoid levels in tissues are antibody-based assays, such as enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) (Liakh et al., 2020, Araujo et al., 2008). However, they are prone to over-estimation of the analyte caused by cross-reactivity of the antibody with related metabolites resulting in reduced selectivity and a lack of specificity, in addition to only analysing one eicosanoid at a time (Araujo et al., 2014, Il'yasova et al., 2004). An alternative method overcoming many of these shortcomings, is LC-MS/MS, which can clearly distinguish between the different analytes and can also analyse a larger suite of eicosanoids at a time (Araujo et al., 2014).

The method used in the work of this thesis for analysis of eicosanoids was an LC-MS/MS method, which was first developed for cod gonad (Janagap et al., 2012). Briefly, this method included crushing the sample to a fine powder in liquid nitrogen, double extraction with acetonitrile (ACN) + chloroform, vacuum drying of extraction solvent, dilution of dried sample in ACN and lastly, injection to a liquid chromatography-ion trap mass spectrometry (LC-IT-MS) system. Though not previously used on liver tissue, the method was deemed to have the potential for use on many different biological tissues. Some minor adjustments were made for further improvement in our work with liver, with the intent of preservation of the sample integrity and increased method sensitivity.

Samples were kept cold (storage at -80°C, pulverisation in liquid nitrogen, weighing on ice) at all times prior to extraction, to ensure no, or at least minimal, post-mortem effects on the tissue eicosanoid levels due to thawing. Out of nine different extraction solvent mixtures[2], a 1:1 mixture of ACN and chloroform had significantly best extraction

[2] dichloromethane, chloroform, ACN, hexane, methanol, ethanol, hexane plus methanol:water (3:1), chloroform plus methanol:water (3:1) and acetonitrile plus chloroform

efficiency (recovery of 99.4 ± 0.4% for PGE_2) (Janagap et al., 2012). Therefore, this solvent was chosen for the present work. An additional filtration step was then included, which resulted in the removal of small particles, thus providing a cleaner sample. This helped prevent clogging of the column and protected the HPLC pump. Both methanol and ACN was tested for re-dissolving of the dried sample. Methanol was found to result in lower background noise than ACN, thus providing increased sensitivity.

The basic principle of liquid chromatography mass spectrometry (LCMS) consist of selecting and fragmenting precursor ions (corresponding to the molecular weight of the analytes minus one proton in the present case) which are separated in time (LC component) and detected in the MS component by their mass-to-charge ratio (m/z). Each analyte creates a characteristic fragmentation pattern even though they have the same molecular weight. However, there are molecules with identical molecular weight and fragmentation pattern, consequently it is vital to separate them in the LC component. For instance, PGD_2 and PGE_2 have identical precursor ion (351 m/z) and fragmentation pattern (333, 315, 271 m/z) and their identification is possible after their separation in the LC component prior to MS analysis. The MS/MS system searches for only one analyte (fragmentation pattern) at a time, and cycles continuously through the analytes specified by the analyst. Searching for many analytes per injection will as a result reduce sensitivity.

As proposed by Janagap et al. (2012), this method definitely has the potential to be used for different tissue types, and in the present thesis was successfully applied on liver samples. We have also successfully applied it on Atlantic salmon head kidney tissue (unpublished data).

3.3 Assessment of stress response in salmon

When quantifying stress, the activity of the HPI axis itself can be measured (the primary response; catecholamines, cortisol, etc.) or the physiological or behavioural responses (secondary and tertiary responses) that changes by the actions of the HPI axis. We analysed plasma cortisol as an indicator of activation of the HPI axis. Cortisol is lipid soluble and cannot be stored in the cells. Therefore, cortisol must be produced prior to

release, a process which takes a few minutes. This means cortisol is unaffected by sampling effects, provided it is sampled within minutes after exposure to the stressor (Ellis et al., 2012). Cortisol should, at least under normal circumstances, leave circulation a few hours after an acute stressor. A chronic stressor can cause a reduced HPI-axis activation (Höglund et al., 2020, Madaro et al., 2015) and thus cause impaired cortisol response to an acute stressor. Increases in plasma glucose from higher catabolic activity for energy and plasma lactate formed during anaerobiosis occur as a secondary response induced by the catecholamines and cortisol (reviewed in Sopinka et al., 2016). Increases in plasma glucose and lactate post-stress can thus be used as proxy measurements of the HPI-axis. Still, care should be exercised when considering glucose as a stress indicator, at least by itself. Many other things, such as time since last feeding, life stage and diet type can affect the blood sugar level (Martínez-Porchas et al., 2009). Cellular stress responses occur simultaneously as these processes aid the fish in handling the stressor. These can be HSP, which have been documented as useful stress markers in some cases, but the response varies according to which stressor is applied (section 1.5.1). Hence, their use for measuring the stress response is justified, but it should be considered whether the HSP used is responsive to the investigated stressor. Many genes are also responsive to stress, and different tools for analysis of gene expression are widely used for interpretation of fish response to stress. A large suite of markers was considered to determine the stress response of the present experiment (**Paper II:** plasma cortisol, plasma chloride, plasma glucose, haematocrit, gene expression, eicosanoids, brain serotonin levels, feed intake). As no single stress indicator by itself can decisively confirm stress, an array of indicators provides a more complete picture of the status of the stress response of the fish.

In the stress trial in **Paper II**, half the fish were exposed to a repeated hypoxia stressor. First exposure to the stressor resulted in considerable decrease in feed intake, indicating a stress response taking place (reviewed in Conde-Sieira et al., 2018). The lack of differences in final weights and other stress indicators suggest that adaptive responses took place. The intent with this trial was not to kill the fish, but to introduce sub-lethal chronic stress effects and see if we could improve the stress response by altering the

dietary FA composition. Possibly, the hypoxia stressor was too mild to induce major stress responses in the fish and therefore so few effects were seen. However, due to delays in delivery of equipment, instruments for measurement of water cortisol level (as a passive stress measurement) in all tanks were installed after the commencement of the trial. Additionally, the repeated stressor involved stopping the flow-through of water in the tanks to induce hypoxia. The cortisol measuring equipment had to be removed each time water flow was stopped, so as not to give erroneous differences between groups when the water was stagnant in one group and flowing freely in the other. This very likely induced unintended stress in the control fish. In support of this, the feed intake prior to the installation had started to differ between control fish and repeatedly stressed fish, with the exception of control fish fed diet 6 whose feed intake was similar to the hypoxia exposed fish. However, this trend disappeared after the fish were disturbed for instalment of the equipment. A combination of a stressor that was too mild and the control fish unfortunately being disturbed during the course of the trial could have led the control fish to having similar stress levels as the repeatedly exposed fish.

3.4 Metabolomics

A metabolite is defined here as a small biochemical compound (partially) water soluble and less than 1 kDa (Beyoglu and Idle, 2013, Kosmides et al., 2013), including small peptides, amino acids, sugars, lipids, steroids, xenobiotics and more. The metabolome is defined as metabolites in a biological matrix (cell, tissue, organ), and represents an organism's metabolic phenotype. A wealth of information can be obtained from all omics approaches. However, the complexity of the investigation increases as we move up the "Omics cascade" (Figure 5), whereas the genome is assembled by four nucleic acid building blocks, proteins are vastly more complex with 20 different amino acids to be constructed from. Proteins still adhere to a relatively simple primary structure that can be directly linked to the genes (Fernie and Stitt, 2012). However, inferring the structure and biochemical properties of metabolites from these same genomic sequences is impossible (Fernie and Stitt, 2012). The metabolome is partly a downstream product of the genome, but it is sensitive to changes in environmental or culturing conditions,

including dietary changes. Analysis of the metabolome after dietary treatment can thus give a momentary metabolic snapshot of the physiological effects of said diets (Alfaro and Young, 2018, Patti et al., 2012). Additionally, most metabolites are not species-specific, as opposed to genomic and amino acid sequences. Consequently, the method does not need to be re-established for every new model species and can also be applied without prior knowledge of the genome. However, the fact that there is no template, as there is for genomic and proteomic approaches, is also a major drawback of this method. Accurate validation of fragmentation patterns and retention times needs to be performed by comparison to already established reference libraries for confident identification of metabolites (Schrimpe-Rutledge et al., 2016). With hundreds to thousands of metabolites in a single dataset, there will undeniably be performed so many statistical analyses that false positives in statistical analysis will occur. This must be corrected for. In the present study, an estimate of the False Discovery Rate (FDR) was calculated for each metabolite to take this into account. The q-value is similar to the p-value, except it is a measure of significance in terms of the FDR and not the false positive rate[3]. For further details in the FDR method used see (Storey and Tibshirani, 2003). A low q-value indicated high confidence in the result. While a high q-value indicated diminished confidence, a significant result was not (and should not) automatically be disregarded due to a higher q-value. Confidence in the result could be augmented by the metabolite being included in the same biochemical family or pathway as other affected compounds. The comprehensive interpretation of significant results gives more confidence than focussing on the results of single metabolites. The exploratory nature of this technique also means that untargeted metabolomics is more of a hypothesis-generating method rather than hypothesis-validating.

[3] "The false positive rate is the rate that truly null features are called significant. The FDR is the rate that significant features are truly null. For example, a false positive rate of 5% means that on average 5% of the truly null features in the study will be called significant. A FDR of 5% means that among all features called significant, 5% of these are truly null on average". Quoted from (STOREY, J. D. & TIBSHIRANI, R. 2003. Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A,* 100**,** 9440-5.

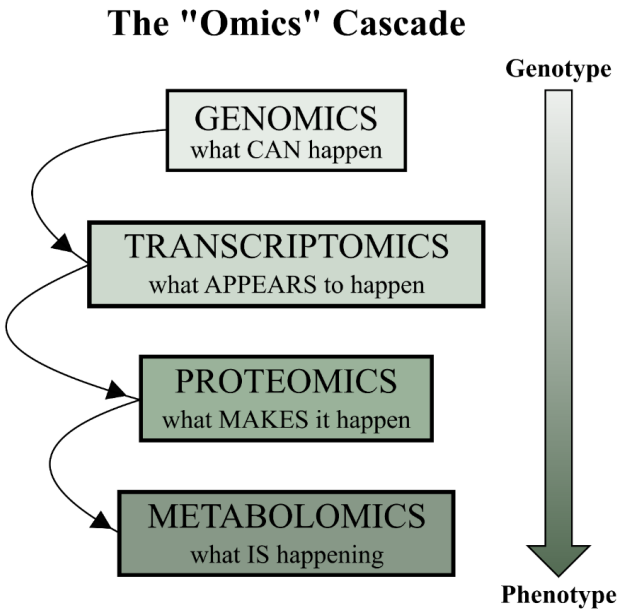

Figure 5: Schematic overview of the hierarchical order from genotype to phenotype of the "Omics" cascade, from genomics, through transcriptomics, proteomics and finally metabolomics. Adapted from Alfaro and Young (2018).

4 INTEGRATED DISCUSSION

4.1 Growth performance and mortality

The current thesis involved two trials (growth trial and stress trial) using the same diets (**Paper I** and **II**), where fish were fed varying relative and absolute levels of n-3 and n-6 FA. A third trial was performed testing the effect of these same diets on the disease progression of amoebic gill disease (AGD) (Selvam et al., 2021). The main aim of the present thesis was to investigate the effect of dietary FA on Atlantic salmon robustness, and investigating what effect dietary n-6 FA would have was a key sub-aim. Due to the role of n-6 FA in eicosanoid production (section 1.3.4), and the indications of the effects on the stress response (section 1.5.2), a negative effect of higher dietary n-6 FA was expected under challenging conditions. When looking at the growth of these three trials, we already here see a confirmation of this hypothesis. In the growth trial, where the fish were kept under calm, stable condition in land-based tanks, no negative effects were seen of increasing the dietary n-6 FA content (stable dietary n-3 FA) (**Paper I**, Figure 6A). Fish fed a dietary n-6/n-3 ratio of 6 even had numerically (but not significantly) better growth than fish fed ratios of 1 and 2 with the same n-3 level, indicating no adverse effects of high dietary n-6 FA levels on growth in this trial (**Paper I**). Similarly, other trials where salmon were fed diets with large variations in n-6 FA and n-6/n-3 ratios did not find differences in growth (Katan et al., 2019, Sissener et al., 2017b, Liland et al., 2013b). The fish in these trials were all kept in tanks under ideal conditions, similar to the fish in **Paper I**. However, fish in the stress trial fed a higher n-6/n-3 ratio performed significantly poorer in terms of growth (**Paper II**, Figure 6B). These growth data support that all fish, control fish included, likely had been subjected to stress, as hypothesised in section 3.3. Likewise, in the AGD trial fish fed the highest dietary n-6/n-3 ratio had a significantly reduced final weight compared to the other diet groups (Selvam et al., 2021)(Figure 6C). These fish were exposed to both to AGD and repeated handling stress due to weekly samplings. AGD and handling stress combined could have been a more severe stressor, leading to the much lower final weight in fish fed the highest n-6/n-3 ratio. This demonstrates that a high dietary n-6 content can have detrimental effects under challenging conditions, as will occur in a commercial setting.

There is also a clear need for testing the dietary impact under challenging conditions to reveal the true effects of dietary alterations.

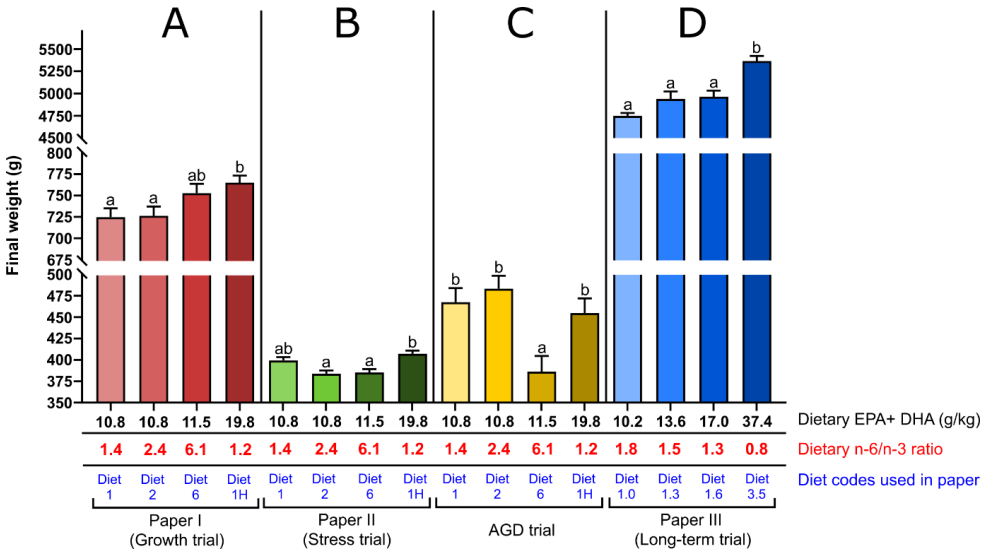

Figure 6: Final weights (g) of Atlantic salmon fed diets with different EPA + DHA in g/kg (black numbers) and n-6/n-3 ratio (red numbers). The diet codes used in the different papers are marked in blue. A) n-6/n-3 growth trial (**Paper I**), B) n-6/n-3 stress trial (**Paper II**), C) AGD trial (Selvam et al., 2021), D) EPA + DHA long-term growth trial (**Paper III**). Statistical difference (within each trial) denoted by different letters.

An increased growth as a result of higher dietary EPA + DHA was found in the work included in the present thesis (**Paper I, II** and **III**). Dietary EPA + DHA at 10 g/kg has been deemed to be at the lower end of requirement range suggested for salmon (section 1.3.1). We found significantly improved growth when increasing dietary EPA + DHA from ~11g/kg to 20 g/kg (**Paper I** and **II**, Figure 6A, B). Further, salmon fed a diet with 35 g/kg EPA + DHA had significantly higher growth than fish fed 16 g/kg (Figure 6D, **Paper III**), also reported in (Lutfi et al., 2022). Therefore, this thesis confirmed the assumption that 10 g/kg EPA + DHA is insufficient for optimal growth under challenging conditions, hence would likely be insufficient under practical farming conditions. However, there were also indications that 16 g/kg EPA + DHA, previously assumed to be sufficient (Bou et al., 2017b, Sissener et al., 2016c), is inadequate for a normally functioning hepatic lipid metabolism (**Paper III)** and optimal growth, welfare, robustness and fillet quality when in open sea cages through a full production cycle

(Lutfi et al., 2022). The difference between dietary groups in **Paper III** were found towards the end of the long-term trial, during a stressful period where the fish were exposed to mechanical delousing and there was a natural outbreak of CMS (Lutfi et al., 2022). After both of these events, fish fed EPA + DHA at 35 g/kg were able to resume normal feeding faster than the lower EPA + DHA dietary groups.

As expected, no mortalities were recorded in the n-6/n-3 growth (**Paper I**) or stress trials (**Paper II**). However, as will be further discussed in later sections, the robustness of fish fed the higher dietary n-6/n-3 ratios was reduced. In commercial settings, this could have caused higher mortality rates. Accordingly, higher mortalities have been registered in salmon fed a high n-6 diet after weighing (Liland, 2014) or transport (Bell et al., 1991a). During the full-scale trials described in **Paper III**, total mortality averaged at 7.5 % and were not significantly affected by diet (Paper III, Lutfi et al., 2022). The observed mortality is low compared to the industry average in Norway of 15 % in the seawater phase (Oliveira et al., 2021). This indicates that although this trial was more commercial-like than many previous trials, it was probably not as challenging as commercial aquaculture. The trial was run in the northern part of Norway, were there is lower summer temperatures, less salmon lice, and also less mortality than found in the southern and western parts of Norway (mortality below 14 % in the north and ~20 % in the southwest) (Oliveira et al., 2021). Despite no differences in mortality in the trial in **Paper III** overall, towards the end of the trial during a CMS outbreak some interesting trends were observed. There was an increase in mortality during this disease outbreak, and a tendency that fish fed dietary EPA + DHA at 35 g/kg had lower mortality caused by CMS compared to fish fed the lower levels of EPA + DHA (10, 13 and 16 g/kg). Cause of death was confirmed by veterinarian (Lutfi et al., 2022). In contrast, there were no differences in mortality in salmon fed 16 or 26 g/kg EPA + DHA in a full-scale trial, despite outbreak of both CMS and pancreatic disease (Sissener et al., 2016c). However, neither of these trials were specifically designed to test whether increased dietary EPA + DHA could improve the resistance to these diseases. Salmon fed functional feeds with lower lipid levels and higher EPA content demonstrated a milder and delayed inflammatory response, and less severe heart lesions both at early

and late stages of CMS (Martinez-Rubio et al., 2014). Nevertheless, histoscoring and gene expression of atria and ventricles from the fish in **Paper III** did not detect any significant diet effects during this disease (Lutfi et al., 2022). Since CMS is an inflammatory disease, it is still likely that EPA can have an effect on the disease progression both through initiation and resolution of inflammation via eicosanoids and resolvins. Further testing is required to see whether a high level of EPA + DHA in the feed will provide significant disease resistance.

Observations of growth and mortality in the present trials emphasise that high dietary n-6 FA can negatively impact growth – but only under challenging conditions (**Paper I** and **II**). Differences in growth due to dietary EPA + DHA will also be found, even under non-challenging conditions, but they will be amplified after stressful events (**Paper I, II and III)**. Mortality in sea cages was further only affected by dietary EPA + DHA after an outbreak of disease (**Paper III**), indicating higher robustness in fish fed EPA + DHA well above the assumed minimum requirement, supporting that the practical requirement is higher. In summary, many beneficial effects of an optimised dietary FA composition are revealed when the fish are faced with fluctuating environmental conditions. This should be kept in mind when future trials are designed.

4.2 Dietary lipids and liver metabolic health

The liver is the metabolic hub for salmon, and is the master regulator for lipid metabolism. It is involved in uptake of dietary lipids, lipogenesis, β-oxidation as well as the synthesis of TAG and lipoproteins for transport to other organs (section 1.2). An imbalance between these processes of utilisation and storage of lipids will cause either depletion or accumulation of hepatic fat. It also means that a change in the hepatic lipid metabolism could affect the entire fish, as the liver is responsible for packaging lipids to be sent to other organs. A recurring result of the changes made to the dietary FA composition in the trials of the present thesis was altered lipid accumulation in the liver (**Paper I, II** and **III**). Increased hepatic lipids were seen with lower dietary EPA + DHA (**Paper I, II** and **III**, Figure 7A, B and C), which has been commonly observed in trials altering the EPA + DHA in the feed (section 1.4). Further, a high dietary n-6 FA content resulted in increases in liver storage lipids (**Paper I** and **II**, Figure 7A, B). An elevated

level of dietary OA was also associated with higher liver fat contents in all trials (**Paper I, II** and **III**). Therefore, by the logic of hepatic lipid accumulation being the result of an imbalance between the processes of lipid storage and utilisation, these results mean that the diets included in this thesis have disrupted the equilibrium between these processes. In mammals, such perturbations of lipid metabolism are strongly linked to the development of metabolic disorders (de Castro and Calder, 2018). Whether this poses a risk to fish welfare has not yet been determined. However, in an experiment keeping salmon in large, open sea cages, unexpected dramatic increases in mortalities in seemingly healthy salmon were found during the winter period after transfer to a feed higher in lipids (Dessen et al., 2021). Moribund fish had significantly higher liver fat, altered hepatic FA composition and elevated plasma alanine aminotransferase (ALAT) and alkaline phosphatase (both markers of liver damage), indicating that such changes to the liver fat levels are related to reduced robustness and welfare. Plasma asparatate aminotransferase (ASAT) and ALAT were measured in the long-term EPA + DHA trial (**Paper III**) and the growth trial (**Paper I,** unpublished data), however no differences between dietary groups where found in any of these studies. The plasma ASAT and ALAT levels ranged from 326 – 364 U/I and 6.1 – 73 U/I, respectively, in fish from **Paper III** (reported in Lutfi et al., 2022), and from 1317 – 1479 U/I and 21.9 – 25.0 U/I, respectively in **Paper I** (unpublished data). Though the differences between the two trials is relatively large, the numbers are within the range of previously reported levels (Sissener et al., 2017a, Sissener et al., 2013).

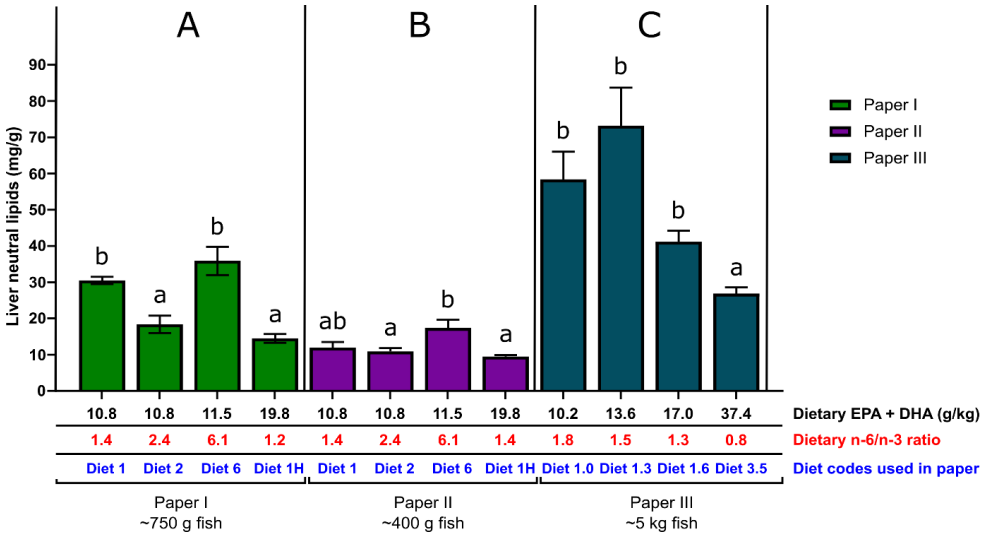

Figure 7: Liver neutral lipids of Atlantic salmon fed diets with different EPA + DHA in g/kg (black numbers) and n-6/n-3 ratio (red numbers). The diet codes used in the different papers are marked in blue. A) n-6/n-3 growth trial (**Paper I**), B) n-6/n-3 stress trial (**Paper II**), C) EPA + DHA long-term growth trial (**Paper III**). Statistical difference (within each trial) denoted by different letters.

Reduced dietary EPA + DHA corresponded to higher hepatic lipid accumulation (**Paper III**). This increase also correlated significantly with liver phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which could indicate a higher occurrence of lipid droplets (**Paper III**). Incidentally, when livers from fish in the same study were investigated histologically, a tendency of increased lipid droplet accumulation was found in fish fed the lowest dietary EPA + DHA (Lutfi et al., 2022). Other studies also found more and larger lipid droplets in liver of salmon fed less EPA + DHA (Sanden et al., 2016). A trial comparing a 100% FO diet to a diet with 100% VO (55 % rapeseed oil, 30 % palm oil, 15 % linseed oil) found significantly increased hepatic TAG stores and reduced plasma VLDL, suggesting a decreased hepatic production of VLDL (Jordal et al., 2007). The observed increase in hepatic lipids and lipid droplets in fish fed lower dietary EPA + DHA in **Paper III** could at least partially be caused by an inability to secrete excess lipids as VLDL. Maintaining constant dietary EPA + DHA while increasing the dietary LA resulted in significant decreases of EPA in liver polar lipids (**Paper I**), corresponding to higher liver fat levels (**Paper I and II**), which could suggest

that the observed effects on liver lipids is related to levels of EPA in polar lipids. The higher dietary LA also resulted in significantly higher ARA in liver polar lipids (**Paper I**) and significant increases in hepatic levels of the ARA derived eicosanoids PGE_2 and PGD_2 (**Paper II**). Both of these eicosanoids inhibit the secretion of VLDL in rat hepatocytes (Perez et al., 2006, Björnsson et al., 1992). There are thus indications that both a lower EPA + DHA and a higher n-6 FA could impede VLDL secretion of the liver.

Reduction in the dietary FO in exchange for other lipid resources alters the FA composition of the feed (see Table 1). This typically leads to reductions in EPA + DHA, and increases in LA and OA, which usually corresponds to prominent increases in liver fat (**Paper I, II, III**, section 1.4). The neutral lipids are more susceptible to dietary influence than the polar lipids (**Paper I, II, III** Sissener et al., 2020, Bou et al., 2017b, Ruyter et al., 2006). Xu et al. (2020) hypothesised that FA commonly found in FO alternatives, such as OA and LA, are inefficiently metabolised, thus resulting in their accumulation. Accordingly, OA, which is common to many VO, is likely metabolised slower than other FA in salmonids (**Paper III**, Caballero et al., 2002) and it accumulates in liver with increasing dietary content (**Paper I** and **III**). Higher TAG accumulation, larger lipid droplets and less active mitochondria were reported in HepG2 cells stimulated with OA compared to control (bovine serum albumin) and palmitic acid (Eynaudi et al., 2021). LA, another prevailing FA in many VO, is deposited in liver when present in high amounts in the diet (**Paper I and III**). Although *in vitro* trials with salmon hepatocytes showed that n-6 FA were good substrates for β-oxidation (Stubhaug et al., 2005b), a 2-fold increase in dietary LA resulted in a 1.7-fold higher deposition of this FA into liver neutral lipids (**Paper I**). When the dietary content of LA increased 6-fold, a 5.3-fold increase of this FA was seen in the liver neutral lipids **(Paper I**). This could suggest that deposition reflects the feed, and the β-oxidation of LA *in vivo* is neither more nor less effective than for other FA. Similarly, 1.2-fold and 1.6-fold increases of dietary OA resulted in exactly 1.2- and 1.6-fold increases of this FA in liver neutral lipids, respectively (**Paper I**). The changes in dietary OA was also mirrored exactly in the liver neutral lipids in **Paper III**. The level of OA in the liver neutral lipids

was further consistently higher than in the diet (**Paper I and III)**, indicating that it accumulates at the expense of other FA.

The FA 16:0, 20:1n-9, 22:1n-9 and 24:1n-9 are more prevalent in FO than VO (Table 1), and are all good substrates for β-oxidation in salmon (Turchini and Francis, 2009, Stubhaug et al., 2005b, Bell et al., 2003). For OA and LA, it is plausible that they are not very efficiently used by the fish even when present in high amount and thus accumulate, in agreement with the proposition by Xu et al. (2020). Correspondingly, in **Paper III** it was found that reducing the dietary EPA + DHA (concomitantly increasing LA and OA) caused disturbances in the hepatic lipid metabolism. The metabolomics data indicated that fish fed 10 g/kg EPA + DHA had an impaired β-oxidation capacity, and had to rely on other pathways for energy production, thus leaving lipids to accumulate (**Paper III**). When the fish were fed 16 g/kg EPA + DHA the metabolic profile improved, however it was more similar to fish fed 10 g/kg than the fish fed 35 g/kg (**Paper III**). Accordingly, clear lipid accumulation was found in the liver of fish fed lower dietary EPA + DHA. We hypothesised that this disturbed energy homeostasis was at least partly a result of perturbation of the mitochondrial membrane, which in mammals has been demonstrated to inhibit hepatic mitochondrial β-oxidation (Hollie et al., 2014).

In summary, disturbances in the dietary balance of FA seem to cause accumulation of lipids in the liver, albeit likely by several different mechanisms. Too much n-6 or too little EPA + DHA seems to cause problems with VLDL secretion, too little EPA + DHA leads to problems with FA oxidation, and too much OA results in its accumulation as it is slowly metabolised. Further, when altering the percentage of one FA, inclusion of other FA will necessarily be modified. Therefore, when the FA composition is out of balance, there will be several lipid factors contributing to accumulation of fat in the liver. Interestingly, in livers of fish fed diet 2 in trial I and II, there was a lower accumulation of neutral lipids compared to diet 1 and 6 (**Paper I, II,** Figure 7A, B), despite higher n-6 levels than diet 1 and higher OA levels than diet 6. It was hypothesised that diet group 1 might have accumulated liver lipids due to high OA, while diet group 6 had more liver fat due to high dietary n-6 FA. Diet 2 meanwhile,

might have a more beneficial balance between these two FA which did not allow either of them to induce excessive lipid accumulation. This once again supports the hypothesis that an ideal balance between FA is important.

4.3 Membrane functionality and fluidity as a function of its fatty acid composition

Phospholipids are the major components of cellular membranes, and their n-3 and n-6 content has significant impacts on the membrane fluidity. The flexibility of the n-3 and n-6 acyl chain is very dissimilar, and elimination of even a single double bond can greatly affect membrane fluidity (Rajamoorthi et al., 2005, Eldho et al., 2003). The diets used in all trials in this thesis greatly affected the n-6 and n-3 FA composition of both storage lipids and cell membranes in all tissues analysed [4] (**Paper I, II, III**). Although the effects were smaller and more selective in membrane lipids than storage lipids, highly significant effects were still found (**Paper I, II, III**). These changes will have affected the physical properties of the cell membrane. In **Paper III**, it was suggested that part of the reason for the impaired hepatic lipid metabolism with low dietary EPA + DHA was perturbation of the mitochondrial membrane, and further that this response could be reinforced at low temperatures. That the effects of decreased levels of EPA + DHA in the feed on liver lipid accumulation are enhanced at low temperatures have been shown previously (Sissener et al., 2017c, Ruyter et al., 2006). This would altogether concur with results of Wijekoon et al. (2021), who investigated the fluidity of membrane in the liver of rainbow trout fed varying dietary EPA + DHA at different temperatures. Their results demonstrated that fish fed higher n-3 level were able to adapt the membranes' physical properties to changing temperature (from 13, to 18, to 13 °C again) much better than fish fed the lowest dietary n-3. Somewhat opposite to intuitive thinking, the low n-3 diet resulted in membranes with *increased* fluidity, but the membrane was unstable with changing temperatures indicating reduced membrane functionality. Notably, the diet lowest in n-3 FA was also highest in n-6 FA, resulting in the corresponding changes to membrane FA composition.

[4] FA composition investigated in liver neutral and polar lipids, whole body, muscle, red blood cells, brain neutral and polar lipids, skin phospholipid classes

Dietary cholesterol will co-vary with dietary EPA and DHA when FO is used as an oil source (Liland et al., 2013a). No cholesterol adjustments were made to the feeds in any of the trials, so dietary cholesterol likely varied in the different diets. Cholesterol is an important factor in the fluidity of the membrane (Holthuis and Menon, 2014), so this could have impacted membrane functionality. However, as dietary cholesterol was not measured, this is pure speculation. FO contain higher SFA levels than commonly used VO, such as rapeseed oil (Table 1), and dietary SFA will therefore often vary with dietary EPA + DHA, as seen in **Paper III**, unless balanced by other lipid sources.

The adjustment of phospholipids and their specific FA combo is important to membrane fluidity when adapting to cold (Arts and Kohler, 2009). Which FA occupy the different positions of the phospholipids can affect the physical properties, and which phospholipid species it is incorporated into will also determine what effect it will have (Arts and Kohler, 2009). For example, cold exposure resulted in specific changes in phospholipid FA composition in liver microsomes of carp (*Cyprinus carpio L.*), with increased fraction of 16:1/22:6 PC and 18:1/22:6 PE (Brooks et al., 2002). Further, fish adapted to cold waters (5-10 °C) have much more unsaturated FA in their liver phospholipids than fish adapted to warm water (25-27 °C), including 2-3-fold and 10-fold increases in PE with 18:1/22:6 and 18:1/20:5, respectively (Dey et al., 1993). In the present thesis, there were significant reductions in nearly all PC and PE containing EPA and DHA in the *sn-2* position in the liver of salmon fed 10 and 16 g/kg EPA + DHA compared to 35 g/kg (**Paper III** - supplementary material). These fish were exposed to low temperatures during the winter months (sampled when water temperature was at 5°C), during which fish fed these two diets will likely have had issues maintaining proper membrane fluidity. Modifying the dietary FA composition could affect membrane functionality. Based on the literature, a low dietary EPA + DHA may lead to more vulnerable cell membranes, particularly at low temperatures. Further testing to specifically investigate the effects of dietary FA composition on the membrane would be required to confirm this.

4.4 Dietary lipids and inflammatory response

Inflammation is a component of the immune system, and is a necessary response to injuries and infections by pathogens (Calder, 2020b, Innes and Calder, 2018). The n-3 and n-6 FA bound to membrane phospholipids play vital roles regulating inflammation, through their use as precursors for eicosanoid production (section 1.3.4)(Calder, 2020b, Innes and Calder, 2018), suggesting that FA nutrition can participate in the regulation of inflammation. A well-functioning inflammation is self-limiting and resolves relatively quickly by pro-resolving mediators such as resolvins, protectins and maresins. The most potent pro-resolving mediators are metabolic products of the n-3 FA EPA and DHA (Calder, 2020b). Meanwhile, ARA is involved in the mediation of many pro-inflammatory responses (Innes and Calder, 2018). These pro-inflammatory effects are absolutely necessary to combat infections. However, an unresolved inflammatory response would lead to chronic inflammation which could eventually cause damage to the host. As already discussed in relation to growth (section 4.1) and liver lipid accumulation (section 4.2), an increase in n-6 FA or decrease in n-3 FA might not be problematic under ideal conditions, but when exposed to challenging conditions it might lead to a less robust fish. Therefore, a decrease in dietary (and thus also tissue) content of n-3 FA, an increase in n-6 FA and, more importantly, a higher content of ARA relative to EPA in the membrane, is expected to affect the fish's immunocompetence by impairing the inflammatory response.

Some conclusions on the effect on the inflammatory status of fish fed either decreasing EPA + DHA (**Paper III)** or increasing LA (**Paper II**) may be drawn from the present thesis. After a full production cycle in open sea cages there were several indications that fish fed 10 g/kg EPA + DHA, and to some degree fish fed 16 g/kg, had an elevated inflammatory status compared to fish fed 35 g/kg EPA + DHA (**Paper III**). Significantly elevated levels of most lysophospholipids analysed, particularly lysophosphatidylcholine (LPC), were detected in livers of fish fed 10 and 16 g/kg EPA + DHA (**Paper III).** LPC plays a pro-inflammatory role in the liver (reviewed in Sevastou et al., 2013), with increases in liver tissue during hepatosteatosis (Han et al., 2008). However, the role in inflammation depends on the chain length and degree of

unsaturation (reviewed in Liu et al., 2020b). For instance, LPC 16:0 have pro-inflammatory effects (Hung et al., 2012). Meanwhile, some PUFA lysophospholipids can counteract inflammatory responses, with LPC of 20:4 and 22:6 reversing the inflammatory effects induced by LPC 16:0 (Hung et al., 2012). The anti-inflammatory effects of LPC 20:4 and 22:6 were associated with reduced production of pro-inflammatory mediators (IL-5, IL-6, NO, 12-HETE and PGE_2), leukocyte extravasation, plasma leakage which were stimulated by LPC16:0. This reduction of pro-inflammatory effects was concurrent with a stronger anti-inflammatory response (higher production of IL-4 and IL-10) (Hung et al., 2012). LPC of 20:5 also show anti-inflammatory effects (Hung et al., 2011). In **Paper III**, LPC containing 20:4 and 20:5 were significantly higher, and LPC with 16:0 lower in fish fed 35 g/kg EPA + DHA compared to the lower groups, indicating a lower inflammatory state. High levels of long-chain acylcarnitines, which can mediate pro-inflammatory responses and stimulate the release of proinflammatory cytokines (McCoin et al., 2015, Rutkowsky et al., 2014), were found to be increased in the low EPA + DHA diet groups, particularly in the fish fed 16 g/kg (**Paper III**). Proinflammatory cytokines induce the hepatic enzyme tryptophan 2,3-dioxygenase and the extrahepatic indoleamine 2,3-dioxygenase, which catalyse the first step of the kynurenic pathway (see review by Höglund et al., 2019). Kynurenine is the first intermediate in the kynurenic pathway, and can be transformed into either kynurenate or 3-hydroxykynurenine (Höglund et al., 2019). Elevated concentration of kynurenine and kynurenate were found in fish fed 10 or 16 g/kg in comparison to 35 g/kg EPA + DHA. While none of these metabolites are pro-inflammatory by themselves (Höglund et al., 2019), they signal a higher inflammatory state. There was also less of the EPA derived eicosanoid 5-HEPE in the fish fed diets with 10 and 16 g/kg EPA + DHA compared to 35 g/kg. By reducing the inflammatory reactions in macrophages in adipose tissue, this EPA derived metabolite reduced hepatic steatosis (Wang et al., 2017, Onodera et al., 2017). As discussed in **Paper III**, this adipo-hepatic relationship could have contributed to the higher liver lipid accumulation observed in the lower EPA + DHA group in **Paper III**.

The feeding of rising levels of LA and its impact on inflammation was also investigated (**Paper II**). Dietary LA is readily converted into ARA in salmon (**Paper I**, Sissener et al., 2020, Katan et al., 2019), which again has been shown to promote production of potent ARA derived eicosanoids (**Paper II**). Primary head kidney cells from salmon had greater expression of prostaglandin d synthase and prostaglandin e synthase when a soy oil diet (LA at 38.2 % of TFA) was used compared to palm oil and rapeseed oil (LA at 11.2 and 14.6 % of TFA, respectively) diets. This coincided with higher expression of the pro-inflammatory cytokine interleukin-1β and elevated PGE_2 after stimulation with the bacterial mimic lipopolysaccharide (Holen et al., 2018). However, a diet with even higher soy oil content (LA at 42.9 % of TFA) was also included in the last study, and these mentioned effects were not seen in head kidney cells of fish fed that diet. This indicates that there is no linearity in the inflammatory response to dietary LA. The authors hypothesised that, at least *in vitro,* the highest dietary n-6 level caused a blunted inflammatory response rather than an excessive one (Holen et al., 2018). In Gjøen et al. (2004), fish fed 50/50 soy oil and FO had a stronger inflammatory response than fish fed a pure FO diet. However, fish fed 100 % soy oil had a reduced inflammatory response compared to fish fed 50/50 (soy oil/FO). In **Paper II**, the diet with an n-6/n-3 ratio of 6 had a remarkably similar FA profile to both the high soy oil diet in Holen et al. (2018) and the 100% soy oil diet in Gjøen et al. (2004). We did not, however, find any indication of a blunted inflammatory response, with higher ARA derived prostaglandin production (PGD_2 and PGE_2) discovered in the liver of fish fed an n-6/n-3 ratio of 6 compared to 1 (**Paper II**). On the other hand, the production of LTB_4 (ARA derived) was lower in the liver of fish fed an n-6/n-3 ratio of 1 than fish fed a ratio of 6 prior to acute stress. Though an hour after acute stress, the fish fed an n-6/n-3 ratio of 6 had a much higher LTB_4 content than fish fed a ratio of 1 (further discussed in section 4.5). The expression of interferon γ tended to be higher in fish fed diet 6 compared to diet 1 ($p = 0.053$). There were no differences in the expression of other inflammatory or anti-inflammatory cytokines based on the diet. In the trial by Selvam et al. (2021) (using the same diets as in **Paper I** and **II**), salmon fed diet 6 had reduced LA and increased ARA in the gills (mostly polar lipids) when challenged with AGD compared to non-challenged fish, indicating a higher ARA production when

challenged, possibly for eicosanoid synthesis. In a series of studies in the nineties, Bell and colleagues (Bell et al., 1993b, Bell et al., 1993a, Bell et al., 1992, Bell et al., 1991b) investigated the effect of dietary LA on the production of eicosanoids in various tissues and cell types in salmon. Generally, they reported that increasing the dietary LA led to higher levels of ARA derived eicosanoids. In some cases, they found decreases in eicosanoids from both ARA and EPA, though in these cases the ratio between ARA/EPA derived eicosanoids still increased. More recently, Sissener et al. (2020) found that in plasma a higher concentration of PGE_2 was found with increasing dietary LA, while in liver reductions in the ARA derived LTB_4 and PGE_2 were found. They found that the relationship LTB_4:LTB_5 increased with higher dietary LA, while no effects were seen on PGE_2:PGE_3. In essence, data from the present thesis and most of the literature support a higher inflammatory state when salmon is fed high levels of LA by up-regulation of pro-inflammatory eicosanoids and cytokines. These trials have however all been relatively short. It remains to be seen what the consequences of feeding diets with plant oils containing high levels of LA would be on a longer-term commercial-like setting.

In the present thesis, it was demonstrated that ARA produced from dietary LA will displace EPA in the cell membrane (**Paper I**). However, maintaining a low ratio between n-6 and n-3 FA when decreasing dietary n-3 avoids this issue (**Paper I**). A feeding trial including dietary EPA from 0.5 to 10 % of TFA (all diets had 5 % DHA of TFA) found that despite drastic increases in EPA, membrane bound ARA was stable (red blood cells, liver polar lipids, head kidney polar lipids (Liland et al., submitted). Furthermore, with increased level of ARA in the liver polar lipids, a general increase in ARA derived prostaglandins is seen (**Paper II**). However, increases in membrane bound EPA with stable ARA induced production of both PGE_2 and PGE_3 in primary liver cells of salmon (Liland et al., submitted). It could appear that an increase in EPA does not contribute to a more anti- or pro-inflammatory environment, but contributes to the general stimulation of eicosanoid production. This could mean that feeding more EPA leads to a salmon more capable of combating pathogens and responding to injuries by induction of eicosanoid pathways, as indicated by disease challenge trials (Hatlen et

al., 2016, Martinez-Rubio et al., 2013, Martinez-Rubio et al., 2012). EPA is also the precursor for pro-resolving mediators called resolvins (Calder, 2020a). Though not much investigated in fish, a positive correlation between increased dietary intake of EPA + DHA and higher plasma or serum levels pro-resolving mediators have been demonstrated multiple times in humans (reviewed in Calder, 2020a). Most studies are based on the intake of both EPA and DHA, but is does imply that the observed effects could also be due to EPA. This would mean that EPA can aid in resolving the inflammatory response and thus avoiding damage to its own tissues through induced production of resolvins. Accordingly, Hatlen et al. (2016) found a tendency of improved recovery in salmon infected with pancreas disease and heart and skeletal muscle inflammatory disease (HSMI) when fed diets high in EPA. The possibility that EPA can promote a healthy inflammatory response and increase disease resistance and recovery is intriguing, and involves complicated processes that needs to be studied further.

4.5 Dietary effects on the stress response

In the n-6/n-3 trial, fish were exposed to both a repeated stressor of induced hypoxia/hypercapnia three times per week over 4 weeks, and towards the end of the trial an acute confinement stressor where the water level was quickly lowered and maintained low for 30 minutes. As explained in section 3.3, the repeated stressor was a mild stressor which the fish likely adapted quickly and easily to. This was supported by the feed intake (measured daily), which dropped significantly after first exposure, but was restored to pre-stressor levels the next day, indicating adaptation (**Paper II**). Subsequent reductions in feed intake after exposure to the repeated stressor was not seen. Also in support of adaptation to the repeated stressor was the fact that both control fish and repeatedly stressed fish were able to mount a cortisol response to the acute stressor (**Paper II**). However, the results of imposing this stressor on the fish were probably confounded by the fact that both control fish and stressor-exposed fish were disturbed by the repeated installation and removal of equipment in the tanks mid-trial. A plethora of stress markers in plasma, brain and liver were analysed to investigate the effect of the confinement stressor (acute stress) and confirmed an acute stress response (**Paper II**). An altered stress response in salmon as a result of alterations in dietary FA

composition was evidenced by data on the liver eicosanoids, with PGD_2 and LTB_4 demonstrating different induction after acute stress depending on dietary n-6/n-3 ratio (**Paper II**). PGD_2 is suggested to play anti-inflammatory roles in the liver of mammals (Cubero, 2016, Fujita et al., 2016), and has also been implicated in the resolution of inflammation in gilthead seabream acidophilic granulocytes (Gómez-Abellán et al., 2015). An acute inflammatory response to stress or injury is a beneficial event, and is also characterised by successful resolution. Persisting response can, however, lead to chronic inflammation. Fish fed both an n-6/n-3 ratio of 1 and 6 had a reduction in liver PGD_2 one hour after acute stress. After 24 hours, fish fed dietary n-6/n-3 of 1 were recuperating, as indicated by their increasing PGD_2 levels, which could mean inflammation resolving responses are underway. However, hepatic PGD_2 levels in fish fed a dietary n-6/n-3 of 6 were still decreasing after 24 hours, suggesting a dysfunctional resolution of the inflammatory response with such high dietary levels of LA.

Unchallenged salmon fed high dietary levels of LA demonstrated reduced LTB_4 compared to fish fed lower levels of LA (constant dietary n-3) (Sissener et al., 2020). Contrarily, challenged fish (or primary cells of fish) fed higher levels of n-6 FA exhibit higher levels of LTB_4 (Alves Martins et al., 2012, Bell et al., 1996). Significantly higher levels of ARA were found in salmon liver polar lipids when increasing the dietary LA content (constant dietary n-3) (**Paper I**). Despite this, there was less of the ARA derived LTB_4 in livers of fish fed higher dietary LA (**Paper II**). Oppositely, after being subjected to a crowding stressor there was more hepatic LTB_4 in fish fed the highest dietary LA (**Paper II**). Salmon cell membranes are normally richer in EPA than ARA, provided no deficiency in dietary n-3 in which case n-6 FA will replace n-3 in the membrane (Bou et al., 2017b). In **Paper I** however, it was discovered that a dietary n-6/n-3 ratio of 6 enriched the membrane with ARA and reduced the content of EPA, resulting in more ARA than EPA (dietary n-6/n-3 at 1 and 6 resulted in ARA/EPA ratios at 0.4 and 1.9, respectively). Similarly, Sissener et al. (2020) found that dietary LA exceeding 30 % of TFA will give an ARA/EPA higher than 1. Possibly, when the access to ARA is exceeding that of EPA, the observed shift in LTB_4 production occurs. LTB_4 is a pro-inflammatory mediator tightly linked to liver damage in mammals (Li et al.,

2015, Martinez-Clemente et al., 2010, Lopez-Parra et al., 2008), and altering the dietary n-6/n-3 ratio could then possibly result in a disproportionately strong response to a stressor in salmon. Despite differences in LTB_4 production as a result of diet, no effect of diet was found on the expression on 5-lipoxygenase.

In the long-term trial feeding graded levels of EPA+DHA, the fish were naturally exposed to many different stressors such as fluctuating temperatures, disease and delousing (**Paper III**). After delousing in August 2018, samples were collected for evaluation of the impact of these diets on the stress response (Selvam et al., 2022). Although the data indicated that the mechanical delousing procedure caused substantial stress for the fish, rising dietary EPA + DHA did not alleviate the stress (Selvam et al., 2022). Interestingly, no differences in plasma cortisol were found, even in the group fed 10 g/kg EPA + DHA (Selvam et al., 2022). Accordingly, EPA + DHA at 9 g/kg did not have an impact on plasma cortisol after exposure to an acute stressor (Sissener et al., 2016a). Not until dietary EPA + DHA was reduced to 5 g/kg feed were increases in plasma cortisol found, and then elevated plasma cortisol was observed both before and after the acute stressor (Sissener et al., 2016a). Neither did we find any results of diet on plasma cortisol with n-6/n-3 ratios ranging from 1 to 6 and varying absolute levels of n-6 and n-3 FA (**Paper II**). These results indicate that plasma cortisol production and release is quite resilient to changes in dietary FA composition, suggesting that it is a highly-prioritised function for the salmon.

4.6 Brain fatty acid composition – relation to the stress response

The brain FA composition was stable despite the dietary changes (**Paper II)**, as expected since the Atlantic salmon brain is known to have a conserved FA composition (Bou et al., 2017b). Yet, decreases in polar lipid n-3 FA was seen with higher dietary n-6/n-3, and polar lipids reflected dietary n-6/n-3 ratio while neutral lipids reflected absolute dietary content (**Paper II**). A higher n-6/n-3 ratio in the feed led to increasing n-6 FA contents in the brain polar lipids, and similar dietary n-6/n-3 ratios led to comparable contents of total n-6 FA despite different absolute dietary contents of n-6 FA (**Paper II**). However, compared to other tissues the changes to brain FA

composition were much smaller (**Paper I and II**). Two FA behaved uniquely in brain polar lipid compared to polar lipids in other tissues. DHA was stable at approximately 26 % of TFA regardless of dietary DHA level (**Paper II**). Relatively stable content of brain DHA despite variations in dietary FA composition has also been seen in other studies (Sissener et al., 2016b, Brodtkorb et al., 1997). Dietary EPA + DHA at 5 g/kg did cause numerically reduced DHA compared to 10 g/kg and higher, but only at 6°C and not 12°C (Sissener et al., 2016b). Curiously, OA was equally stable at around 20.5 % of TFA for all dietary groups, despite large variations in dietary level (from 18.2 – 37.1 % of TFA). The studies of Sissener et al. (2016b) and Brodtkorb et al. (1997) also demonstrated this behaviour of brain OA. This is unlike any other tissue in salmon, neutral or polar, as the level of OA in tissues normally is greatly affected by its dietary content. Deuterated dietary OA was not recovered in the rat brain, meaning that it did not cross the brain barrier (Edmond et al., 1998). This indicates that the brain OA does not have a dietary origin. If similar in Atlantic salmon, this would mean that the brain is not reliant on dietary OA, but synthesises OA to fulfil its own needs, indicating an important role for OA in the brain. Phospholipids, particularly PE, with OA in the sn1 position has been shown to be an important factor in cold adaptation of membranes in brain cells of carp (*Cyprinus carpio*) (Buda et al., 1994). Generally low levels of OA in the natural feed of salmon might partly explain this specific requirement of the salmonid brain to synthesise its own. The brain is an overarching regulator of the primary stress response. Mammalian studies indicate that FA can interact with the HPI axis (reviewed in Maes et al., 2009). There was a significant interaction effect between diet and repeated stress in **Paper II**, with a minor reduction in the cortisol production after acute stress of fish fed an n-6/n-3 ratio of 6 compared to 1 (equal dietary n-3 FA). However, the plasma cortisol levels of fish fed the high n-6/n-3 ratio was not different from the fish fed diet with an n-6/n-3 ratio of 1 but twice the dietary n-3 FA (**Paper II).** The effect on cortisol might therefore have been random. The fact that the brain FA composition was remarkably conservative in the face of large dietary variations could support that the HPI axis is resilient to effects of dietary FA composition, as discussed in section 4.5. It seems more likely that the dietary FA can modify the stress response at the level of secondary and tertiary stress responses (**Paper II**).

5 CONCLUDING REMARKS – How does the dietary n-3, n-6 and n-6/n-3 ratio affect the robustness of Atlantic salmon under challenging conditions?

The overall goal of the present doctoral thesis was to investigate whether dietary n-3 fatty acids EPA and DHA and n-6 fatty acids impacted the robustness of Atlantic salmon under challenging environmental conditions. Based on data presented in this thesis, the short answer is "yes". Improved growth was demonstrated with higher dietary levels of EPA + DHA both under ideal and challenging conditions. Meanwhile, rising feed content of n-6 FA negatively affected growth, but only when exposed to stress or disease. It was demonstrated that reductions in dietary EPA + DHA and increases in LA and OA can lead to accumulation of liver lipids, which in turn likely is a symptom of a less robust fish. Tissue levels of n-3 and n-6 FA are highly dependent on dietary level. However, polar lipids reflect the relative level between dietary n-3 and n-6 FA, while neutral lipids reflect absolute levels. The stress response was also different in fish fed different n-6/n-3 ratios, with a higher n-6/n-3 ratio causing a different eicosanoid response after stress (PGD_2 and LTB_4) and possibly a slower recovery (PGD_2). The dietary levels of EPA, DHA and LA were also clearly linked to the inflammatory response in the liver. Dietary LA significantly affected the production of potent ARA derived eicosanoids (higher PGD, PGE_2; lower LTB_4). The metabolomic profile of liver in fish fed reduced levels of EPA + DHA indicated a higher state of inflammation (increased lysophospholipids, increased long-chain acylcarnitines, increased kynurenine and kynurenate, lower 5-HEPE), but also a dysregulation of lipid metabolism. All data considered, the present thesis indicates that a higher level of dietary EPA + DHA (preferably above 16 g/kg, but at least above 10 g/kg) is beneficial for salmon robustness. Further, a lower dietary n-6/n-3 ratio (ideally 1 or probably less) is preferable for better utilisation of dietary EPA. A high level of n-6 FA in the feed, also in relation to n-3 FA, additionally seems to cause adverse effects on stress, inflammation and liver lipid accumulation, thus giving several reasons for why attention should be given to n-6 FA levels in salmonid feeds.

Below follow specific answers to each of the sub-aims presented earlier in the thesis.

I: Maintaining constant dietary EPA and DHA while increasing the dietary level of LA (increasing the n-6/n-3 ratio) caused reductions in EPA concentrations in polar lipids (liver, red blood cells, brain, skin). There were significant increases in polar lipid ARA with more LA in the diet. This demonstrated production of ARA, which further dislocated EPA from the polar lipids. DHA was not significantly affected by rising dietary inclusion of LA. Thus, preserving a low dietary n-6/n-3 ratio can achieve a better utilisation of EPA.

II: FA composition analyses were performed on liver (polar and neutral fractions), red blood cells (polar), different phospholipids classes of the skin (polar), whole fish homogenate (mainly neutral), muscle (Norwegian quality cut - NQC) (mainly neutral) and brain (polar and neutral fractions). They all consistently displayed the same pattern; in polar lipids, the relative amount of dietary n-6 and n-3 FA had the greatest effect on FA composition. While polar lipid EPA decreased with higher feed LA, DHA was only affected by its dietary level. Contrarily, in neutral lipids, the absolute dietary content of n-3 and n-6 FA was reflected.

III: Fish fed a high dietary n-6/n-3 ratio appeared phenotypically healthy after the exposure to stressors, and they were all able to mount a stress response to the acute stressor. However, the eicosanoid production after stress was different depending on the dietary relationship between n-6 and n-3 FA. It indicated a poorer capability to recover after stress in fish fed n-6/n-3 ratio of 6 compared to 1, based on the PGD_2 production after acute stress. Furthermore, the high ratio diet resulted in rising levels of LTB_4 in the fish liver one hour after acute stress compared to a decline seen in fish fed the low ratio. As LTB_4 is strongly implicated in inflammatory responses in salmon, this could be an unfortunate situation leading to a less robust fish. Maintaining a low dietary n-6/n-3 ratio will thus likely be beneficial for the stress coping ability of salmon.

IV: EPA + DHA at 10 g/kg feed resulted in a dysregulated hepatic lipid metabolism in Atlantic salmon under challenging conditions in a full production cycle, where

the salmon demonstrated a reduced ability to use lipids for energy. Other pathways such as BCAA catabolism and the pentose phosphate pathway were activated to compensate. Some amelioration was seen in fish fed EPA + DHA at 16 g/kg, however further significant improvements were found in fish fed 35 g/kg compared to all other diets. This indicates that even higher levels than what has been used commercially the past few years will provide additional benefits and a more robust fish.

6 FUTURE PERSPECTIVES

The feed is an important part of salmon robustness, and the fish' capability to handle stressors, disease and pathogens. This further means that we need a good understanding of the salmon dietary requirement to face oncoming challenges. This thesis (together with Lutfi et al. (2022) and Selvam et al. (2022)) demonstrate that the practical requirement for EPA + DHA likely is higher than 16 g/kg, which was previously assumed to be sufficient. However, the conditions were probably less challenging than in commercial production, as shown by the relatively low mortality compared to commercial production. The determination of practical dietary requirement of EPA + DHA between 16 and 35 g/kg is necessary.

The inflammatory system of salmon is complex, and eicosanoids derived from both the n-3 and n-6 family are involved in these processes. We need better knowledge on the eicosanoids as inflammatory markers and what functions they have in inflammation and immunity of Atlantic salmon. Several eicosanoids (PGE_2, PGD_2, LTB_4 and LTB_5) were found to be implicated in the acute stress response of salmon. PGE_2, PGD_2 and LTB_4 were also significantly affected by the dietary n-6 content. The production of eicosanoids was also differently affected by a stressor depending on the dietary FA composition (PGD_2 and LTB_4). However, there is little knowledge on the functions of different eicosanoids in salmon. Which changes in eicosanoids are causing negative effects, and which might be positive? Despite higher dietary n-6 FA resulting in an altered stress response, the fish were phenotypically healthy, albeit with lower final weight. The stress trial was quite short, and did not include as many stressors as the fish would face in open sea cages. If modifying the dietary n-6 FA content results in a fish less resilient to stressors, disease and pathogens needs verification in a longer-term, larger-scale trial. Additionally, as it was shown that increasing inclusion of LA negatively impact salmon robustness, a "safe level" of dietary LA should be quantified. Determination of the normal range of plasma eicosanoids would be very useful to permit use of these metabolites as markers of both the stress response and inflammation. In regards to stress, the development non-invasive tests would be very useful to monitor the stress status of fish in aquaculture. Skin mucus cortisol levels have been identified

as a useful indicator for stress in other species such as yellow croaker (*Larimichthys crocea*), rainbow trout and gilthead seabream (Cai et al., 2020, De Mercado et al., 2018, Guardiola et al., 2016), and might be a possible candidate in salmon as well.

The metabolomics analysis indicated that a low dietary EPA and DHA resulted in a reduced hepatic β-oxidation through a disturbed mitochondrial membrane. This would be interesting to pursue further. For instance, a significantly higher level of lysophospholipids was found with low dietary EPA + DHA – perhaps this disrupted the mitochondrial membrane, as has been found in mammals (Tan et al., 2020). As several of the enzymes for mitochondrial β-oxidation are membrane bound, it might be that measuring the activity of these enzymes in fish fed low dietary EPA and DHA could reveal part of the answer to this.

A possible adipo-hepatic relationship in regards to adipose tissue inflammation and liver lipids was hypothesised. To investigate this further, co-culture cell models of adpiocytes, head kidney cells and liver cells where inflammation is only induced in the adipocytes would be interesting. Would adipose tissue inflammation lead to liver lipid accumulation?

This thesis has shown that practical requirements of EPA + DHA are higher than previously thought, while the global supply of these FA are limited. Alternative sources of these FA must be found to ensure sufficient supply. There are, essentially, two main options for *de novo* production of marine EPA and DHA – microalgae and genetically modified organisms (Tocher et al., 2019). As sources for EPA and DHA, a major drawback is that they mostly have a very one-sided FA composition with mainly EPA or DHA (Tocher et al., 2019). However, combining several of these new feed resources can provide an optimal dietary FA composition. A caveat for many algal biomasses is that they often contain high levels of carbohydrate and protein, limiting the amount that can be directly incorporated into feeds before negative effects. Pure algal oils have also been tested in salmonids with promising results (Santigosa et al., 2020). However, the FA composition is still skewed compared to FO, with much more DHA compared to EPA and a higher level of long-chain n-6 PUFA. Good results have also been found using a genetically modified high-DHA plant oil in salmon diets (Ruyter et al., 2019).

Although preliminary results using these alternative oils are promising, further optimisation of feed resources are required.

The present thesis clearly demonstrated that higher inclusion of dietary n-6 FA (higher n-6/n-3 ratio) will decrease the membrane bound EPA. As the use of VO has increased, knowledge on the role of EPA is thus important. There are some indications of a specific requirement for EPA in Atlantic salmon. Fish fed functional diets high in EPA apparently perform better after disease (Hatlen et al., 2016, Martinez-Rubio et al., 2014, Martinez-Rubio et al., 2012), and high DHA/low EPA diets resulted in a slightly higher liver lipid accumulation despite dietary EPA + DHA levels which should have been sufficient (Kousoulaki et al., 2020). This role of EPA could very well be related to its bioactive role as a precursor for eicosanoids and resolvins. Studies specifically looking at the requirement and role of EPA in disease for Atlantic salmon are required.

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Paper I

Hundal BK, Liland NS, Rosenlund G, Bou M, Stubhaug I & Sissener NH

Increasing dietary n-6 fatty acids while keeping n-3 fatty acids stable decreases EPA in polar lipids of farmed Atlantic salmon (Salmo salar)

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Increasing dietary *n*-6 fatty acids while keeping *n*-3 fatty acids stable decreases EPA in polar lipids of farmed Atlantic salmon (*Salmo salar*)

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Abstract

There is an increased use of vegetable oils containing *n*-6 fatty acids (FA) in aquafeeds, and several trials indicate that there might be an increased requirement of EPA and DHA for Atlantic salmon when they are fed higher dietary *n*-6 FA. With a limited supply of EPA and DHA for production of aquafeeds, it is important to know how to efficiently use these FA to maintain growth and health of the fish. In the present trial, three diets containing equal amounts of *n*-3 FA (about 7·7 % of total FA) and different *n*-6:*n*-3 FA ratios (about 1, 2 and 6), as well as one diet with *n*-6:*n*-3 FA ratio at about 1 but twice as much *n*-3 FA, were fed to Atlantic salmon. Despite constant dietary *n*-3, increasing dietary *n*-6 led to significantly reduced *n*-3 in tissue polar lipids. Interestingly, EPA was significantly reduced while DHA was not. Maintaining a stable *n*-3 content in the polar lipids when increasing dietary *n*-6 FA was only obtained by simultaneously increasing the dietary *n*-3 content and with this maintaining the same *n*-6:*n*-3 FA ratio. Polar lipid *n*-6 FA in tissues thus primarily reflected the dietary *n*-6:*n*-3 FA ratio and not the absolute dietary *n*-6 FA content. Neutral lipids, on the other hand, reflected the dietary absolute levels of both *n*-3 and *n*-6 FA. This study indicates that a better use of dietary EPA is achieved by keeping the dietary *n*-6:*n*-3 FA ratio low.

Key words: ***n*-6:*n*-3 ratio: *n*-6 Fatty acids: *n*-3 Fatty acids: Polar lipids: Neutral lipids: Lipids: Atlantic salmon**

Increasing replacement of fish oil (FO) with vegetable oils (VO) in aquafeeds is now common due to limited availability of FO worldwide. VO are completely lacking in the LC PUFA (EPA, 20 : 5*n*-3; DHA, 22 : 6*n*-3; arachidonic acid, 20 : 4*n*-6, ARA) considered essential for Atlantic salmon (*Salmo salar*)[1], but are rich in their 18-carbon precursors *α*-linolenic acid (18 : 3*n*-3) and linoleic acid (18 : 2*n*-6)[2]. Furthermore, VO usually contain much more 18 : 2*n*-6 than 18 : 3*n*-3, leading to a very different *n*-6:*n*-3 fatty acids (FA) ratio than in FO (*n*-6:*n*-3 FA ratio of rapeseed oil is 2·9, while it in capelin oil is 0·07[3]). Including VO in fish feed at the expense of FO therefore greatly affects the dietary content of the essential long-chain PUFA, as well as leading to considerable changes in the *n*-6:*n*-3 FA ratio of the feed. Multiple trials have shown that partially replacing FO with oils of non-marine origin can be done without reducing growth for salmonids[4–8], provided that minimum requirements of nutrients are still covered. FA profiles in tissues of Atlantic salmon are, however, influenced by dietary FA composition and often reflect dietary differences in FA[2,9]. An increasing dietary concentration of *n*-6 FA will cause increased tissue level of these FA[10–14], while decreasing dietary *n*-3 FA causes decreased tissue levels[1,15]. However, as most previous studies have replaced FO with VO, thus increasing *n*-6 FA and decreasing *n*-3 FA at the same time, it is hard to conclude on how dietary *n*-6 FA affect tissue incorporation of *n*-3 FA, and whether the absolute amounts of these FA or the ratio between them are the most important in determining tissue FA composition.

Feeding the freshwater fish Murray cod (*Maccullochella peelii*, Mitchell 1838) diets with 18 : 3*n*-3 and 18 : 2*n*-6 at a ratio of 1:1 combined with varying the absolute content of these FA showed that absolute levels played a vital role in the FA metabolism (*β*-oxidation, Δ6-desaturase activity)[16]. A follow-up study kept the absolute content of the sum of 18 : 2*n*-6 + 18 : 3*n*-3 constant, while changing the ratio between the two. Fillet FA profile was clearly impacted by dietary ratio, with higher dietary 18 : 3*n*-3/18 : 2*n*-6 ratio being reflected in the fillet and resulting

Abbreviations: ARA, arachidonic acid; CF, condition factor; FA, fatty acid; FAPV, fatty acid productive value; FCR, feed conversion ratio; FO, fish oil; HSI, hepatosomatic index; NQC, Norwegian Quality Cut; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; SGR, specific growth rate; VO, vegetable oil; VSI, viscerasomatic index.

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in higher EPA and DHA levels[17]. These trials demonstrate that both ratio and absolute levels can influence the final FA make up but does not reveal which of the two matters more.

Based on several studies using VO in aquafeeds, there are some indications of an increased requirement of EPA and DHA for Atlantic salmon with higher dietary proportions of *n*-6 FA (pers. Comm N. Sissener). That changes in the concentration of one FA can affect the requirement of another FA has been shown in other species, like the freshwater fish Eurasian Perch (*Perca fluviatilis* Linnaeus 1758), where excessive dietary 18 : 2*n*-6 caused an apparent deficiency of 18 : 3*n*-3[18]. Senadheera *et al.*[17] found that for Murray cod, a freshwater fish, increasing the dietary 18 : 2*n*-6 with constant EPA, and DHA had a negative effect on EPA and DHA tissue deposition. It should thus be possible to lower the dietary EPA and DHA without losing the beneficial effects of these FA, if you simultaneously reduce the dietary *n*-6 FA content.

A major concern when changing the *n*-3 and *n*-6 FA composition of the cellular membranes of Atlantic salmon is whether the production of eicosanoids will change. Eicosanoids are highly biologically active molecules with functions in inflammation and immunity[19], with the potency of the *n*-6 FA derived ones being higher than the *n*-3 FA derived ones[20]. Manipulating the dietary *n*-6 and *n*-3 FA levels can affect the production of eicosanoids. Atlantic salmon fed increased 18 : 2*n*-6 levels had higher levels of ARA-derived eicosanoids[21,22] and feeding high 18 : 3*n*-3 compared with high 18 : 2*n*-6 caused increased membrane bound EPA with reduced production of ARA-derived eicosanoids[23]. These studies suggest that manipulation of dietary *n*-6 and *n*-3 FA can affect the stress and immune responses of Atlantic salmon by regulating the eicosanoid production.

Surveillance of commercial Norwegian aquafeeds reveals that current *n*-6:*n*-3 FA ratios of salmonid diets are on average at 0·9 (range from 0·7 to 1·1)[24]. The most commonly used VO in Norway today is rapeseed oil[25], which has a moderate *n*-6 FA content at 20 %[3]. However, should the need to use other VO with higher *n*-6 contents arise (such as soya oil with *n*-6 FA at 54 %[3]), then the dietary *n*-6:*n*-3 FA ratio could easily rise to 2. Hence, the purpose of this trial was to distinguish between the effects of absolute *n*-3 and *n*-6 FA level and the *n*-6:*n*-3 FA ratio on the requirement of EPA and DHA and their effects on tissue FA composition. A feeding experiment was conducted with three diets containing equal absolute amounts of *n*-3 FA and *n*-6:*n*-3 FA ratios ranging from 1·0 to 6·0 as well as a final diet with double absolute *n*-3 FA content and *n*-6:*n*-3 FA ratio of 1·0. This gives this trial the possibility to separate between the effects of ratio and absolute amounts of *n*-3 on tissue FA levels. This trial was performed in a controlled experimental facility, where feed intake and growth were closely monitored for a maximised precision in collected data. A trial in such a setting does, however, also mean that the fish are protected from most external stressors such as diseases and temperature or oxygen fluctuations. The effect of the same changes in dietary and tissue FA composition on fish exposed to such external stressors in a large-scale commercial trial will be important to complement the current findings and to detect possible effects on fish robustness due to these dietary changes.

Materials and methods

Experimental design

Four diets were produced with different absolute contents of *n*-6 and *n*-3 FA and different *n*-6:*n*-3 FA ratios. The first three diets were formulated to contain *n*-3 at approximately 8 % of total FA, but to differ in their *n*-6 FA contents to create *n*-6:*n*-3 FA ratios of 1, 2 and 6. An *n*-6:*n*-3 FA ratio of 6 is extreme compared with current commercial Norwegian salmonid feeds, averaging at 0·9[24], but was included to provoke possible effects of *n*-6 FA inclusion. In these three diets, roughly half of the *n*-3 FA was provided as 18 : 3*n*-3 and half as EPA + DHA, resulting in EPA + DHA contents at around 1 % of the total diet. The fourth diet was formulated to contain twice as much *n*-3 FA as the first three diets (EPA + DHA at 2·0 % of feed), but with an *n*-6:*n*-3 FA ratio of 1, like the first diet. The diets will be referred to according to their planned dietary *n*-6:*n*-3 FA ratios for the rest of the paper. Hence, they will be called diets 1, 2 and 6, and with the final diet 1H due to its higher absolute contents of *n*-3 and *n*-6 FA compared with the first diet. The dietary formulation is given in Table 1. The only difference between them is the combination of oils used to adjust the *n*-3 and *n*-6 FA content. A graphical overview of the experimental design is given in Fig. 1.

Feeding trial

The feeding trial was performed at Skretting ARC Research Station at Lerang, Norway, and was conducted according to the guidelines of the Norwegian State Commission for Laboratory Animals (approval ID number 13576). A common extruded basal pellet was produced by Skretting ARC Feed Technology Plant (Stavanger, Norway) and coated with different oil blends to give the experimental diets. The experiment started 11 November 2017 with a pre-feeding period, to allow tissues to stabilise according to dietary FA composition. Atlantic salmon (SalmoBreed, Erfjord, Stamfisk AS, hatched February 2017) with an average weight of 80 g were distributed randomly between four circular tanks (3 m diameter, 7000 litres, 735 fish/tank) supplied with running sea water at 8°C and exposed to 24 h light. The fish were a mixed population of both sexes. During the pre-feeding period, the four experimental diets (3 mm pellet size) were fed to one tank each. Dietary composition of pre-feeding diets can be found in online Supplementary Table S1.

On 26 February 2018, the fish (mean weight 256 ± 2 g) were transferred to 12 experimental tanks (1 m diameter, 450 litres, thirty-two fish per tank, triplicate tanks per diet) and fed the experimental diets (4 mm pellets, increased from pre-feeding due to fish size). Tanks were supplied with flow through sea water at 11·8 ± 0·1°C, and the photoperiod was 24 h light. Diets were distributed in slight excess of expected feed intake using automatic feeders (Hølland Teknologi AS). All tanks were equipped with feed collectors for control of daily feed intake. Standard husbandry procedures at the research station were applied.

Sampling

At the start of the trial (i.e. after the pre-feeding period), all fish were weighed, and fork length was measured. The sampled fish

Table 1. Feed formulation in g/100 g of the four diets used in the feeding trial (4 mm pellet size)*

	Diet 1	Diet 2	Diet 6	Diet 1H
Wheat	7·2	7·2	7·2	7·2
Soya protein concentrate	28·2	28·2	28·2	28·2
Sunflower meal	6·0	6·0	6·0	6·0
Wheat gluten	18·0	18·0	18·0	18·0
Faba beans, dehulled	2·0	2·0	2·0	2·0
FM, North Atlantic	10·0	10·0	10·0	10·0
Linseed oil	1·2	1·2	1·3	2·7
Sunflower oil	0·6	4·8	19·3	5·1
Olive oil	12·2	7·7	0·0	1·6
Coconut oil	0·7	1·0	0·3	0·0
FO, North Atlantic	0·0	0·0	3·9	4·9
FO, Capelin	10·0	10·0	0·0	10·5
Premixes	3·7	3·7	3·7	3·7
Yttrium	0·1	0·1	0·1	0·1

FM, fishmeal; FO, fish oil.
* Diet 1/Diet 2/Diet 6/Diet 1H, diet codes according to dietary *n*-6:*n*-3 fatty acid ratio. The final diet is labelled 1H due to its higher absolute contents of *n*-3 and *n*-6 fatty acids compared with the first diet.

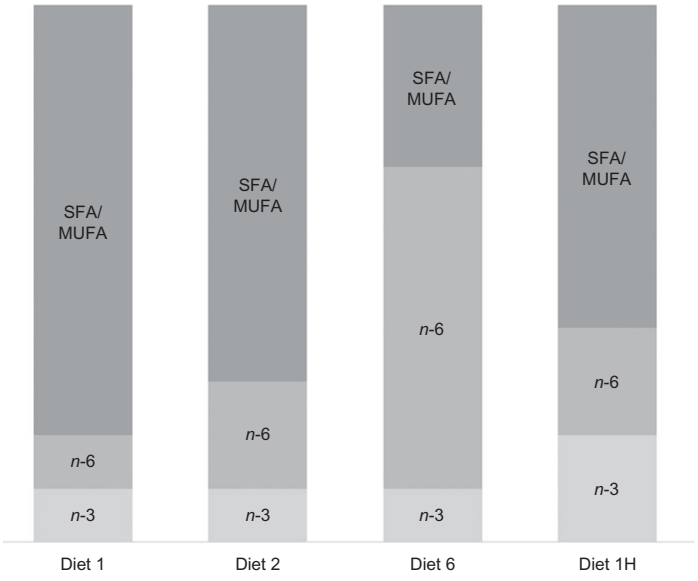

Fig. 1. Overview of the experimental design. The first three diets had the same *n*-3 fatty acid (FA) content with increasing *n*-6 content to increase the *n*-6:*n*-3 FA ratio. The last diet had double *n*-3 FA content, with an equal *n*-6 content, thus maintaining a low ratio. Diets are named according to their planned dietary *n*-6:*n*-3 FA ratio, with the final diet named 1H due to its higher absolute contents of *n*-3 and *n*-6 FA compared with the first diet.

were euthanised with an overdose of anaesthetic (Tricanine Pharmaq, 0·3 g/l). Totally, fifteen whole fish were collected from each pre-feeding tank, pooled in three samples (five fish per sample) and homogenised for FA analysis. The final sampling was conducted on 24 May 2018 after eighty-five feeding days. Before the final sampling, the fish were starved for 12 h. All fish were weighed, and fork length was measured before blood was sampled from the caudal vein using EDTA-coated vacutainers. The blood was centrifuged for 7 min at 2500 ***g*** to separate the plasma fraction from the erythrocytes. The erythrocytes were washed three times using physiological saltwater. From each tank, erythrocyte samples were collected from three fish and plasma samples from six fish. For liver lipid class and FA composition, the samples were pooled from six individual fish per tank. Pooled fillet samples (Norwegian quality cut) were taken from the same six fish as the liver samples. From three fish per tank, a section (approximately 8 × 8 cm) immediately anterior to the sampled Norwegian quality cut was sampled and frozen for skin samples. The samples of skin with muscle attached were sent frozen on dry ice to Nofima, who semi-thawed the samples,

pulled the skin off the muscle and removed any remaining subcutaneous muscle from the skin samples before pooling samples from each tank. Eight whole fish from each tank were homogenised and pooled for proximate and FA composition analysis. Organ samples were flash frozen in liquid nitrogen, then put on dry ice and stored at –80°C until analysed. Erythrocytes, plasma and pooled samples (whole fish and fillet) were frozen on dry ice, then stored at –80°C until analyses. The sampled tissues were selected due to their known effects on general health and metabolism as well as their known dependence on dietary FA composition[15,26,27].

Analytical procedures

Total nitrogen was measured in whole fish using a Vario Macro Cube (Elementar Analysensysteme GmbH) nitrogen analyser according to the Association of Official Analytical Chemists[28], and total protein calculated as N × 6·25. Total fat in whole fish was determined gravimetrically after extraction with ethyl acetate. Ash content was determined gravimetrically by heating the sample to 550°C overnight in a muffle furnace (Thermolyne F 30, 430 CM).

Liver lipids were extracted using chloroform/methanol 2:1 (Merck) with 1 % added (2,6-di-tert-butyl-methylphenol; Sigma-Aldrich). The samples were analysed for absolute and relative amounts of different lipid classes using high-performance thin layer chromatography as described in Torstensen *et al.*[29].

Whole fish, liver and fillet (Norwegian quality cut) were analysed for FA composition using GC as described in Torstensen *et al.*[30]. In short, the lipids were extracted from the samples by addition of chloroform/methanol 2:1. The FA 19 : 0 was added as an internal standard for later quantification of the FA. The extracted samples were filtered and evaporated before saponification and methylation using 12 % BF_3 in methanol. The separation of FA was performed on either an AutoGC (Autosystem XL, Perkin Elmer Inc.) or a Scion 436-GC (Scion Instruments) with a flame ionisation detector. The FA were identified by their retention time using a standard mixture of methyl esthers (Nu-Chek-Prep, Elysian), and the FA composition (area %) was determined. The software Chromeleon® version 7.2 (Thermo Scientific) was used to integrate the samples. The method used for FA composition at the Institute of Marine Research is accredited. For each run, control samples are also analysed, which must fall within the range of a well-established control card for the sample run to be approved. Liver FA were determined in both the polar and neutral lipids after separation by solid-phase extraction prior to saponification as described in Sissener *et al.*[31]. The FA analysis of the feed was performed by Skretting ARC. The FA were methylated using methanolic HCl and extracted in hexane before separation by GC and is further described in Sissener *et al.*[15]. The FA composition of the erythrocytes was analysed using ultra-fast GC, which is a faster method than the aforementioned FA analysis. This method is slightly more limited as MUFA are not separated depending on their double-bond position and are reported collectively as 16 : 1, 18 : 1, 20 : 1 and 22 : 1. It does, however, allow for analyses of a larger number of samples, as the methylation with NaOH and BF_3 can be performed directly on the sample, thus omitting the extraction procedure. The FA composition by ultra-fast GC was performed as described in Sissener *et al.*[31].

The analyses of the FA composition of skin samples were performed by Nofima on pooled samples of three fish per tank. Total lipids were extracted from homogenised skin following the method described by Folch *et al.*[32]. A sample of 0·5 ml from the chloroform-methanol phase was used for analysis of FA composition of total lipids. The FA composition was performed using the method described by Mason & Waller[33]. A fuller description of the method can be found in Bou *et al.*[26]. The HP Chem Station software was used, and individual FA methyl esters were identified by referring to well-characterised standards. The relative amount of each FA was expressed as a percentage of the total amount of FA in the analysed sample, and the absolute amount of FA per g tissue was calculated using C23 : 0 methyl ester as the internal standard.

The lipid class composition of skin was performed by Nofima as described in Bou *et al.*[26]. Briefly, a part of the Folch chloroform lipid extract was evaporated and then re-dissolved in hexane before separation by TLC. The spots corresponding to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) were scraped off into glass tubes and trans-methylated following the FA composition method by Nofima.

Calculations

$$\text{Feed conversion ratio (FCR): FCR} = \frac{\text{feed eaten (g)}}{\text{weight gain (g)}}$$

$$\text{Condition factor (CF): CF} = \frac{\text{body weight (g)}}{\text{length}^3\text{(cm)}} \times 100^{(34)}$$

$$\text{Specific growth rate (SGR): SGR} = \frac{\ln w_2 - \ln w_1}{d} \times 100$$

where w_2 and w_1 are final and initial weights in grams and d the sum of experimental days.

$$\text{Hepatosomatic index (HSI): HSI} = \frac{\text{liver weight (g)}}{\text{body weight (g)}} \times 100$$

$$\text{Viscerasomatic index (VSI): VSI} = \frac{\text{viscera weight (g)}}{\text{body weight (g)}} \times 100$$

Fatty acid productive value (FAPV)[8]:

$$\text{FAPV} = \frac{\text{g FA per tank at end of trial} - \text{g FA per tank at start of trial}}{\text{g FA eaten in total per tank for the duration of the trial}} \times 100$$

Statistics

Based on considerable previous experience, the hypothesised effect sizes were expected to be >1·5 SD. The experimental power was calculated *post hoc* using the power calculation tool for one-way ANOVA in the software Statistica (version 13.5.0.17).

An α value of 0·05 and average effect size of approximately 1·5 SD were applied to a selection of response variables. For example, FA composition in erythrocytes had a power level ($1-\beta$) of 0·999 and final weight a power level ($1-\beta$) of 0·817.

All other statistical analyses were performed using the free software environment R[35]. Differences between the groups were detected by one-way ANOVA when no tank effects were found. When tank effect (random effect) was found to be present, nested one-way ANOVA was used. Tukey's honestly significant difference *post hoc* test was used when one-way ANOVA resulted in significant differences, using the packages *nlme*[36] and *multcomp*[37]. Correlation analyses were performed using the function *cor.test*. The data were analysed for homogeneity of variances using Levene's test (Brown Forsythe modification) and for normality using Shapiro Wilk's test, in addition to using QQ-plots for graphical evaluation of normality. A P value of $\leq$0·05 was considered statistically significant. Results are presented as means with standard deviations.

Results

Dietary composition

As planned, diets 1, 2 and 6 had the same *n*-3 FA content (7·7–7·8 % of total FA). 18 : 3*n*-3 ranged from 2·9 to 3·1 % of FA in the same three diets. Diets 1 and 2 contained equal amounts of EPA at 2·4 % of total FA, while diet 6 had a little less at 2·0 % of total FA. Diet 6 had slightly more DHA than groups 1 and 2 (1·8 % of total FA in diet 6 *v.* 1·5 % in diets 1 and 2), making the sum of EPA + DHA similar for these three diets (3·9, 3·9 and 3·8 % of total FA). As planned, diet 1H had approximately twice as much 18 : 3*n*-3, EPA and DHA compared with the three other diets.

Sunflower oil, containing large amounts of 18 : 2*n*-6 (62·5 %[3]), was used to increase the dietary *n*-6 FA content and thus determine the *n*-6:*n*-3 FA ratio. Hence, 18 : 2*n*-6 varied from 10·8 to 46·7 % with diets 2 and 1H having similar contents at 17·9 and 17·6 % of total FA. The *n*-6:*n*-3 FA ratios ended up slightly higher than planned, but were still close to the planned 1, 2, 6 and 1 at 1·4, 2·4, 6·1 and 1·2, respectively. The FA 18 : 2*n*-6 was the major *n*-6 FA in all diets. Dietary SFA were stable across all diets at around 20 % of total FA, except for in diet 6 which due to a high content of sunflower oil got a reduced saturated fat content (16·6 % of total FA). MUFA was the main group of FA reduced when *n*-6 FA increased, due to the replacement of the MUFA-rich olive oil with *n*-6 rich sunflower oil, thus being highest in diet 1 (59 % of total FA) and the lowest in diet 6 (27 % of total FA). Proximate and FA composition of the 4 mm pellet is given in Table 2, while the results from the same analyses for the 3 mm pellet are given in online Supplementary Table S2.

Growth

Feeding diet 1H resulted in a significantly higher final weight and fork length of the fish than when feeding diets 1 and 2. The SGR for fish fed diet 1H was significantly higher than fish fed diet 2.

Table 2. Analysed dietary proximate composition (g/100 g), fatty acid (FA) composition (% of total FA) and total FA (g/100 g diet) of the four diets used in the feeding trial (4mm pellet size)*

	Diet 1	Diet 2	Diet 6	Diet 1H
Proximate composition (g/100 g)				
Lipids	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
FA (% of total FA)				
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*n*-7	3·8	3·7	1·5	5·1
18 : 1*n*-7	2·2	2·0	1·1	2·0
18 : 1*n*-9	37·1	30·1	23·1	18·2
20 : 1*n*-9	6·4	6·4	0·7	7·0
22 : 1*n*-11	7·7	7·7	0·7	8·5
Σ*n*-6	11·1	18·2	46·9	18·4
18 : 2*n*-6	10·8	17·9	46·7	17·6
20 : 4*n*-6 (ARA)	0·1	0·1	0·1	0·2
Σ*n*-3	7·7	7·7	7·8	15·1
18 : 3*n*-3	2·9	2·9	3·1	5·7
20 : 5*n*-3 (EPA)	2·4	2·4	2·0	4·4
22 : 6*n*-3 (DHA)	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
Total FA (g/100 g diet)	27·1	27·3	29·7	25·5

ARA, arachidonic acid.
* Diet 1/Diet 2/Diet 6/Diet 1H, diet codes according to dietary *n*-6:*n*-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of *n*-3 and *n*-6 FA compared with the first diet.

Table 3. Growth performance and organ indices of Atlantic salmon fed diets with varying dietary *n*-6:*n*-3 ratios and absolute levels of *n*-6 fatty acids (FA) and *n*-3 FA*
(Mean values and standard deviations in three tanks per diet)

	Diet 1		Diet 2		Diet 6		Diet 1H	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Start weight (g)	254	22	257	22	259	23	255	22
Final weight (g)	726[a]	101	727[a]	107	754[a,b]	107	766[b]	82
Final length (cm)	36·2[a]	1·7	36·1[a]	1·8	36·6[a,b]	1·8	37·0[b]	1·5
Feed intake (g per fish)	367[b]	4	362[a,b]	17	379[a,b]	8	393[a]	6
SGR	1·232[a]	0·008	1·222[a]	0·035	1·256[a,b]	0·008	1·292[b]	0·010
FCR	0·781	0·006	0·770	0·013	0·770	0·021	0·769	0·005
CF	1·5	0·1	1·5	0·1	1·5	0·1	1·5	0·1
HSI	1·2	0·2	1·2	0·1	1·2	0·1	1·1	0·1
VSI	12·2	3·2	12·6	1·1	13·4	3·2	12·6	1·0

SGR, specific growth rate; FCR, feed conversion ratio; CF, condition factor; HSI, hepatosomatic index; VSI, viscerasomatic index.
[a,b] Significantly different means are denoted by unlike superscript letters.
* Weight, fork length and CF from all fish at the final sampling (*n* 96/97), start weight, feed intake for the 85 d experimental period, SGR and FCR is given per tank (*n* 3), HSI and VSI are from six fish per tank (*n* 18). Diet 1/Diet 2/Diet 6/Diet 1H, diet codes according to dietary *n*-6:*n*-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of *n*-3 and *n*-6 FA compared with the first diet.

Feed intake for the whole period was significantly lower in fish fed diet 1 than fish fed diet 1H. Neither FCR, CF, HSI nor VSI was affected by any of the diets (Table 3). There was no mortality in any of the dietary groups during the experiment.

Whole fish proximate composition

The experimental diets did not lead to any differences in total protein or ash in whole fish; however, a significantly higher fat content was found in the fish fed diet 1 compared with fish fed diets 6 and 1H (Table 4).

Fatty acid composition and fatty acid productive value in whole fish

The FA composition of whole fish is presented as % of total FA (Table 5), although mg/g was also calculated. The mg/g data were used to calculate the FAPV. The whole fish FA composition generally reflected the dietary composition, with SFA and MUFA following the dietary composition closely. The sum of PUFA in whole fish was slightly reduced compared with the dietary contents of this group of FA. Fish fed diet 6 had the largest reduction in tissue PUFA, followed by fish fed diets 1H, 2 and 1. The sum of *n*-6 FA was reduced relative to the feed for all groups with the biggest reduction occurring in fish fed diet 6, whereas the sum of *n*-3 FA increased in whole fish compared with the diets for all groups. A reduction in *n*-6 FA and an increase in *n*-3 FA in the tissues compared with the diets thus led to a slight reduction of the *n*-6:*n*-3 FA ratios in the fish tissues compared with the diets (1·3, 2·0, 5·0 and 1·1 in the tissues compared with 1·4, 2·4, 6·1 and 1·2 in the diets). In whole fish, it was the absolute dietary contents of *n*-3 and *n*-6 FA which were reflected.

The FA composition of the Norwegian quality cut was almost identical to the FA composition in whole fish, though the total amount of fat was lower in muscle than in whole fish (online Supplementary Table S3).

The FAPV for DHA ranged from 122 to 144 % (Fig. 2) showing a net production of DHA for all dietary groups and no significant effects of diets. FAPV of 18 : 3*n*-3 ranged from 55 to 69 % and EPA from 44 to 56 % (Fig. 2), with group 1H having significantly higher FAPV than the other groups for both FA. FAPV for SFA (82·9 ± 4·3 %, all dietary groups combined) and MUFA (83·4 ± 4·0 %) did not show any dietary effects. Correlation analyses revealed no relation between dietary MUFA and whole fish content of EPA, DHA or the sum of EPA + DHA (mean *P* value 0·98, mean correlation coefficient = –0·006).

When calculating FAPV, the dietary content of each FA is required. Some FA are present in the diet at low levels (≤1 %) at which point the uncertainty in the FA analysis becomes very large. This can affect the retention calculations drastically and induce variation in retention values that is random rather than

Table 4. Total fat, protein and DM (g/100 g) in homogenised whole Atlantic salmon fed the diets with varying dietary *n*-6:*n*-3 ratios and absolute levels of *n*-6 fatty acids (FA) and *n*-3 FA*
(Mean values with their standard deviations of three tanks per diet with eight fish pooled per tank (*n* 3 per diet))

	Diet 1		Diet 2		Diet 6		Diet 1H	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Crude fat	16·7[a]	0·1	16·3[a,b]	0·2	16·2[b]	0·1	16·0[b]	0·3
Crude protein	16·5	0·3	16·7	0·2	16·5	0·3	16·5	0·2
DM	35·3	0·6	35·0	1·0	34·7	0·6	34·7	0·6

[a,b] Significantly different means are denoted by unlike superscript letters.
* Diet 1/Diet 2/Diet 6/Diet 1H, diet codes according to dietary *n*-6:*n*-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of *n*-3 and *n*-6 FA compared with the first diet.

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Table 5. Fatty acid (FA) composition (% of total FA) of whole Atlantic salmon fed diets with varying dietary *n*-6:*n*-3 ratios and absolute levels of *n*-6 FA and *n*-3 FA*
(Mean values and standard deviations of three tanks per diet with eight fish pooled per tank, *n* 3)

	Diet 1		Diet 2		Diet 6		Diet 1H	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ΣSFA	19·4^{c}	0·2	19·1^{b}	0·1	16·7^{a}	<0·1	19·9^{d}	0·1
ΣMUFA	58·8^{d}	0·2	52·3^{c}	0·2	29·5^{a}	0·1	43·8^{b}	0·1
Σ*n*-6	11·0^{a}	0·1	17·9^{b}	0·1	43·2^{c}	0·2	18·0^{b}	0·1
18 : 2*n*-6	9·3^{a}	0·1	15·3^{c}	0·1	38·1^{d}	0·1	15·7^{b}	0·1
20 : 2*n*-6	0·7^{a}	<0·1	1·0^{b}	<0·1	2·2^{c}	<0·1	0·9^{b}	<0·1
20 : 3*n*-6	0·5^{a}	<0·1	0·8^{b}	<0·1	1·5^{c}	0·1	0·4^{a}	<0·1
20 : 4*n*-6 (ARA)	0·3^{a}	<0·1	0·4^{a}	<0·1	0·6^{b}	<0·1	0·6^{b}	<0·1
Σ*n*-3	8·6^{a}	0·1	8·7^{a}	0·1	8·6^{a}	0·1	15·7^{b}	<0·1
18 : 3*n*-3	2·0^{a}	0·1	2·1^{a,b}	<0·1	2·2^{b}	<0·1	4·5^{c}	0·1
20 : 5*n*-3 (EPA)	1·4^{a,b}	0·1	1·4^{b}	<0·1	1·2^{a}	<0·1	2·8^{c}	0·1
22 : 6*n*-3 (DHA)	3·4^{a,b}	0·1	3·3^{a}	0·1	3·6^{b}	0·1	5·0^{c}	0·1
EPA + DHA	4·8^{a}	<0·1	4·7^{a}	0·1	4·8^{a}	<0·1	7·9^{b}	<0·1
ΣPUFA	19·8^{a}	0·2	26·7^{b}	0·1	51·9^{d}	0·3	33·9^{c}	0·1
n-6:*n*-3	1·3^{b}	<0·1	2·0^{c}	<0·1	5·0^{d}	<0·1	1·1^{a}	<0·1
Total FA (mg/g tissue)	164·8	4·7	159·4	3·3	161·0	2·8	157·7	4·5

ARA, arachidonic acid.
^{a,b,c,d} Significantly different means are denoted by unlike superscript letters.
* Diet 1/Diet 2/Diet 6/Diet 1H, diet codes according to dietary *n*-6:*n*-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of *n*-3 and *n*-6 FA compared with the first diet.

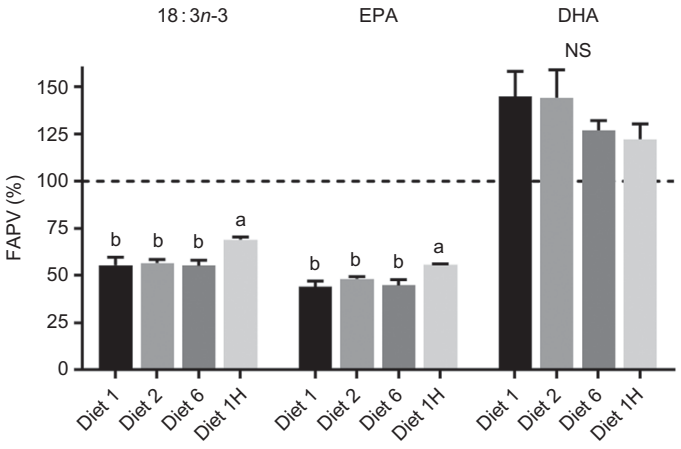

Fig. 2. Fatty acid productive value (FAPV) (%) of 18 : 3*n*-3, EPA and DHA of whole Atlantic salmon fed diets with varying dietary *n*-6:*n*-3 fatty acid (FA) ratios and absolute levels of *n*-6 FA and *n*-3 FA. Values above the dashed line (100 %) indicate a net production, while values below indicate net consumption. Homogenates of whole fish were pooled from five fish per tank (*n* 3) at the initial sampling and pooled from eight fish per tank (*n* 3) at final sampling. Values are means with vertical bars representing standard deviations. ^{a,b} Significantly different means are denoted by unlike letters (one-way ANOVA, followed by Tukey's honestly significant difference multiple comparison). Diet 1/Diet 2/Diet 6/Diet 1H, diet codes according to dietary *n*-6:*n*-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of *n*-3 and *n*-6 FA compared with the first diet.

caused by the diets. The *n*-6 FA 20 : 2*n*-6 and 20 : 3*n*-6 were not present in the diet, and ARA was present at ≤1 % which makes it difficult to calculate accurate FAPV. However, 20 : 2*n*-6 and 20 : 3*n*-6 were present in whole fish, and ARA was present at higher amounts in whole fish than in the diets, thus indicating an endogenous production of these FA.

Liver lipid classes

Generally, the liver content of polar lipids remained quite stable across dietary groups, while there were significant differences in the neutral lipid content (Table 6). TAG was significantly higher in the liver of fish fed diets 1 and 6 compared with fish fed diets 2 and 1H, also causing a significant difference in the sum of neutral lipids and total lipids.

Fatty acid composition of the liver

Increasing the dietary *n*-6:*n*-3 FA ratio (diets 1, 2 and 6) resulted in a significant reduction of EPA in the polar lipids of the liver, while DHA remained unaffected (Fig. 3(A) and (B)). Every other *n*-3 FA analysed (18 : 4*n*-3, 20 : 4*n*-3, 21 : 5*n*-3, 22 : 5*n*-3 and

Table 6. Liver lipid classes (mg/g) of Atlantic salmon fed diets with varying dietary *n*-6:*n*-3 fatty acid (FA) ratios and absolute levels of *n*-6 FA and *n*-3 FA*
(Mean values and standard deviations of three tanks per diet with six fish pooled per tank, *n* 3)

	Diet 1		Diet 2		Diet 6		Diet 1H	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LPC	1·9	0·2	1·9	0·3	1·9	0·2	1·8	0·1
SM	1·5	0·2	1·5	0·2	1·8	0·1	1·7	0·1
PC	22·4	0·2	24·4	2·8	25·3	1·5	23·1	0·3
PS	3·4	0·5	2·9	0·7	3·3	0·9	3·6	0·2
PI	3·0	0·2	2·9	0·3	2·9	0·2	2·9	0·1
CL	1·0	0·1	0·8	0·1	0·8	0·1	0·8	<0·1
PE	4·60	0·8	5·4	1·6	4·8	1·7	4·1	0·3
Sum polar	37·8	0·6	39·1	2·1	40·1	0·9	38·1	0·6
DAG	0·1	0·1	0·2	0·1	0·2	0·1	0·2	0·1
CHOL	3·4	<0·1	3·3	0·6	3·3	0·4	3·5	0·1
NEFA	ND	–	ND	–	ND	–	ND	–
TAG	26·9[a]	1·7	14·9[b]	4·8	32·4[a]	7·3	10·8[b]	2·1
CE	ND	–	ND	–	ND	–	ND	–
Sum neutral	30·5[a]	1·8	18·4[b]	4·2	35·9[a]	6·8	14·5[b]	2·0
Sum lipids	68·3[a,b]	2·3	57·5[b,c]	6·3	76·1[a]	7·7	52·5[c]	2·3

LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin; PE, phosphatidylethanolamine; DAG, diacylglycerol; CHOL, cholesterol; ND, not detected; CE, cholesteryl ester.
[a,b,c] Significantly different means are denoted by unlike superscript letters.
* Diet 1/Diet 2/Diet 6/Diet 1H, diet codes according to dietary *n*-6:*n*-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of *n*-3 and *n*-6 FA compared with the first diet.

24 : 6*n*-3), except 18 : 3*n*-3, was also significantly reduced in the liver polar lipids when the dietary *n*-6:*n*-3 FA ratio was increased. There were no effects of dietary *n*-6:*n*-3 FA ratio on the content of any *n*-3 FA in the neutral lipids (diets 1, 2 and 6), but an increase in dietary *n*-3 FA led to higher contents of every *n*-3 FA (diet 1H). The fish fed diet 1H had significantly higher amounts of every *n*-3 FA compared with the other dietary groups. The fish fed diet 2 had a slightly elevated content of some of the *n*-3 FA (EPA, docosapentaenoic acid (22 : 5*n*-3), DHA and 24 : 6*n*-3) compared with the fish fed diet 6 (neutral lipid data in online Supplementary Table S4). The ratio of EPA:ARA in the liver polar lipids was strongly influenced by *n*-6:*n*-3 FA ratio of the diets and was the highest in diet 1 and diet 1H (Fig. 3(C)). The content of SFA was quite stable in the liver polar lipids at about 25 % of total FA, but significantly lower in fish fed diet 6 (23·5 % of total FA, online Supplementary Table S4). The MUFA in liver polar lipids were much lower than dietary MUFA, lying between 10 and 20 % of total FA (lowest in fish fed diet 6 and highest in fish fed diet 1). The neutral lipid MUFA and SFA were higher and lower than their levels in the diet, respectively, but followed the same fluctuations as in the diets (online Supplementary Table S4).

Increasing the dietary *n*-6:*n*-3 FA ratio led to increasing *n*-6 FA contents in the polar lipids (diets 1, 2 and 6), and similar dietary *n*-6:*n*-3 ratios led to similar contents of total *n*-6 FA despite different absolute dietary contents (diets 1 and 1H, Fig. 5(A)). Hence, indicating that in the polar lipids, dietary *n*-6:*n*-3 FA ratio had a greater effect than absolute dietary amount of these FA. Additionally, all intermediary FA in the production of ARA (and some longer chain *n*-6 FA) were present in all dietary groups, despite not being present in the feed. The ARA content of the liver polar lipids of fish fed diet 1H was intermediate between the content in fish fed diet 1 and diet 2 (Fig. 4(A)–(D)). Increased dietary content of *n*-6 FA also caused higher *n*-6 FA in the neutral lipids, but in contrast to the polar lipids, the absolute amounts appeared to be of greater importance here than the *n*-6:*n*-3 FA ratio. For example, fish fed diet 2 and fish fed diet 1H had the same total *n*-6 FA in the liver neutral lipids, even though they had different dietary *n*-6:*n*-3 FA ratios (Fig. 5(A)). All intermediates from 18 : 2*n*-6 to ARA were present, but 18 : 3*n*-6 and 20 : 3*n*-6 seemed to follow the *n*-6:*n*-3 FA ratio rather than absolute dietary content. The 18 : 3*n*-6, 20 : 2*n*-6, 20 : 3*n*-6 were also present at much lower amounts than in the polar lipids. ARA, however, seemed to simply reflect the dietary content and was present at lower concentrations than most of the intermediates of its biosynthesis (online Supplementary Table S4).

Compared with EPA and DHA feed content, these FA were present at considerably higher concentrations in the liver polar lipids. Contrarily, 18 : 2*n*-6 was reduced manifold in the liver polar lipids compared with the feed, while a smaller reduction was seen in the neutral lipids. In the liver neutral lipids, DHA was similar to the dietary content, whilst EPA was reduced relative to the feed. Consequently, the liver *n*-6:*n*-3 FA ratio ranged from 0·3 to 1·1 in the polar lipids and from 1·4 to 10·2 in the neutral lipids (Fig. 5(B)).

Fatty acid composition of erythrocytes

The FA composition of the erythrocytes was more dominated by *n*-3 FA than the other tissues (42–53 % of total FA) and had a stable SFA content at about 31 % of total FA. The MUFA was much lower than in the diets, ranging from 7 to 13 % of total FA (online Supplementary Table S5). A higher *n*-6:*n*-3 FA ratio significantly affected the EPA content of the erythrocytes, while DHA remained more resilient towards change (diets 1, 2 and 6, Fig.

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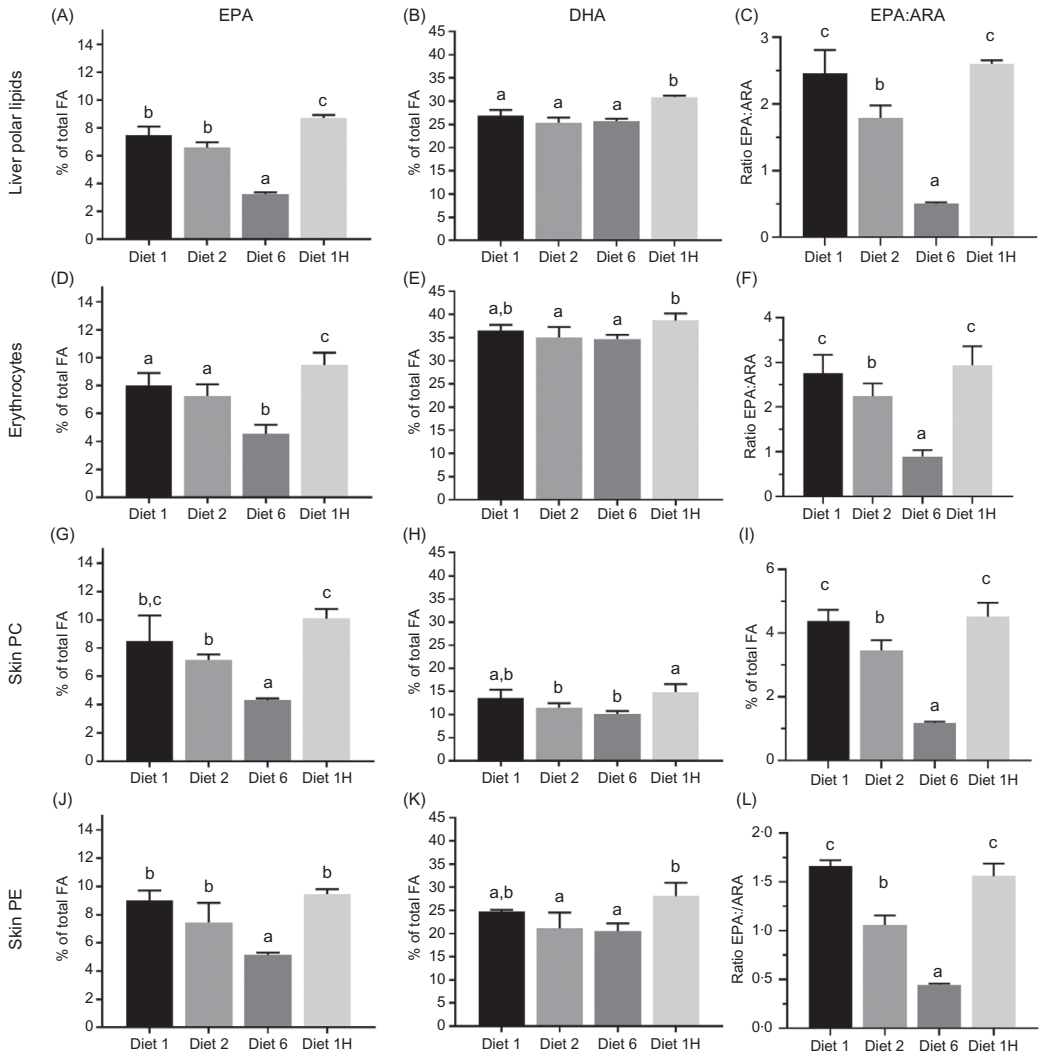

Fig. 3. Content of EPA, DHA (% of total fatty acids (FA)) and the ratio of EPA:ARA in polar lipids of the liver (A–C), erythrocytes (D–F), and in phosphatidylcholine (PC) (G–I) and phosphatidylethanolamine (PE) (J–L) in the skin of Atlantic salmon fed diets with varying dietary *n*-6:*n*-3 FA ratios and absolute levels of *n*-6 FA and *n*-3 FA. Six liver samples were pooled from each tank (n 3), erythrocyte samples from three fish from each tank diet (n 9), skin samples from three fish were pooled from each tank (n 3). Values are means with vertical bars representing standard deviations. ^{a,b,c} Significantly different means are denoted by unlike letters (one-way ANOVA, followed by Tukey's honestly significant difference multiple comparison). Diet 1/Diet 2/Diet 6/Diet 1H, diet codes according to dietary *n*-6:*n*-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of *n*-3 and *n*-6 FA compared with the first diet. ARA, arachidonic acid.

3(D) and (E)). However, although a numeric decrease in EPA was seen when the dietary *n*-6:*n*-3 FA ratio was increased from 1 to 2, it was not significant. Only when raising the *n*-6:*n*-3 FA ratio to 6 was a significant decrease in EPA content seen. The EPA:ARA ratio in erythrocytes was highly influenced by dietary *n*-6:*n*-3 FA ratio with similarly high values in diet 1 and diet 1H, and lower values in the two other dietary groups (Fig. 3(F)). The other *n*-3 FA analysed (20 : 4*n*-3 and 22 : 5*n*-3) were numerically decreased when increasing the dietary *n*-6:*n*-3 FA ratio to 2 and significantly decreased when the *n*-6:*n*-3 FA ratio was raised to 6 (online Supplementary Table S5). Fish fed diet 1H exhibited the highest percentage of every *n*-3 FA in the erythrocytes.

An increase in the dietary *n*-6:*n*-3 FA ratio led to higher levels of 18 : 2*n*-6, 20 : 2*n*-6 and 20 : 3*n*-6 (diets 1, 2 and 6) in the erythrocytes; however, similar *n*-6:*n*-3 FA ratios but different absolute amounts of dietary *n*-6 FA (diets 1 and 1H) resulted in the same

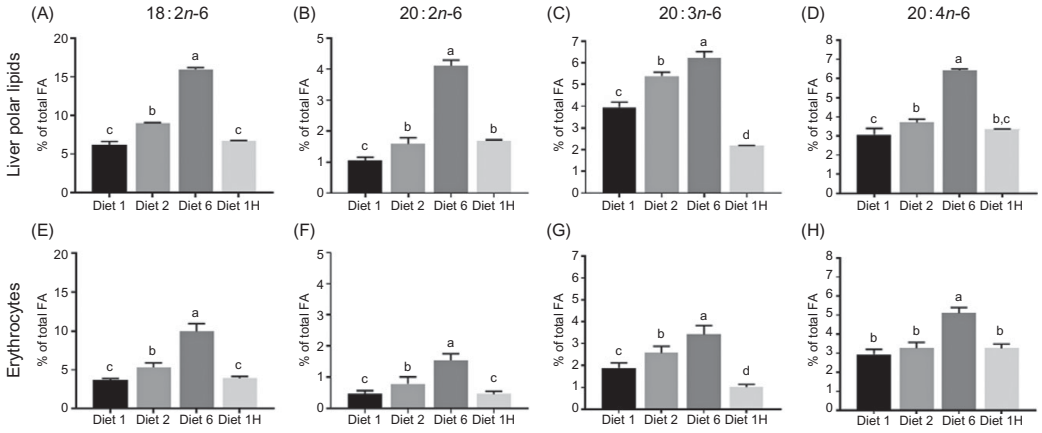

Fig. 4. Content of 18:2*n*-6, 20:2*n*-6, 20:3*n*-6 and 20:4*n*-6 (% of total fatty acids (FA)) in liver (A–D) and erythrocytes (E–H) of Atlantic salmon fed diets with varying dietary *n*-6:*n*-3 ratios and absolute levels of *n*-6 FA and *n*-3 FA. Six liver samples were pooled from each tank (*n* 3), erythrocytes sampled from three fish from each tank (*n* 9). Values are means with vertical bars representing standard deviations. [a,b,c,d] Significantly different means are denoted by unlike letters (one-way ANOVA, followed by Tukey's honestly significant difference multiple comparison). Diet 1/Diet 2/Diet 6/Diet 1H, diet codes according to dietary *n*-6:*n*-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of *n*-3 and *n*-6 compared with the first diet.

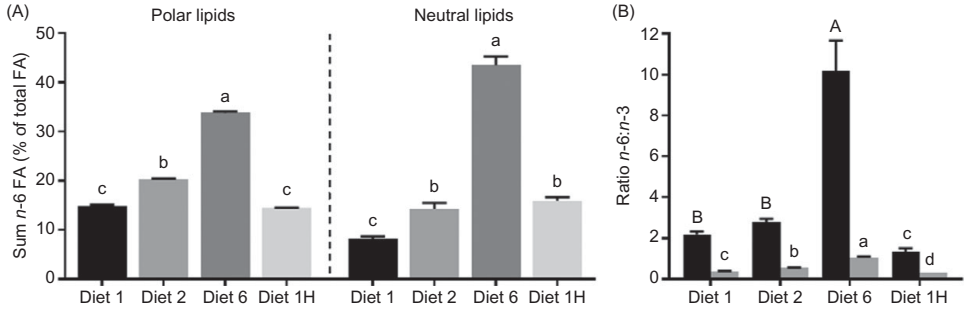

Fig. 5. Comparison of sum *n*-6 (% of total fatty acids (FA)) and *n*-6:*n*-3 FA ratio between neutral (■) and polar (■) lipids in the liver of Atlantic salmon fed diets with varying dietary *n*-6:*n*-3 FA ratios and absolute levels of *n*-6 FA and *n*-3 FA. Six liver samples were pooled from each tank (*n* 3). Values are means with vertical bars representing standard deviations. [a,b,c,d;A,B] Significantly different means are denoted by unlike letters (one-way ANOVA, followed by Tukey's honestly significant difference multiple comparison). Diet 1/Diet 2/Diet 6/Diet 1H, diet codes according to dietary *n*-6:*n*-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of *n*-3 and *n*-6 FA compared with the first diet.

amount of that *n*-6 FA in the erythrocytes (Fig. 4(E)–(G)). In ARA, however, only fish fed diet 6 stood out, with significantly higher content than the other three diets (Fig. 4(H)).

Fatty acid composition of phospholipid classes in skin

Regardless of diet, each of the PL classes was characterised by their own compositional pattern of FA. PC was characterised by the highest relative levels of 16 : 0 and 18 : 1*n*-9. The PS fraction had a high proportion of 18 : 0 and DHA, while PI had a large fraction of 18 : 0 and the highest relative amount of ARA. Lastly, DHA was the FA with the highest concentration in PE, which also had relatively high amounts of 18 : 1*n*-9 (Fig. 6). The SFA content was generally higher than in the diets at approximately 30–40 % of total FA, except for in PE which had lower levels (approximately 15 % of total FA). MUFA followed the same pattern as in the diets, being highest in fish fed diet 1 and the lowest in the fish fed diet 6, but mostly lying at lower levels than in the diets (10–40 % of total FA).

PC and PE were the PL classes most affected by the diets, with almost every FA analysed significantly affected (online Supplementary Table S6). A decrease in PC and PE content of EPA was seen with increasing the *n*-6:*n*-3 FA ratio (diets 1, 2 and 6). Feeding the fish diet 1H resulted in the significantly highest content of EPA and DHA in the PC (Fig. 3(G) and (H)) and PE (Fig. 3(J) and (K)) of the skin. The ratio between EPA:ARA in the PC and PE of the skin was also highly influenced by the dietary *n*-6:*n*-3 diets, with the fish fed diet 1 and diet 1H having similar EPA:ARA ratios (Fig. 3(I) and (L)). The increasing dietary *n*-6:*n*-3 FA ratio caused a significant increase in 18 : 2*n*-6, 20 : 2*n*-6,

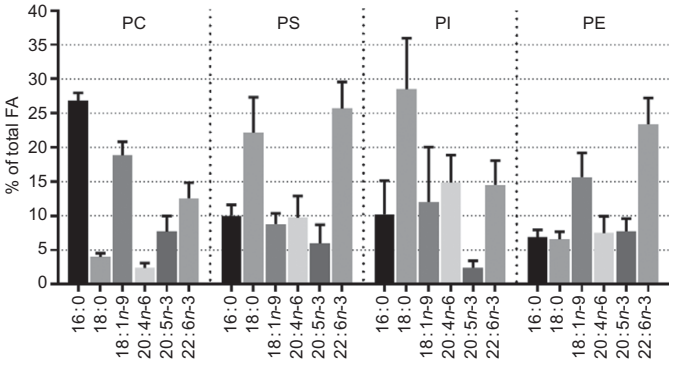

Fig. 6. Selected fatty acids (FA) that characterise the different phospholipid classes in the skin of Atlantic salmon fed diets with varying dietary *n*-6:*n*-3 ratios and absolute levels of *n*-6 FA and *n*-3 FA. Values for each FA are pooled for all dietary groups and given as means with vertical bars representing standard deviations. Diet 1/Diet 2/Diet 6/Diet 1H, diet codes according to dietary *n*-6:*n*-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of *n*-3 and *n*-6 FA compared with the first diet. PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

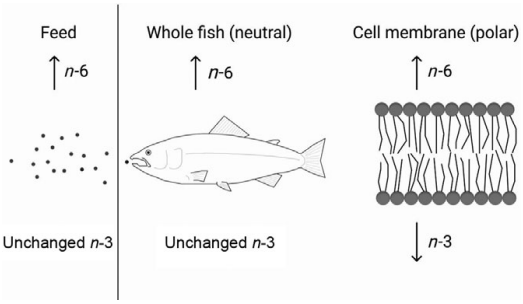

Fig. 7. Constant dietary *n*-3 fatty acids (FA) with increasing dietary *n*-6 FA will lead to higher *n*-6 FA content in the fish and its cell membranes. However, while the *n*-3 FA levels in the whole fish will not be affected by the increasing dietary *n*-6 FA, the cell membrane *n*-3 FA content will decrease despite constant dietary *n*-3 FA.

20 : 3*n*-6 and ARA in both PC and PE (diets 1, 2 and 6, online Supplementary Table S6). In PC, dietary *n*-6:*n*-3 FA ratio was reflected in 18 : 2*n*-6 content and ARA reflected absolute dietary *n*-6 FA. Contrastingly in PE, 18 : 2*n*-6 reflected absolute dietary *n*-6 and ARA reflected dietary *n*-6:*n*-3 ratio. ARA and 20 : 3*n*-6 were significantly increased by higher dietary *n*-6 content in the PI. In PS, 18 : 2*n*-6, 20 : 2*n*-6 and 20 : 3*n*-6 reflected dietary *n*-6:*n*-3 FA ratio. No other FA was significantly affected in PS and PI.

The *n*-6:*n*-3 FA ratio in the total FA of the skin was increased by the dietary ratio (data not shown). The different PL classes were much less impacted, though once again it was clear that the PI and PS were more resistant to change, their ratios barely affected even with 6-fold increases in dietary ratio. Though more resistant to change than the total FA, PE and PC were more influenced than PI and PS (online Supplementary Table S6). Thus, PI and PS were the most conserved PL classes and the least affected by dietary differences.

Discussion

The requirement of EPA and DHA has been extensively studied in fish; however, it has been sparsely studied in relation to the dietary content of *n*-6 FA. To test the possibility of a higher requirement for EPA and DHA with increasing dietary *n*-6 FA is of high relevance to the aquaculture industry. Knowing how to keep the beneficial effects of EPA and DHA even at low dietary levels is crucial with the limited global availability of these nutrients. Important functions of these FA, like maintaining membrane fluidity and function as well as their roles in the inflammatory system through eicosanoid production, depend on the correct levels of these FA in the polar lipid fraction. It is therefore important to be able to formulate fish diets that reduce the metabolic losses of the valuable EPA and DHA and by this maintaining fish health by maximising the positive output of these FA. There is general agreement that a higher dietary *n*-6 FA content will result in increased fish tissue levels of *n*-6 FA[10–12,38]. Additionally, altering the ratio between *n*-3:*n*-6 FA has also been shown to affect *n*-6 FA tissue levels[11,17]. Nevertheless, it has remained unclear whether it is the ratio or absolute contents that play the greater role in deciding the fate of the ingested FA. The present trial, especially designed to be able to distinguish between ratio or absolute contents of *n*-3 and *n*-6 FA, demonstrated that both *n*-6:*n*-3 FA ratio and absolute contents affected the lipid composition of Atlantic salmon. However, neutral and polar lipids were not affected the same way by these dietary variations. Whole fish and muscle (dominated by neutral lipids[11,39]) and liver neutral lipids all reflected the dietary absolute content of *n*-6 and *n*-3 FA. Liver polar lipids, erythrocytes (dominated by polar lipids) and PL of the skin, on the other hand, generally responded more to the dietary *n*-6:*n*-3 FA ratio. Fig. 7 shows a schematic overview of the main results. This is in accordance with earlier reports, where the FA composition of the polar lipids remained more conserved than that of the neutral lipids[9,40,41].

Failure to provide sufficient dietary *n*-3 LC-PUFA can result in reduced growth for salmonids[40]. In the present trial, fish fed

diets with constant EPA + DHA (11 g/kg feed) and increasing *n*-6:*n*-3 FA ratio did not differ significantly in their final weights. However, fish fed the diet with double absolute *n*-3 LC-PUFA (20 g/kg feed) had a significantly higher final weight. The present trial ended when the fish were about 750 g. Bou *et al.*[26] found significantly lower weight in fish provided with 10 g/kg dietary EPA + DHA than fish given 17 g/kg when allowed to grow to about 1·2 kg. This could indicate a slightly higher requirement for EPA and DHA than 10–11 g/kg feed for smaller fish.

The fillet FA composition of Atlantic salmon generally reflects the FA composition of the diets[2,9], and this trial was no exception. What was demonstrated in the present trial, however, was that the absolute amounts of dietary *n*-6 and *n*-3 FA was reflected, not dietary *n*-6:*n*-3. Whole fish also reflected the absolute dietary *n*-6 and *n*-3 FA. Neutral lipids constitute the largest lipid fraction of both Atlantic salmon muscle[39] and whole fish[11]; hence, it is likely that at least the neutral lipid fraction mirrors the dietary absolute *n*-6 and *n*-3 FA. It was unfortunate that the fillet reflected the absolute dietary contents of *n*-3 and *n*-6 FA and not *n*-6:*n*-3 FA ratio. This means that Atlantic salmon fillet content of these FA cannot be modified simply by manipulating the *n*-6:*n*-3 FA ratio of the feed. Mice given salmon fed soya oil as the main lipid source (high 18 : 2*n*-6) developed insulin resistance, accumulated fat in the liver and became overweight[42]. So, although a higher dietary *n*-6:*n*-3 for Atlantic salmon will not reduce fillet EPA and DHA levels, it will cause elevated levels of *n*-6 FA which could reduce the lowering effect eating salmon has on consumer's *n*-6:*n*-3 ratio.

The FAPV values for 18 : 3*n*-3, EPA and DHA were not affected by dietary *n*-6:*n*-3 ratio; however, a higher dietary *n*-3 FA content significantly increased the FAPV for both 18 : 3*n*-3 and EPA, probably indicating a lowered use of these FA for DHA biosynthesis when more DHA was provided in the feed. It could also mean less 18 : 3*n*-3 and EPA being used for β-oxidation. Dietary DHA is known to inhibit its own biosynthetic pathway[43,44], with inhibition occurring mainly at the final Δ6-desaturation step (24 : 5*n*-3 to 24 : 6*n*-3) and the second elongation step (22 : 5*n*-3 to 24 : 5*n*-3)[44] It has been suggested that increased dietary *n*-6 FA could have a positive effect on the endogenous production of DHA[14,45], and Caballero *et al.*[45] hypothesised that this could be due to activation of the Δ5- and Δ6-desaturases (dietary DHA at 2·9–6·7 in % of total FA). Sissener *et al.*[14] found a positive correlation between dietary 18 : 2*n*-6 and FAPV of DHA in Atlantic salmon (dietary DHA at 2·3–2·8 in % of total FA). However, a recent trial feeding Atlantic salmon fry/parr an EPA and DHA-free diet from start feeding found that high dietary 18 : 2*n*-6 inhibited the biosynthesis of long-chain PUFA[46]. The present experiment did not show any effect of dietary 18 : 2*n*-6 on DHA FAPV. Although DHA production was evident due to all FAPV being above 100 (ranging from 122 to 145), there were no significant differences between the groups in FAPV despite large differences in dietary 18 : 2*n*-6. MUFA and SFA are good sources of energy and their dietary content may therefore have a sparing effect on EPA and DHA. A potential sparing effect of SFA was not possible to assess in this trial, due to quite stable SFA contents in all the diets. Correlation analyses did, however, show a clear lack of relation between dietary MUFA and tissue EPA and DHA, thus rejecting the hypothesis of a MUFA-sparing effect. In short, there were no effects of dietary *n*-6:*n*-3 FA ratio or absolute *n*-6 FA on the FAPV of 18 : 3*n*-3, EPA and DHA, but a higher dietary *n*-3 led to significantly higher FAPV and consequently higher conservation of 18 : 3*n*-3 and EPA.

Using VO as the main lipid source for Atlantic salmon can cause an increase in liver TAG content[11,42,47], which is considered a general sign of dietary imbalance that can originate from several sources. Multiple trials have also demonstrated that low dietary EPA + DHA can result in increased liver fat in Atlantic salmon[26,48], which could explain the lower liver TAG content seen in the fish fed the highest dietary *n*-3 FA of the present trial (diet 1H). Bransden *et al.*[11], Alvheim *et al.*[42] and Ruyter *et al.*[47] used VO very high in 18 : 2*n*-6, which could suggest 18 : 2*n*-6 as the culprit for the high liver TAG content. This would correspond well with the high liver TAG seen in the fish fed diet 6 in this experiment. Feeding trials using rapeseed oil (high 18 : 1*n*-9)[49] and cell studies using 18 : 1*n*-9 in the medium[50] suggest that 18 : 1*n*-9 too can contribute to a fatty liver. Diet 1 contained the highest amount of 18 : 1*n*-9 in the present trial, this could help explaining the high liver TAG found in this group. The fish fed diet 1 additionally had the highest content of crude fat in whole fish. A generally high fat level (high visceral fat, liver fat) has previously been seen when Atlantic salmon has been fed rapeseed oil as the main oil source[30]. Fish fed diet 2, however, ended up with significantly lower liver TAG than fish fed diets 1 and 6 and similar TAG levels as fish fed diet 1H. Diet 2 had less 18 : 1*n*-9 than diet 1 and less *n*-6 than diet 6. It is possible that diet 2 had a more beneficial balance between 18 : 1*n*-9 and 18 : 2*n*-6 than diet 1 and diet 6, leading to the significantly lower liver TAG. This means that there were likely several causes to the varying liver TAG levels. Too high dietary 18 : 1*n*-9 caused liver TAG, and too high dietary *n*-6 FA did the same, while higher dietary content of EPA and DHA led to lower liver TAG.

It is a known phenomenon that DHA is preferentially accumulated in salmonid tissues, particularly at low dietary levels, while EPA is more utilised for energy production or biosynthesis of DHA[51,52]. In the present study, while EPA was significantly reduced by an increasing *n*-6:*n*-3 FA ratio (constant *n*-3 FA) in the liver polar lipids, the erythrocytes and the PC and PE of the skin, DHA remained relatively unchanged in these tissues. In fact, all *n*-3 FA analysed other than DHA and 18 : 3*n*-3 were reduced by increasing the dietary *n*-6:*n*-3 ratio. A possible explanation could be that DHA is more critical than EPA as a structural component, while EPA is believed to have a bioactive role in processes related to inflammation and immunity[53,54]. In line with our results, Bell *et al.*[12] found reduced EPA but stable DHA in liver PC and PE (the two largest PL fractions) in Atlantic salmon fed a diet containing sunflower oil (dietary *n*-6:*n*-3 FA ratio at 4·4) compared with when fed a FO diet (dietary *n*-6:*n*-3 FA ratio at 0·1). However, in the study by Bell *et al.*[12], the FO diet contained more EPA and DHA than the sunflower oil diet, while in our study it was kept constant in diets 1, 2 and 6. Our results demonstrate that this reduction of tissue EPA caused by increased dietary *n*-6:*n*-3 FA ratio occurs even when dietary EPA and DHA are kept unchanged. On the contrary, in the neutral lipids of the liver, an increasing *n*-6:*n*-3 FA ratio did

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not reduce the *n*-3 FA as in the polar lipids. The only dietary change that clearly caused a significant effect on the *n*-3 FA in this tissue was an increased absolute dietary content of *n*-3 FA. In theory, there could be a few reasons why a higher dietary *n*-6:*n*-3 could cause a rise in polar lipid *n*-6 FA at the expense of EPA (and the other *n*-3 FA except DHA and 18 : 3*n*-3) in Atlantic salmon. It could mean that there has been a reduced biosynthesis of EPA due to increased competition for enzymes shared by *n*-6 and *n*-3 FA, or a competition for incorporation into the polar lipids. It could also be an increased requirement for EPA, and its anti-inflammatory eicosanoids, due to higher levels of *n*-6 FA and lower EPA/ARA ratio causing an increased inflammatory status. In whole fish (mainly neutral lipids[11]), the dietary absolute contents of the *n*-3 and *n*-6 FA were reflected; meaning no reduction of whole fish EPA (or other *n*-3 FA) by increased dietary *n*-6:*n*-3 ratio. As the effects were only seen in the polar lipids, it is most likely not an 18 : 2*n*-6 induced inhibition of *n*-3 PUFA biosynthesis. A competition between *n*-6 and *n*-3 FA for incorporation into the phospholipids seems more probable. DHA appeared exempted in this competition, as it was the only FA spared the response seen for the other *n*-3 FA. However, although a higher dietary *n*-6:*n*-3 FA ratio caused a reduction in most polar lipid *n*-3 FA in our study, increasing the *n*-3 FA content concomitantly with *n*-6 FA trumped this. Not only were the adverse effects of increasing dietary *n*-6 FA on polar lipid *n*-3 FA negated, but a higher content of all *n*-3 FA in polar lipids was seen. The fact that DHA was the only *n*-3 FA to remain stable in polar lipid types despite increasing dietary *n*-6:*n*-3 FA ratios emphasises its importance and shows that dietary *n*-6 FA is not a decisive factor in deciding the DHA levels of polar lipids. Based on this, using oils with higher *n*-6 FA content would not be a large issue. However, the amount of EPA was notably reduced when increasing the dietary *n*-6:*n*-3 FA ratio. Not only do we not know the consequences of this should there be a higher need for EPA when the fish are exposed to challenging conditions (stress, illness, etc.), it also means poor utilisation of a valuable nutrient. Hence, the results of this study indicate that by keeping a low *n*-6:*n*-3 FA ratio, a better utilisation of the dietary EPA content can be achieved.

The dietary *n*-6 FA content is reflected in the tissue *n*-6 FA of Atlantic salmon[11,12,14]. Hence, it was unsurprising that constant dietary *n*-3 with increasing dietary *n*-6 FA (mainly 18 : 2*n*-6) caused an increase in *n*-6 FA in all tissues (both neutral and polar lipids). The neutral lipids accumulated much more 18 : 2*n*-6 than the polar lipids of the liver. This corresponds to the findings of Berge *et al.*[55] in Atlantic salmon vertebrae where fish fed a diet high in 18 : 2*n*-6 led to larger increase of 18 : 2*n*-6 in TAG than in PL. In the present study, the neutral lipids of the liver incorporated more 18 : 2*n*-6 and less EPA, docosapentaenoic acid and DHA than the polar lipids, resulting in *n*-6:*n*-3 FA ratios from 1·4 to 10·2 and 0·3 to 1·1 in the neutral and polar lipids, respectively. Although this highlights a stricter control of the polar lipids, increases in polar lipids *n*-6 FA were still seen with higher dietary *n*-6 FA, particularly in the longer chain *n*-6 FA. PL are major components of cellular membranes, and their *n*-6 and *n*-3 FA content has a strong influence on membrane fluidity[20]. The *n*-6 and *n*-3 FA acyl chain flexibility differ substantially and the removal of even one double bond can significantly alter membrane fluidity[56,57]. Hence, the alteration of membrane *n*-6 and *n*-3 FA content could affect the physical properties of the cell membrane, and thereby alter cell function.

There was also a clear difference in the distribution of the different *n*-6 FA in the neutral lipids and polar lipids in the present study. In the neutral lipids, the total *n*-6 FA was mostly composed of 18 : 2*n*-6, with a little 20 : 2*n*-6 and 20 : 3*n*-6, and only miniscule amounts of ARA which merely reflected the dietary content. In the polar lipids, on the other hand, there was a higher amount present of all intermediates from 18 : 2*n*-6 to ARA, but also of ARA itself. However, though the dietary *n*-6:*n*-3 FA ratio increased, it did not lead to an equally large increase in polar lipid ARA. Therefore, it seems to be some restricting mechanism to avoid extreme accumulation of ARA in polar lipids when facing very high dietary *n*-6:*n*-3 FA ratios. The present study mostly found less ARA than EPA in PL of Atlantic salmon, which has been seen before[58,59]. Less membrane ARA than EPA is provided no deficiency of EPA + DHA, as *n*-6 FA will then replace the *n*-3 FA in the PL[9]. However, in the fish fed the extreme diet in the present study (dietary *n*-6:*n*-3 FA ratio at 6), the relationship was inversed with more ARA than EPA. ARA is readily incorporated into membrane PL of fish[60], from which it can be released again mainly by cytosolic phospholipase 2 to be further metabolised by enzymes such as cyclo-oxygenase 2 or lipoxygenase into eicosanoids. Eicosanoids are particularly bioactive compounds involved in, e.g. inflammatory responses and tissue homeostasis[61]. Since ARA is preferentially incorporated into fish PLs[60], increasing dietary 18 : 2*n*-6 will lead to a shift in the balance between EPA and ARA incorporated into the PL, as seen in this trial. Additionally, Atlantic salmon has a preference for using ARA over EPA for eicosanoid production[62]. As ARA-derived eicosanoids are generally known to be more potent than EPA derived ones[20], it is possible that an excessive *n*-6 FA intake could cause negative eicosanoid-mediated responses. The fish in this trial were kept under controlled, stable conditions and did not seem impaired by these diets. However, it is possible that exposure to more challenging conditions common to the aquaculture industry such as transport and handling could result in eicosanoid-mediated responses with a negative impact on fish performance.

PS and PI of the skin were only marginally affected by the dietary FA variations, but this is in line with previous results in skin[27], liver, heart[12], gill and leucocytes[22]. PC and PE, however, had significantly reduced EPA. Cheng *et al.*[27] suggested that a higher content of EPA and DHA in the PL of the skin could improve the inflammatory and protective-barrier capacities in the skin of fish, though they found no significant differences in epidermal thickness or mucus cell density due to high variation. This could mean that increased dietary *n*-6:*n*-3 FA ratio could negatively affect the protective capabilities of the skin by reducing the PL content of EPA and DHA.

The hypothesis that an increased dietary *n*-6:*n*-3 FA ratio and/or higher dietary *n*-6 FA could affect the requirement of EPA and DHA in Atlantic salmon seems to be at least partly true. The reduction of EPA in polar lipids with increasing dietary *n*-6:*n*-3 FA ratio in this study indicates that a better utilisation of EPA can be achieved when the dietary *n*-6:*n*-3 FA ratio is kept low. DHA was not significantly decreased. The content of EPA

and DHA in fillet was only affected by absolute dietary *n*-3 FA, but so was the content of all *n*-6 FA. This means that a feed with higher dietary *n*-6:*n*-3 FA ratio does not reduce the EPA and DHA for human consumption, but the *n*-6 FA content will increase. While these diets led to considerable changes in tissue FA composition, the fish in this trial did not seem to suffer from it. However, the fish were kept under ideal, stable conditions, which did not necessarily represent the challenging conditions under which fish in aquaculture are kept. The effects of altering the Atlantic salmon tissue FA composition with diets with varying *n*-6:*n*-3 FA ratio and absolute *n*-6 FA under non-ideal conditions (e.g. fish exposed to disease, non-optimal oxygen levels or temperature, stress due to handling) need to be tested.

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The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114520002494

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Supplementary table 1: Feed formulation in g/100g for the 3mm pellet size.

Pellet size	3 mm			
	Diet 1	Diet 2	Diet 6	Diet 1H
Wheat	8.8	8.8	8.8	8.8
Soya protein concentrate	31.5	31.5	31.5	31.5
Sunflower meal	6.0	6.0	6.0	6.0
Wheat gluten	18.2	18.2	18.2	18.2
Faba beans dehulled	2.0	2.0	2.0	2.0
FM North Atlantic	10.0	10.0	10.0	10.0
Linseed oil	1.0	1.1	1.1	2.3
Sunflower oil	0.5	4.0	16.3	4.3
Olive oil	10.2	7.1	0.0	2.0
Coconut oil	0.7	0.3	0.0	0.0
FO North Atlantic	0.0	0.0	3.2	4.6
FO Capelin	8.2	8.2	0.0	7.6
Premixes	3.2	3.2	3.2	3.2

Diet 1/Diet 2/Diet 6/Diet 1H, diet codes are set according to dietary n-6/n-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of n-3 and n-6 FA compared to the first diet

Supplementary table 2: Analysed dietary proximate (g/100g) and full fatty acid composition (% of total FA, total FA in mg/g) of the four diets at both pellet sizes.

	3 mm				4 mm			
	Diet 1	Diet 2	Diet 6	Diet 1H	Diet 1	Diet 2	Diet 6	Diet 1H
Proximate composition g/100g								
Lipid	25.5	24.6	25.8	25.5	29.8	30.0	28.6	28.8
Protein	46.6	47.0	45.5	46.3	44.5	44.1	44.5	44.8
Ash	5.3	5.5	5.7	5.5	5.1	5.2	5.5	5.4
Fatty acids (% of total area)								
ΣSFA	20.3	18.2	16.0	19.9	19.7	19.7	16.6	19.8
8:0	0.2	0.1	≤LOQ	≤LOQ	0.2	0.2	0.1	≤LOQ
10:0	0.2	0.1	≤LOQ	≤LOQ	0.2	0.2	0.1	≤LOQ
12:0	1.4	0.7	0.0	0.1	1.3	1.7	0.4	0.1
14:0	3.5	3.1	1.4	4.1	3.3	3.5	1.5	4.4
15:0	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.2
16:0	11.6	10.7	9.5	11.8	11.2	10.4	9.4	11.5
18:0	2.4	2.6	3.6	2.8	2.5	2.6	3.7	2.7
20:0	0.4	0.3	0.3	0.3	0.4	0.3	0.3	0.3
22:0	0.5	0.6	0.9	0.6	0.5	0.6	0.9	0.6

24:0	0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.1
ΣMUFA	57.7	52.8	27.5	41.9	59.4	52.2	27.3	43.5
14:1n-5	0.1	0.1	≤LOQ	0.1	0.1	0.1	≤LOQ	0.1
16:1n-7	3.8	3.6	1.5	4.8	3.8	3.7	1.5	5.1
16:4n-1	0.2	0.2	0.1	0.3	0.2	0.2	0.1	0.3
18:1n-5	0.2	0.2	≤LOQ	0.2	0.2	0.2	≤LOQ	0.2
18:1n-7	2.2	2.0	1.1	2.0	2.2	2.0	1.1	2.0
18:1n-9	36.1	31.2	22.7	19.6	37.1	30.1	23.1	18.2
20:1n-7	0.3	0.3	≤LOQ	0.3	0.3	0.3	≤LOQ	0.3
20:1n-9	6.1	6.2	0.8	6.0	6.4	6.4	0.7	7.0
20:1n-11	0.3	0.3	0.1	0.4	0.3	0.4	0.1	0.4
21:5n-3	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.2
22:1n-7	0.1	0.1	≤LOQ	0.1	0.1	0.1	≤LOQ	0.1
22:1n-9	0.9	0.9	0.1	0.9	0.9	0.9	0.1	1.0
22:1n-11	7.3	7.5	0.9	7.3	7.7	7.7	0.7	8.5
24:1n-9	0.3	0.3	0.1	0.4	0.3	0.3	0.1	0.4
Σn-6	11.7	19.0	47.4	20.2	11.1	18.2	46.9	18.4
16:2n-6	0.2	0.1	0.1	0.3	0.2	0.2	0.1	0.3
18:2n-6	11.3	18.7	47.1	19.4	10.8	17.9	46.7	17.6
18:3n-6	≤LOQ	≤LOQ	≤LOQ	0.1	≤LOQ	≤LOQ	≤LOQ	0.1
20:2n-6	0.1	0.1	≤LOQ	0.1	0.1	0.1	≤LOQ	0.1
20:3n-6	≤LOQ	≤LOD	≤LOQ	≤LOQ	≤LOQ	≤LOQ	≤LOQ	≤LOQ
20:4n-6 (ARA)	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.2
Σn-3	8.3	7.8	7.7	15.1	7.7	7.7	7.8	15.1
18:3n-3	3.0	3.0	3.0	5.8	2.9	2.9	3.1	5.7
18:4n-3	0.6	0.5	0.4	0.9	0.5	0.5	0.4	0.9
20:3n-3	≤LOQ	≤LOQ	≤LOQ	≤LOQ	≤LOQ	≤LOQ	≤LOQ	≤LOQ
20:4n-3	0.1	0.1	0.1	0.3	0.1	0.1	0.2	0.3
20:5n-3 (EPA)	2.5	2.3	2.0	4.3	2.4	2.4	2.0	4.4
22:5n-3	0.2	0.2	0.3	0.5	0.2	0.2	0.3	0.5
22:5n-6	≤LOQ	≤LOQ	≤LOQ	0.06	≤LOQ	≤LOQ	≤LOQ	0.1
22:6n-3 (DHA)	1.7	1.6	1.8	3.2	1.5	1.5	1.8	3.1
EPA + DHA	4.3	3.9	3.8	7.4	3.9	3.9	3.8	7.5
ΣPUFA	22.1	29.0	56.5	38.3	20.9	28.1	56.1	36.8
n-6/n-3	1.4	2.4	6.2	1.3	1.4	2.4	6.1	1.2
Sum FA (mg/g)	227.3	223.2	253.4	223.3	270.7	272.7	296.5	255.3

Diet 1/Diet 2/Diet 6/Diet 1H, diet codes are set according to dietary n-6/n-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of n-3 and n-6 FA compared to the first diet; FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids; LOQ: limit of quantification (0.1% of total FA)

Supplementary table 3: Fatty acid composition (% of total FA) of the Norwegian quality cut (NQC; fillet) of Atlantic salmon fed diets with varying dietary n-6/n-3 ratios and absolute levels of n-6 FA and n-3 FA.
(Mean values with their standard deviations of three tanks per diet with six fish pooled per tank, n = 3. Significantly different means are denoted by different superscript letters.)

	Diet 1		Diet 2		Diet 6		Diet 1H	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ΣSFA	19.5 ^{b}	<0.1	19.2 ^{b}	0.1	16.8 ^{a}	0.1	19.9 ^{c}	0.2
ΣMUFA	57.9 ^{d}	0.2	51.7 ^{c}	0.2	28.8 ^{a}	0.3	43.3 ^{b}	0.1
Σn-6	11.2 ^{a}	<0.1	17.9 ^{b}	0.2	43.3 ^{c}	0.5	18.1 ^{b}	<0.1
18:2n-6	9.5 ^{a}	<0.1	15.4 ^{b}	0.1	38.3 ^{d}	0.5	16.0 ^{c}	<0.1
20:2n-6	0.3 ^{a}	<0.1	1.0 ^{b}	<0.1	2.0 ^{c}	0.1	0.9 ^{b}	<0.1
20:3n-6	0.5 ^{a}	<0.1	0.8 ^{b}	0.1	1.5 ^{c}	<0.1	0.4 ^{a}	<0.1
20:4n-6 (ARA)	0.4 ^{c}	<0.1	0.4 ^{b}	<0.1	0.6 ^{a}	<0.1	0.6 ^{a}	<0.1
Σn-3	8.8 ^{a}	0.1	8.6 ^{a}	0.1	8.6 ^{a}	0.2	15.5 ^{b}	0.2
18:3n-3	2.0 ^{a}	<0.1	2.1 ^{a}	<0.1	2.2 ^{b}	<0.1	4.5 ^{c}	<0.1
20:5n-3 (EPA)	1.4 ^{b}	<0.1	1.4 ^{b}	<0.1	1.2 ^{a}	<0.1	2.8 ^{c}	0.1
22:6n-3 (DHA)	3.5 ^{a}	0.1	3.3 ^{a}	<0.1	3.6 ^{a}	0.2	5.1 ^{b}	0.2
EPA + DHA	4.9 ^{a}	0.1	4.7 ^{a}	0.1	4.9 ^{a}	0.2	7.8 ^{b}	0.1
ΣPUFA	20.0 ^{a}	0.2	26.6 ^{b}	0.1	51.9 ^{d}	0.4	33.8 ^{c}	0.2
n-6/n-3	1.3 ^{a}	<0.1	2.1 ^{b}	<0.1	5.0 ^{c}	0.1	1.2 ^{a}	<0.1
Total fatty acids (mg/g)	106.1	3.6	102.8	3.1	104.8	6.7	110.3	1.3

Diet 1/Diet 2/Diet 6/Diet 1H, diet codes are set according to dietary n-6/n-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of n-3 and n-6 compared to the first diet; FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids

Supplementary table 4: Fatty acid composition (% of total FA) of the polar and neutral lipids of the liver in Atlantic salmon fed diets with varying dietary n-6/n-3 FA ratios and absolute levels of n-6 and n-3 FA.

(Mean values with standard deviation of three tanks per diet with 6 fish pooled per tank, n = 3. Significantly different means are denoted by different superscript letters.)

	Diet 1		Diet 2		Diet 6		Diet 1H	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Polar lipids								
ΣSFA	24.9 ^{ab}	1.0	25.4 ^{b}	0.9	23.5 ^{a}	0.2	25.6 ^{b}	0.2
ΣMUFA	20.6 ^{d}	0.2	17.3 ^{c}	0.2	10.4 ^{a}	<0.1	14.2 ^{b}	0.2
Σn-6	14.8 ^{a}	0.3	20.3 ^{b}	0.2	33.8 ^{c}	0.3	14.5 ^{a}	0.1
18:2n-6	6.2 ^{a}	0.4	9.0 ^{b}	0.1	16.0 ^{c}	0.2	6.7 ^{a}	0.1
20:2n-6	1.1 ^{a}	0.1	1.6 ^{b}	0.2	4.1 ^{c}	0.2	1.7 ^{b}	<0.1
20:3n-6	4.0 ^{b}	0.2	5.4 ^{c}	0.2	6.2 ^{d}	0.3	2.2 ^{a}	<0.1
20:4n-6 (ARA)	3.1 ^{a}	0.3	3.7 ^{b}	0.2	6.4 ^{c}	0.1	3.3 ^{ab}	<0.1
Σn-3	38.4 ^{c}	0.9	35.8 ^{b}	0.5	31.4 ^{a}	0.5	44.2 ^{d}	0.3
18:3n-3	0.5 ^{a}	<0.1	0.4 ^{a}	<0.1	0.4 ^{a}	<0.1	0.9 ^{b}	0.1
20:4n-3	0.7 ^{b}	0.1	0.6 ^{b}	<0.1	0.4 ^{a}	<0.1	0.9 ^{c}	<0.1

20:5n-3 (EPA)	7.5 [b]	0.6	6.6 [b]	0.4	3.3 [a]	0.1	8.7 [c]	0.2
22:5n-3	2.2 [bc]	0.1	2.1 [b]	0.2	1.2 [a]	0.1	2.4 [c]	0.1
22:6n-3 (DHA)	26.8 [a]	1.3	25.4 [a]	1.1	25.7 [a]	0.5	30.9 [b]	0.3
ΣPUFA	53.3 [a]	0.6	56.2 [b]	0.6	65.2 [d]	0.3	58.8 [c]	0.3
n-6/n-3	0.4 [b]	<0.1	0.6 [c]	<0.1	1.1 [d]	<0.1	0.3 [a]	<0.1
Neutral lipids								
ΣSFA	17.0 [b]	0.9	17.4 [a]	1.3	13.5 [a]	1.1	18.7 [b]	<0.1
ΣMUFA	69.7 [d]	0.8	62.0 [c]	0.3	37.8 [a]	05.	51.9 [b]	1.3
Σn-6	8.2 [a]	0.5	14.3 [b]	1.1	43.5 [c]	1.8	15.9 [b]	0.7
18:2n-6	6.4 [a]	0.3	11.1 [b]	0.8	34.2 [c]	1.0	12.8 [b]	0.7
20:2n-6	0.7 [a]	0.1	1.3 [a]	0.3	5.4 [b]	0.9	1.5 [a]	0.2
20:3n-6	0.6 [a]	<0.1	1.2 [c]	0.1	2.6 [d]	<0.1	0.8 [b]	<0.1
20:4n-6 (ARA)	0.2 [a]	0.1	0.4 [b]	<0.1	0.7 [c]	<0.1	0.7 [c]	<0.1
Σn-3	3.8 [a]	0.4	5.1 [a]	0.1	4.3 [a]	0.5	11.9 [b]	1.2
18:3n-3	0.9 [a]	0.1	1.0 [a]	0.1	1.4 [b]	<0.1	2.9 [c]	0.2
20:5n-3 (EPA)	0.7 [ab]	0.1	0.9 [b]	0.1	0.6 [a]	0.1	2.2 [c]	0.2
22:6n-3 (DHA)	1.5 [b]	0.2	2.3 [b]	0.1	1.7 [b]	0.4	4.5 [a]	0.8
ΣPUFA	12.2 [a]	0.8	19.7 [b]	1.3	48.0 [d]	1.5	28.1 [c]	1.2
n-6/n-3	2.2 [b]	0.1	2.8 [b]	0.2	10.2 [c]	1.5	1.4 [a]	0.2

Diet 1/Diet 2/Diet 6/Diet 1H, diet codes are set according to dietary n-6/n-3 ratio. The final diet is labelled 1H due to its higher absolute contents of n-3 and n-6 compared to the first diet; FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids

Supplementary table 5: Fatty acid composition (% of total FA) of the RBC in Atlantic salmon fed diets with varying dietary n-6/n-3 FA ratios and absolute levels of n-6 FA and n-3 FA.

(Mean values with standard deviation of three tanks per diet with samples from three fish per tank, n = 3. Significantly different means are denoted by different superscript letters.)

	Diet 1		Diet 2		Diet 6		Diet 1H	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ΣSFA	30.4	1.4	31.5	3.0	30.6	1.1	30.8	1.2
ΣMUFA	12.6 [c]	1.1	10.4 [b]	0.4	7.1 [a]	0.6	8.0 [a]	0.7
Σn-6	9.0 [a]	0.4	12.0 [b]	1.0	20.0 [c]	0.8	8.7 [a]	0.4
18:2n-6	3.7 [a]	0.2	5.3 [b]	0.6	10.0 [c]	1.0	3.9 [a]	0.2
20:2n-6	0.5 [a]	0.1	0.8 [b]	0.2	1.5 [c]	0.2	0.5 [a]	0.1
20:3n-6	1.9 [b]	0.2	2.6 [c]	0.3	3.4 [d]	0.4	1.0 [a]	0.1
(ARA)	2.9 [a]	0.3	3.3 [a]	0.3	5.1 [b]	0.3	3.3 [a]	0.2
20:4n-6								
Σn-3	48.4 [c]	0.9	46.1 [b]	2.3	42.2 [a]	0.7	52.6 [d]	1.1
20:4n-3	1.0 [b]	0.1	0.9 [b]	0.1	0.6 [a]	0.1	1.1 [b]	0.1
(EPA)	8.0 [b]	0.9	7.3 [b]	0.9	4.5 [a]	0.7	9.5 [c]	0.9
20:5n-3								
22:5n-3	2.9 [b]	0.3	2.9 [b]	0.3	2.4 [a]	0.3	3.4 [c]	0.4
(DHA)	36.5 [ab]	1.2	35.0 [a]	2.3	34.7 [a]	0.9	38.7 [b]	1.5
22:6n-3								
ΣPUFA	57.4 [a]	0.8	58.1 [a]	2.8	62.3 [b]	0.8	61.2 [b]	1.3

n-6/n-3	0.2 [b]	<0.1	0.3 [c]	<0.1	0.5 [d]	<0.1	0.2 [a]	<0.1

Diet 1/Diet 2/Diet 6/Diet 1H, diet codes are set according to dietary n-6/n-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of n-3 and n-6 FA compared to the first diet; RBC, red blood cells; FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids

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Supplementary table 6: Fatty acid composition (% of total FA) of PC, PE, PI and PS in the skin of Atlantic salmon fed diets with varying dietary n-6/n-3 FA ratios and absolute levels of n-6 and n-3 FA. (Mean values with standard deviation of three tanks per diet with three skin samples pooled per tank, n = 3. Significantly different means are denoted by different superscript letters.)

	PC								PE							
	Diet 1		Diet 2		Diet 6		Diet 1H		Diet 1		Diet 2		Diet 6		Diet 1H	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ΣSFA	32.1	2.4	33.6	0.3	33.6	0.6	34.0	0.5	13.0	0.9	15.4	2.4	14.7	0.8	14.7	2.0
ΣMUFA	30.3 ^{b}	2.2	28.5 ^{b}	0.5	23.6 ^{a}	0.7	25.6 ^{a}	1.4	27.8 ^{b}	0.7	25.9 ^{b}	4.9	16.4 ^{a}	0.4	20.5 ^{ab}	0.9
Σn-6	11.1 ^{a}	0.5	15.3 ^{b}	0.9	25.6 ^{c}	0.1	10.3 ^{a}	0.5	13.9 ^{a}	0.6	20.2 ^{c}	0.7	33.5 ^{d}	0.3	16.2 ^{b}	1.1
18:2n-6	5.7 ^{a}	0.3	8.6 ^{b}	0.5	15.3 ^{c}	0.5	5.7 ^{a}	0.3	5.6 $^{a\|}$	0.4	8.5 ^{b}	0.1	16.0 ^{c}	0.6	7.5 ^{b}	0.4
20:2n-6	0.8 ^{a}	<0.1	1.3 ^{b}	0.2	2.3 ^{c}	0.1	1.0 ^{a}	<0.1	0.8 ^{a}	<0.1	1.2 ^{b}	0.1	2.3 ^{c}	0.1	1.3 ^{b}	0.1
20:3n-6	2.4 ^{b}	0.1	3.1 ^{b}	0.4	3.9 ^{c}	0.5	1.4 ^{a}	0.2	2.0 ^{b}	0.1	2.8 ^{c}	0.2	3.3 ^{d}	0.2	1.3 ^{a}	0.1
(ARA) 20:4n-6	1.9 ^{a}	0.3	2.1 ^{a}	0.2	3.7 ^{b}	<0.1	2.1 ^{a}	0.3	5.4 ^{a}	0.1	7.4 ^{b}	0.2	11.5 ^{c}	0.2	6.1 ^{a}	0.1
Σn-3	**24.3 ^{bc}**	**4.1**	**20.6 ^{ab}**	**1.2**	**15.8 ^{a}**	**0.9**	**27.6 ^{c}**	**2.1**	**36.2 ^{bc}**	**0.7**	**33.1 ^{b}**	**1.5**	**27.1 ^{a}**	**1.9**	**40.3 ^{c}**	**3.0**
18:3n-3	0.5 ^{a}	0.1	0.5 ^{a}	<0.1	0.5 ^{a}	<0.1	0.8 ^{b}	0.1	0.3 ^{b}	<0.1	0.3 ^{b}	<0.1	<0.1 ^{a}	0.1	0.5 ^{c}	<0.1
(EPA) 20:5n-3	8.5 ^{bc}	1.8	7.2 ^{b}	0.4	4.3 ^{a}	0.1	10.1 ^{c}	0.7	9.0 ^{bc}	0.7	8.1 ^{b}	0.5	5.1 ^{a}	0.2	9.5 ^{c}	0.4
22:5n-3	1.8 ^{a}	0.4	1.5 ^{a}	0.1	1.0 ^{a}	0.1	1.8 ^{a}	0.1	2.0 ^{b}	0.2	2.0 ^{b}	0.1	1.4 ^{a}	0.1	2.3 ^{b}	0.1
(DHA) 22:6n-3	13.5 ^{ab}	1.9	11.5 ^{a}	1.0	10.1 ^{a}	0.7	14.8 ^{b}	1.7	24.8 ^{ab}	0.3	22.7 ^{a}	1.2	20.5 ^{a}	1.7	28.1 ^{b}	2.9
ΣPUFA	35.3 ^{a}	4.4	35.9 ^{a}	0.4	41.4 ^{b}	1.0	37.9 ^{ab}	1.8	50.2 ^{a}	0.8	50.4 ^{a}	5.8	60.7 ^{b}	1.6	56.5 ^{ab}	2.4
n-6/n-3	0.5 ^{a}	0.1	0.7 ^{b}	0.1	1.6 ^{c}	0.1	0.4 ^{a}		0.4 ^{a}		0.6 ^{b}		1.2 ^{c}	0.1	0.41 ^{a}	0.1
	PI								PS							
	Diet 1		Diet 2		Diet 6		Diet 1H		Diet 1		Diet 2		Diet 6		Diet 1H	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ΣSFA	33.5	7.6	41.8	6.5	46.6	1.2	43.1	3.7	36.1	4.0	33.7	5.8	31.2	3.8	35.4	5.7
ΣMUFA	38.2 ^{b}	8.2	15.7 ^{a}	2.8	8.3 ^{a}	0.2	12.4 ^{a}	1.4	14.9 ^{b}	0.5	14.1 ^{bc}	0.9	10.3 ^{a}	1.5	11.7 ^{ac}	1.6
Σn-6	15.8	4.0	22.2	1.4	27.1	0.9	22.0	2.0	17.5	2.3	17.0	1.5	23.3	0.7	15.6	4.3
18:2n-6	3.9	2.3	3.4	1.1	3.8	0.4	2.1	0.2	1.9 ^{a}	0.2	3.6 ^{a}	1.1	7.4 ^{b}	1.7	2.6 ^{a}	0.8

20:2n-6	0.7 [a]	0.6	1.3 [ab]	0.2	1.7 [b]	0.4	1.3 [ab]	0.2	0.8 [a]	<0.1	1.1 [b]	0.1	1.8 [c]	0.1	1.0 [ab]	0.1
20:3n-6	1.4	1.4	3.0	0.5	2.9	0.1	1.9	0.5	3.3 [b]	0.4	3.6 [b]	0.4	4.5 [c]	0.3	1.8 [a]	0.1
(ARA) 20:4n-6	9.9 [a]	4.7	14.5 [ab]	2.0	18.7 [b]	1.4	16.6[b]	1.4	11.4	2.9	8.5	3.0	9.2	1.5	10.1	4.8
Σn-3	15.8	6.2	19.5	5.3	17.7	0.8	20.8	3.5	30.9	6.4	34.8	6.9	34.9	2.1	36.7	10.1
18:3n-3	<LOQ	-	<LOQ	-	<LOQ	-	0.2	0.4	<LOQ	-	0.1	0.2	0.3	0.1	0.2	0.3
(EPA) 20:5n-3	2.9	0.5	2.9	1.3	1.1	0.2	2.6	0.2	4.6	2.0	6.4	3.1	5.6	1.3	6.9	3.9
22:5n-3	0.8	1.4	1.5	1.0	1.8	0.6	1.9	0.6	2.9	0.3	2.7	0.3	2.2	0.3	3.0	0.7
(DHA) 22:6n-3	11.9	5.3	15.1	3.9	14.8	1.0	16.0	0.6	23.5	4.2	25.7	3.6	26.8	0.8	26.6	5.7
ΣPUFA	25.8 [a]	2.8	41.7 [b]	5.7	44.8 [b]	1.2	42.8 [b]	4.5	48.4	4.2	51.8	5.4	58.2	2.7	52.3	5.9
n-6/n-3	1.0	0.2	1.2	0.3	1.5	0.1	1.1	0.2	0.6	0.2	0.5	0.1	0.7	<0.1	0.5	0.3

Diet 1/Diet 2/Diet 6/Diet 1H, diet codes are set according to dietary n-6/n-3 FA ratio. The final diet is labelled 1H due to its higher absolute contents of n-3 and n-6 FA compared to the first diet; FA, fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid;

Paper II

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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 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

Abbreviations: 5-HIAA, serotonin metabolite; 5-HT, serotonin; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; FO, fish oil; HCT, haematocrit; HPA/I, hypothalamic-pituitary adrenal/interrenal; IGF-1, insulin-like growth factor 1; LA, linoleic acid; LPL, lipoprotein lipase; LTB4, leukotriene B4; LTB5, leukotriene B5; NL, neutral lipids; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PGE3, prostaglandin E3; PL, polar lipids; RBC, red blood cells; TAG, triacylglycerol; TFA, total fatty acids; TRP, tryptophan; VLDL, very-low-density lipoprotein; VO, vegetable oil.

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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 Mastermix (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 ± 0.06 (mean ± 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%
PGE_2 (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; PGE_2 EP4 – prostaglandin E_2 (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.

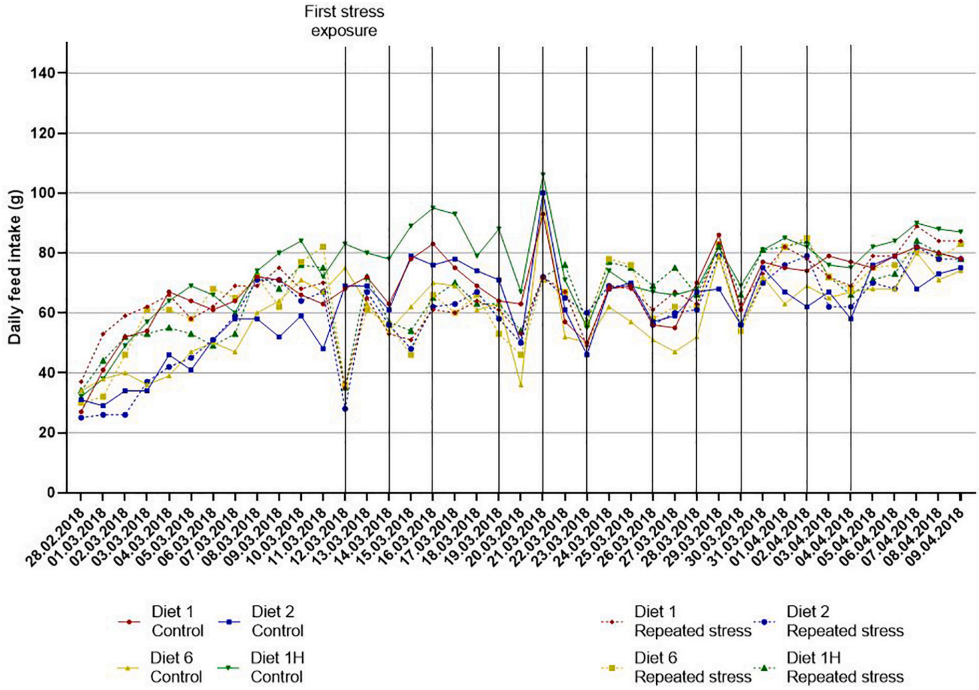

Fig. 1. Daily feed intake (g) of Atlantic salmon fed diets with varying dietary n-6/n-3 FA ratios and absolute levels of n-6 and n-3 FA. The fish were either exposed to a repeated stressor (hypoxia 3 times per week, 4 weeks, marked with black lines) or left undisturbed. Diet codes are given by dietary 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

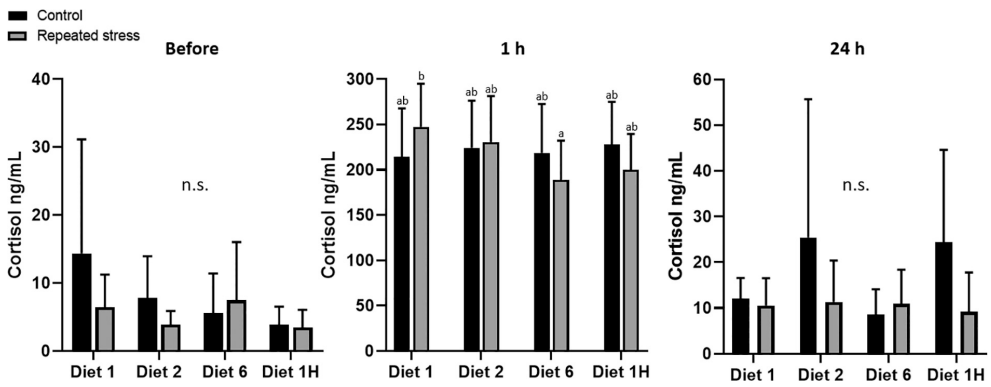

Fig. 2. Plasma cortisol (ng/mL) of Atlantic salmon before, 1 h after and 24 h after an acute stressor ($n = 15$ per diet/repeated stress/acute stress, hence per bar in figure). The fish had been fed diets with varying dietary n-6/n-3 ratios and absolute levels of n-6 and n-3 FA. The fish were either 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.

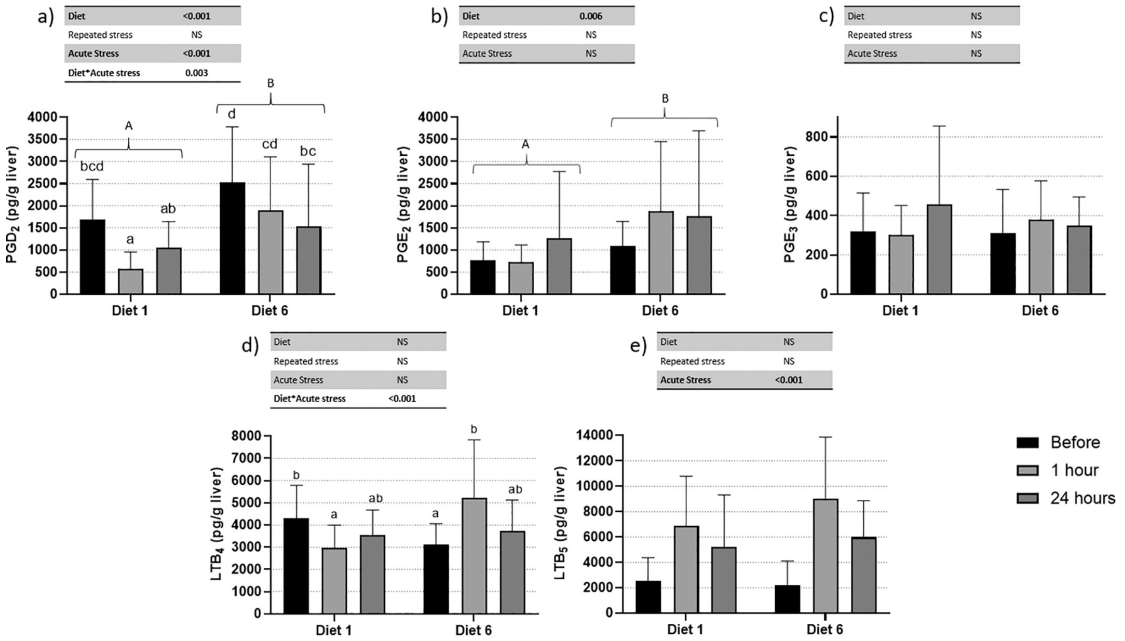

Fig. 3. Level of prostaglandins (PGD2, PGE2, PGE3, a, b and c respectively) and leukotrienes (LTB4, LTB5, d and e, respectively) in liver (pg/g) of Atlantic salmon fed varying dietary n-6/n-3 FA ratios and total n-6 and n-3 FA, and exposed to repeated and/or acute stressors. Repeatedly stressed fish 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 ± 0.0 ^{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

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1 Appendix

Supplementary table 1: Fatty acid composition of RBC (% of TFA) in Atlantic salmon fed varying dietary n-6/n-3 ratios and absolute levels of n-6 and n-3 FA and exposed to repeated stressor or not. Data are pooled over repeatedly stressed and control fish. Three fish sampled from each tank (n = 6). Different letters denote significant statistical difference ($p < 0.05$, two-way ANOVA with Tukey HSD post hoc). Numbers are mean with standard deviation.

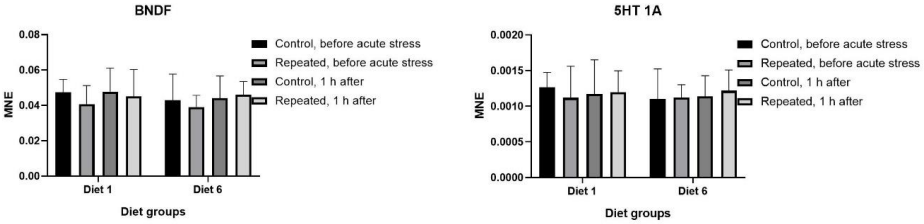
	Diet 1	Diet 2	Diet 6	Diet 1H
Fatty acids (% of TFA)				
SFA	29.9 ± 2.3	30.7 ± 1.7	29.2 ± 1.5	31.0 ± 1.5
MUFA	13.1 ± 1.1d	11.6 ± 0.8c	8.0 ± 1.0a	9.7 ± 0.9b
18:2n-6 (LA)	3.4 ± 0.4a	5.0 ± 0.5b	10.3 ± 0.9c	4.0 ± 0.5a
20:2n-6	0.6 ± 0.1a	0.7 ± 0.1b	1.5 ± 0.3c	0.5 ±0.1a
20:3n.6	1.7 ± 0.2b	2.1 ± 0.3c	2.9 ± 0.3d	0.8 ± 0.1a
20:4n-6 (ARA)	3.2 ± 0.4a	3.7 ± 0.6b	5.1 ± 0.4c	3.3 ± 0.2a
Total n-6	8.8 ± 0.6a	11.5 ± 0.7b	19.8 ± 1.2c	8.5 ± 0.7a
20:4n-3	1.0 ± 0.2b	0.9 ± 0.2b	0.6 ± 0.1a	1.0 ± 0.1b
20:5n-3 (EPA)	8.7 ± 0.9c	7.4 ± 1.1b	5.2 ± 0.7a	9.6 ± 0.8c
22:5n-3	2.6 ± 0.3c	2.3 ± 0.3b	2.0 ± 0.3a	2.8 ± 0.2c
22:6n-3 (DHA)	35.8 ± 2.1	35.5 ± 2.1	35.2 ± 1.2	37.4 ±1.8
Total n-3	48.1 ± 1.6c	46.1 ± 1.5b	43.0 ± 1.3a	50.7 ± 1.5d
FA ratios				
n-6/n-3	0.18 ± 0.02b	0.25 ± 0.02c	0.46 ± 0.04d	0.17 ± 0.02a
ARA/EPA	0.4 ± 0.1a	0.5 ± 0.1b	1.0 ± 0.1c	0.3 ± 0.0a
Sum FA (mg/g)	29.9 ± 2.3	30.7 ± 1.7	29.2 ± 1.5	31.0 ± 1.5

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, ARA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, FA: fatty acid, TFA: total fatty acids, 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

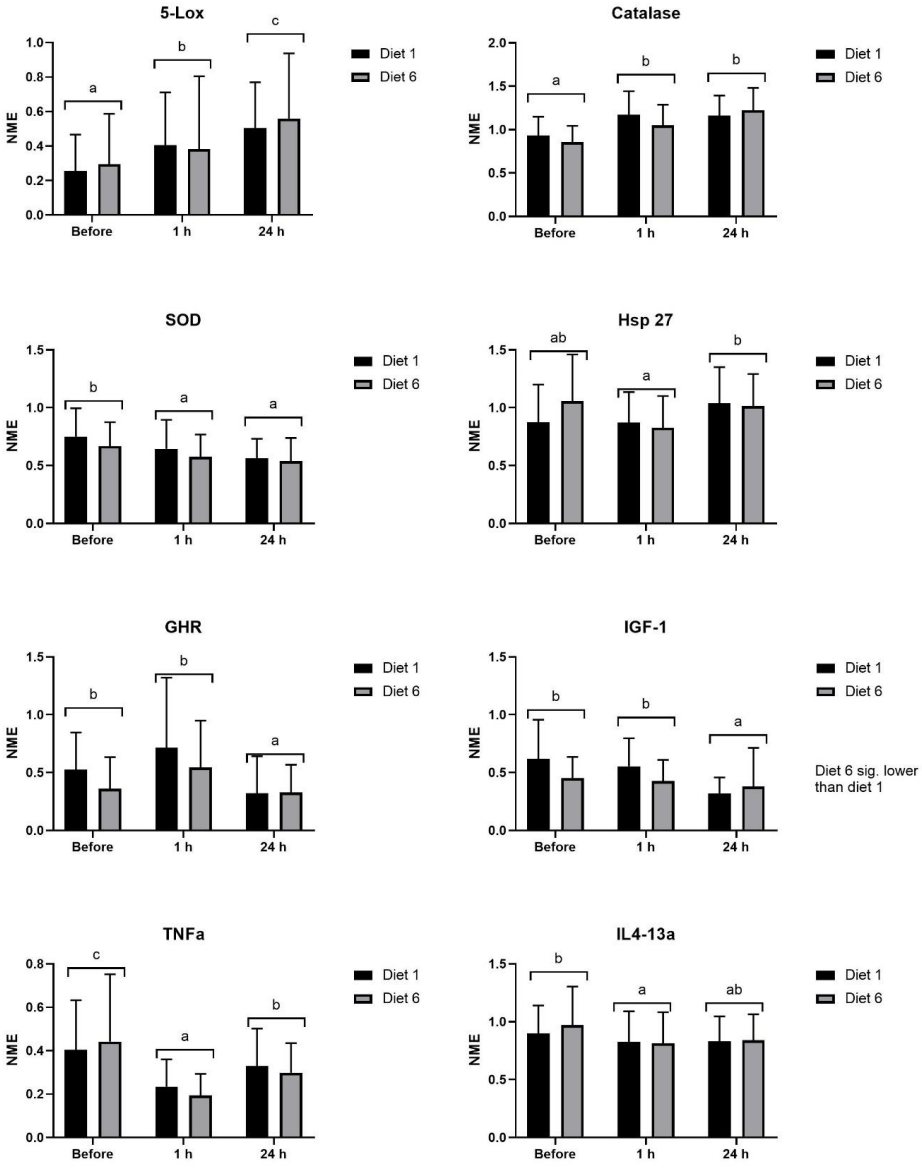
Supplemetary Table 2. Hematocrit (Hct), plasma glucose and plasma chloride in Atlantic salmon before (B), 1 hour after (1 h) and 24 h after (24 h) acute stress, for the four different diet groups and for fish not exposed to repeated stress (Control) and fish exposed to repeated stress. Mean and standard deviation, n=3. No statistically significant effects of diet, repeated stress or acute stress were seen.

	Diet 1						Diet 2						Diet 6						Diet 1H					
	Control			Repeated			Control			Repeated			Control			Repeated			Control			Repeated		
	B	1h	24h	B	1h	24h	B	1h	24h	B	1h	24h	B	1h	24h	B	1h	24h	B	1h	24h	B	1h	24h
Glucose	4.35 ± 0.24	7.42 ± 0.09	5.28 ± 0.45	4.86 ± 0.28	7.75 ± 0.10	5.24 ± 0.39	4.57 ± 0.38	7.50 ± 0.13	4.83 ± 0.25	4.12 ± 1.04	8.11 ± 0.69	5.06 ± 0.21	4.77 ± 0.22	7.21 ± 0.82	5.17 ± 0.12	4.59 ± 0.29	8.04 ± 0.93	5.25 ± 0.22	4.39 ± 0.62	7.73 ± 0.45	5.13 ± 0.26	4.14 ± 0.47	8.05 ± 0.57	5.21 ± 0.43
Chloride	143.86 ± 8.75	167.84 ± 3.40	158.02 ± 0.30	152.72 ± 1.54	165.29 ± 0.23	153.34 ± 0.91	149.90 ± 6.26	165.77 ± 3.05	157.08 ± 2.91	141.71 ± 23.17	166.78 ± 2.44	157.92 ± 0.85	150.05 ± 6.12	161.70 ± 2.62	154.28 ± 1.70	148.43 ± 6.20	165.72 ± 9.42	156.69 ± 2.12	142.19 ± 12.81	165.59 ± 3.40	155.77 ± 1.67	139.68 ± 10.54	166.38 ± 5.16	157.50 ± 3.40
HCT	39.57 ± 3.01	41.79 ± 2.61	40.20 ± 3.14	38.47 ± 2.64	40.20 ± 2.18	39.27 ± 2.87	38.60 ± 2.90	40.93 ± 2.27	38.73 ± 2.52	39.27 ± 1.58	40.36 ± 2.10	37.93 ± 2.15	38.47 ± 2.64	39.47 ± 2.00	39.20 ± 0.94	39.27 ± 2.15	39.47 ± 2.97	38.67 ± 2.55	39.25 ± 3.14	42.73 ± 4.71	39.40 ± 1.84	39.93 ± 1.39	40.73 ± 2.09	40.60 ± 2.29

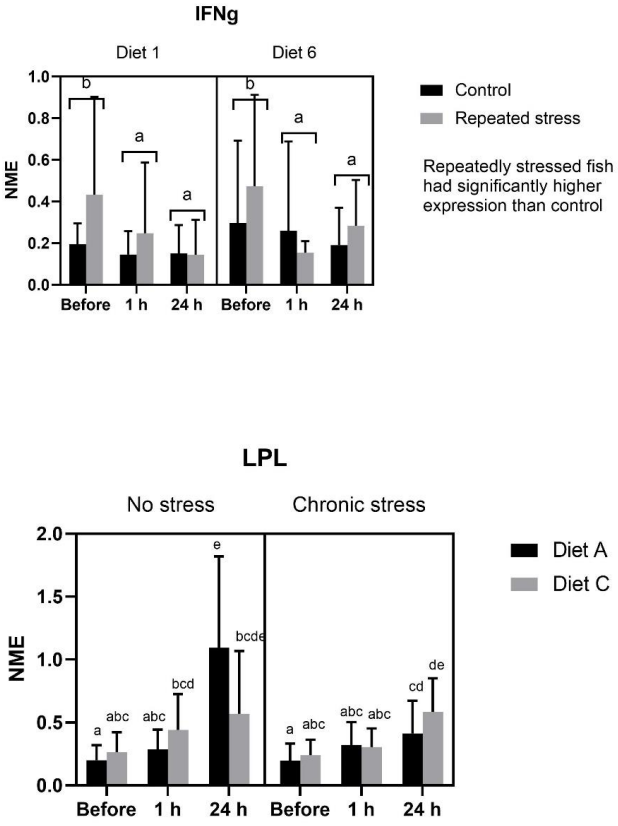
Supplementary Figure 1. Effects on gene expression in the brain of Atlantic salmon fed dietary n-6/n-3 ratios of ~1 or ~6 (diet 1 and diet 6, respectively), and exposed to repeated stressor or not, before and 1 h after an acute stressor was applied. Genes in brain were normalized relative to S20. BDNF – brain derived neurotrophic factor; 5-HT1Aα – serotonin 1Aα receptor. No statistically significant effects were seen, neither of diet, repeated stress or acute stress.



Supplementary Figure 2. Effects on gene expression in the liver of Atlantic salmon fed dietary n-6/n-3 ratios of ~1 or ~6 (diet 1 and diet 6, respectively), before and 1 h and 24 h after an acute stressor was applied. As no effects of repeated stress in the weeks before the acute stress was seen for these genes, the data for control and repeated stress were pooled in these figures. Statistically significant differences ($p<0.05$) are denoted by letters in the figure. 5-Lox – 5-lipoxygenase; SOD – superoxide dismutase; Hsp27 – heat shock protein 27; GHR – growth hormone receptor; 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.


5-Lox
Catalase
SOD
Hsp 27
GHR
IGF-1
TNFa
IL4-13a
NME
Before
1 h
24 h
Diet 1
Diet 6
Diet 6 sig. lower than diet 1

Supplementary Figure 3. Effects on gene expression in the liver of ~~in~~ Atlantic salmon fed dietary n-6/n-3 ratios of ~1 or ~6 (diet 1 and diet 6, respectively), with or without repeated stress over several weeks (control vs chronic/repeated stress), before and 1 h and 24 h after an acute stressor was applied. Statistically significant differences ($p<0.05$) are denoted by letters in the figure.



Paper III

Hundal BK, Lutfi E, Sigholt T, Rosenlund G, Liland NS, Glencross B &

Sissener NH

A piece of the puzzle - possible mechanisms for why low dietary EPA and DHA cause hepatic lipid accumulation in Atlantic salmon (Salmo salar)

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Article

A Piece of the Puzzle—Possible Mechanisms for Why Low Dietary EPA and DHA Cause Hepatic Lipid Accumulation in Atlantic Salmon (*Salmo salar*)

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Abstract: The present study aimed at elucidating the effects of graded levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the hepatic metabolic health of Atlantic salmon reared in sea cages. Diets containing 10, 13, 16 and 35 g/kg EPA + DHA (designated diets 1.0, 1.3, 1.6 and 3.5, respectively) were fed in triplicate through a full production cycle from an average starting weight of 275 g to slaughter size (~5 kg). Feeding low dietary EPA + DHA altered the hepatic energy metabolism, evidenced by reductions in tricarboxylic acid cycle intermediates originating from β-oxidation, which was compensated by elevated activity in alternative energy pathways (pentose phosphate pathway, branched chain amino acid catabolism and creatine metabolism). Increases in various acylcarnitines in the liver supported this and indicates issues with lipid metabolism (mitochondrial β-oxidation). Problems using lipids for energy in the lower EPA + DHA groups line up well with observed increases in liver lipids in these fish. It also aligns with the growth data, where fish fed the highest EPA + DHA grew better than the other groups. The study showed that diets 1.0 and 1.3 were insufficient for maintaining good liver metabolic health. However, diet 3.5 was significantly better than diet 1.6, indicating that diet 1.6 might also be suboptimal.

Keywords: lipid metabolism; EPA; DHA; robustness; Atlantic salmon; metabolomics

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1. Introduction

The dietary substitution of fish oil (FO) by vegetable oils (VO) in feed for Atlantic salmon (*Salmo salar)* is a common practice in aquaculture due to the limited availability of FO. This causes a distinct reduction in eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) and typically increases in linoleic acid (18:2n-6), α-linolenic acid (18:3n-3) and oleic acid (18:1n-9). Multiple reports have shown that exchanging FO for some types of VO (such as rapeseed, soy oil and sunflower oil) results in hepatic lipid accumulation in salmon over time [1–10]. In humans, non-alcoholic fatty liver disease (NAFLD) is strongly associated with obesity, diabetes, dyslipidaemia, cardiovascular disease, hypertension and, ultimately, the metabolic syndrome [11,12]. Demonstrating that hepatic lipid accumulation is detrimental and linked to reduced fish robustness has been difficult, as many trials have been short-term, land-based and/or under con-

trolled conditions. Even so, there are indications that a higher liver lipid content is a symptom of a less robust fish. Salmon kept in large, open sea cages have suddenly experienced large increases in mortality, with the only common denominators between moribund fish being fatty liver combined with increased plasma alanine aminotransferase and alkaline phosphatase levels, both being marker enzymes of liver damage [13]. A trial feeding salmon 2, 10 and 17 g/kg EPA + DHA found significantly higher liver lipid contents in fish fed the two lowest levels of EPA + DHA [2]. Additionally, there were higher mortality rates in fish fed these two diets. Another trial in sea cages found increased mortality combined with a considerably fattier liver (10 vs. 6% fat in liver) in salmon fed a diet where 70% of the added oil was rapeseed compared to 30% (2.8% vs. 7% EPA + DHA in feed, respectively) [14]. The authors posited that low temperature was one of the reasons for this lipid accumulation, combined with the dietary fatty acid (FA) composition. Indeed, salmon kept at 6 °C gained more fat in the liver with decreasing dietary EPA + DHA, though no such relationship was seen at 12 °C [5]. Similar results of temperature were found in Reference [7]. No clear definition of how much liver fat is normal or healthy in salmon exists as of yet, though, as described, a higher liver lipid content seemingly cooccurs with reduced robustness.

High dietary EPA and DHA is known to affect hepatic lipid metabolism in mammals, resulting in decreased liver fat with higher dietary levels of these FA [15]. However, the lipid-lowering effects of EPA and DHA appear to differ from one another [16,17]. For salmon, dietary EPA + DHA have been proposed to inhibit lipogenesis when sufficiently high [18,19] and increase FA oxidation [20–22]. Incubating salmon hepatocytes with EPA resulted in an increased mitochondrial area and a reduced secretion of glycerolipids, suggestive of higher mitochondrial β-oxidation [23]. EPA has also been reported to increase the number of hepatic mitochondria [24]. Salmon fed diets with high DHA and very low EPA (algal oil) had a higher occurrence of pale livers and a higher total lipid level in the liver than fish fed FO. The authors hypothesised the reason to be mild chronic stress in the liver caused by a lack of EPA [25]. DHA increases the peroxisomal β-oxidation capacity in salmon [26]. The peroxisomal β-oxidation capacity in salmon liver is high [27] and can account for a considerable amount of the hepatic β-oxidation [20]. In contrast to the aforementioned results, few or no effects on the hepatic β-oxidation capability were seen in salmon fed diets with 100% of the added oil being vegetable oil (rapeseed oil or a vegetable oil blend) compared to fish oil during the seawater phase [28]. Exchanging FO with VO blends (rapeseed, linseed and palm oil) was thought to cause fat accumulation in the liver by inhibiting the production of very low-density lipoproteins (VLDL), which reduced the secretion of lipids and, hence, caused lipid deposition [29]. Thus, EPA and DHA have several ways of influencing hepatic lipid storage.

The exact mechanisms behind hepatic fat accumulation remains unclear, as there are many pathways, with different regulatory steps, that have been proposed to contribute to this phenotype. As mentioned, there can be an increase in lipogenesis, the inhibition of FA oxidation, increased triacylglycerol (TAG) synthesis, an altered uptake of FA into the liver or a decreased clearance of lipids from the liver due to an imbalanced FA composition. However, other dietary components or environmental factors can also have an effect. The use of metabolomics approaches in fish nutrition is a relatively recent field and provides an unbiased investigation of the effects of the feed on the metabolism [30,31]. Nutrigenomics, namely the investigation of the relationship between nutrients and gene expression, has provided valuable knowledge in the field of fish nutrition. However, it is limited by not studying downstream post-translational effects or protein activities [30]. Metabolomics focuses on the metabolites present in the sample, hence giving an overview of the metabolic activities at a given time point [30], greatly aiding in explaining the observed phenotype.

While 10 g of EPA + DHA per kg feed is considered too low for salmon [2], with significantly higher mortality rates after delousing compared to higher levels, an increase in dietary EPA + DHA from 16 to 26 g/kg yielded no differences in robustness [32]. Despite

the fish in this study going through multiple lice treatments and facing significant disease outbreaks of, i.e., pancreas disease and gill infections, no significant difference in mortality was observed. Hence, the current requirements of EPA and DHA for salmon are thought to be between 10 and 16 g per kg of formulated feed (for commercial growing out diets with 35–39% lipids). Currently, Norwegian salmon feed contains, on average, 24 g of EPA + DHA per kg feed (surveillance of commercial Norwegian salmon feeds [33]); the lowest dietary level found was 16.6 g/kg. This is above the expected minimum requirement of salmon at 10–16 g/kg. However, due to the limited global supply of FO and fishmeal, the pressure for reduced inclusion is ever increasing. Therefore, the present study investigated dietary EPA + DHA at 10, 13, 16 and 35 g/kg (corresponding to 2.8, 3.8, 4.7 and 10.2% of the total FA (TFA), respectively) fed to Atlantic salmon during a full growing out phase in sea cages. The final diet at 35 g/kg was included to reveal whether beneficial effects of EPA + DHA higher than the current commercial levels could be seen. The goal of this paper was to investigate the relationship between hepatic lipid metabolism and EPA + DHA in the feed by using an untargeted metabolomics approach.

2. Results

2.1. Dietary Fatty Acids and Proximate Composition

The dietary proximate and FA composition are presented in Table 1. The diets will be referred to by their planned percentage of EPA + DHA in the diet (Diet 1.0, diet 1.3, diet 1.6 and diet 3.5). All diets were isoproteic (~33%), isolipidic (~38%) and isoenergetic (~26 MJ/kg). Dietary EPA + DHA increased nicely across the diets, as planned. Concurrently, reductions in 18:1n-9, 18:2n-6 and 18:3n-3 and a slight increase in saturated FA (SFA) were seen.

Table 1. Analysed proximate and FA composition (% of TFA) of the final feed batch (9 mm, fed September 2018 to January 2019). Also published in Lutfi et al. [34].

	Dietary EPA + DHA Levels (%)			
	1.0	**1.3**	**1.6**	**3.5**
PROXIMATE COMPOSITION				
Moisture (%)	6.0	6.0	6.0	6.0
Energy–crude (MJ/Kg)	26.2	26.2	26.2	26.1
Protein–crude (%)	33.4	33.4	33.4	33.5
Fat–crude (%)	38.7	38.7	38.6	38.4
Ash (%)	4.4	4.4	4.4	4.4
FATTY ACIDS				
14:0	0.9	1.1	1.4	2.8
16:0	7.0	7.2	7.5	9.6
18:0	2.8	2.7	2.7	3.0
20:0	0.7	0.6	0.6	0.5
ΣSFA [1]	12.6	13.1	13.3	17.0
16:1 n-7	1.0	1.3	1.8	3.6
18:1 n-7	2.8	3.0	3.0	3.2
18:1 n-9	46.5	45.8	43.8	33.0
20:1 n-9	1.6	1.9	2.4	4.2
20:1 n-11	0.3	0.4	0.5	1.1
22:1 n-9	0.2	0.2	0.2	0.3
22:1 n-11	0.6	0.9	1.3	3.1
24:1 n-9	0.2	0.2	0.2	0.2
Σ MUFA [2]	53.2	53.7	53.1	48.8
16:2 n-6	0.1	0.1	0.2	0.3
18:2 n-6	20.0	18.7	16.9	12.5

18:3 n-3	8.1	8.0	7.7	5.1
20:4 n-3	0.1	0.2	0.3	0.7
20:5 n-3	1.5	2.0	2.4	5.1
22:5 n-3	0.2	0.3	0.3	0.7
22:6 n-3	1.4	1.8	2.3	5.0
EPA + DHA	2.8	3.8	4.7	10.2
ΣPUFA	31.6	31.4	30.4	30.1
Σn-3 [3]	11.5	12.5	13.3	17.2
Σn-6 [4]	20.2	19.0	17.2	13.1
n-6/n-3	1.8	1.5	1.3	0.8
TFA mg/g feed	354.4	356.5	357.7	362.6

[1] Includes 15:0, 17:0, 22:0 and 24:0. [2] Includes 16:1 n-5, 16:1 n-9, 17:1 n-7, 18:1 n-11, 20:1 n-7. 22:1 n-7. [3] Includes 20:3 n-3. [4] Includes 18:3 n-6. DHA—docosahexaenoic acid; EPA—eicosapentaenoic acid; FA—fatty acid; MUFA—monounsaturated fatty acid; PUFA—polyunsaturated fatty acid; SFA—saturated fatty acid; TFA—total fatty acids.

2.2. Growth Is Higher and Hepatosomatic Index Is Lower with Higher Dietary EPA + DHA

The average fish size at the final sampling was roughly 5 kg; however, fish that had been fed diet 3.5 had significantly higher final weights, a specific growth rate (SGR) and thermal growth coefficient (TGC) compared to fish fed the other diets (Table 2). The feed intake was similar in all diet groups. Further details on the growth performance, mortality, welfare scores and fillet quality were thoroughly discussed in Lutfi et al. [34]. The hepatosomatic index (HSI) decreased significantly with increasing dietary EPA and DHA (Table 2).

Table 2. Growth performance and hepatosomatic index for Atlantic salmon fed diets with increasing dietary EPA + DHA. Data are shown as the mean ± SEM. Weight, SGR and TGC based on average values per cage, giving $n = 3$. HSI was calculated for 15 fish per cage, $n = 45$. Different letters within each row indicate significant differences between values determined using a one-way ANOVA with Tukey's HSD post hoc; Kruskal–Wallis was used for HSI.

	Diet 1.0	**Diet 1.3**	**Diet 1.6**	**Diet 3.5**
Initial weight (g)	275.4 ± 1.4	275.2 ± 2.9	276.4 ± 0.6	277.1 ± 0.7
Final weight (g)	4748.5 ± 33.4 [a]	4938.0 ± 85.6 [a]	4963.6 ± 68.4 [a]	5364.6 ± 56.9 [b]
SGR	0.68 ± 0.0003 [a]	0.69 ± 0.005 [a]	0.69 ± 0.003 [a]	0.70 ± 0.002 [b]
TGC	3.1 ± 0.016 [a]	3.1 ± 0.031 [a]	3.1 ± 0.023 [a]	3.3 ± 0.017 [b]
HSI	1.15 ± 0.02 [c]	1.12 ± 0.02 [bc]	1.08 ± 0.02 [ab]	1.05 ± 0.02 [a]

HSI—hepatosomatic index; SGR—specific growth rate; TGC—thermal growth coefficient.

2.3. Lipid Classes—Significantly More Fat in the Livers of Fish Given Diet 1.0 or 1.3 Than Fish Given Diet 3.5

The fish fed diet 1.3 had significantly higher levels of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in the liver than fish fed diet 3.5. The same dietary groups also showed a significantly higher sum of liver polar lipids (Table 3). The mean levels of PE, PC and sum polar lipids were numerically high in the 1.0 diet group but not statistically different from the other groups due to larger individual variations. In all dietary groups, there was an unusually high content of free fatty acid (FFA) combined with less TAG than expected for fish of this size (data not shown). This likely indicates thawing of the samples at some point, allowing the release of FFA from TAG [35]. Hence, data for the individual neutral lipid classes are not provided. However, the results on the sum of neutral lipids are still valid. The fish fed diet 3.5 had significantly lower sum neutral lipids than the other diet groups (Table 3). The standard deviations were large in fish fed diets 1.0 and 1.3, where almost all outliers were found. There was a significant correlation between PC and PE with sum neutral lipids (Figure 1). When analysing within the dietary

groups, the correlations were still significant within diet group 1.0 (R = 0.76, $p = 0.0002$ and R = 0.75, $p = 0.0004$ for PC and PE, respectively) and 1.3 (R = 0.74, $p = 0.0005$ and R = 0.5, $p = 0.034$ for PC and PE, respectively).

Table 3. Liver lipid classes (mg/g) in the liver of Atlantic salmon fed diets with increasing dietary EPA + DHA. Data are the mean ± standard deviation of three tanks per diet group with 6 fish per tank ($n = 18$). Different letters denote significant statistical differences ($p < 0.05$ one-way ANOVA with Tukey's HSD post hoc). Numbers are the mean with standard deviation.

	Diet 1.0	Diet 1.3	Diet 1.6	Diet 3.5
PC	12.0 ± 7.6 ^{ab}	11.6 ± 2.7 ^{b}	10.1 ± 1.2 ^{ab}	9.1 ± 1.1 ^{a}
PS	1.5 ± 0.9	1.4 ± 0.3	1.5 ± 0.4	1.4 ± 0.2
PI	2.4 ± 1.5	2.2 ± 0.5	2.1 ± 0.4	2.2 ± 0.5
PA/CL/PG	1.2 ± 0.6	1.1 ± 0.4	1.3 ± 0.3	1.1 ± 0.2
PE	7.2 ± 4.9 ^{ab}	6.8 ± 1.4 ^{b}	5.8 ± 0.9 ^{ab}	5.3 ± 0.6 ^{a}
Sum polar lipids	32.3 ± 19.1 ^{ab}	30.7 ± 5.9 ^{b}	27.5 ± 4.2 ^{ab}	24.6 ± 2.4 ^{a}
Sum neutral lipids	58.4 ± 32.2 ^{b}	73.2 ± 44.7 ^{b}	41.2 ± 12.6 ^{b}	26.9 ± 7.0 ^{a}
Sum lipids	90.8 ± 48.6 ^{b}	103.8 ± 48.7 ^{b}	68.7 ± 15.5 ^{ab}	51.9 ± 8.8 ^{a}

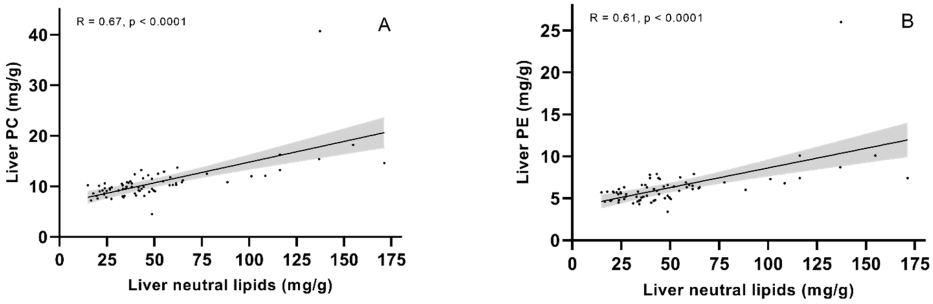

Figure 1. Correlations between sum neutral lipids and (**A**) phosphatidylcholine (PC) or (**B**) phosphatidylethanolamine (PE) in the liver of Atlantic salmon fed diets with increasing dietary EPA + DHA. Spearman's rank correlation coefficient.

2.4. FA Composition in Neutral and Polar Lipids—Neutral Lipids Accumulate Oleic Acid When Dietary EPA + DHA Is Low

The FA composition of the liver polar lipids is presented in % of TFA (Table 4). EPA + DHA increased significantly in liver polar lipids with a dietary content. EPA + DHA was higher in polar lipids than in the feed. The sum of n-6 FA decreased with increasing EPA + DHA in the feed, concurrent with the dietary n-6 content. However, arachidonic acid (ARA, 20:4n-6) showed the opposite direction. The SFA were stable in diet groups 1.0, 1.3 and 1.6, while fish fed diet 3.5 had significantly more than the other diet groups. SFA were also higher in the polar lipids than in the feed. Monounsaturated FA (MUFA) decreased significantly with more dietary EPA + DHA, concurrent with the dietary content of the MUFA oleic acid.

Full FA acid composition of polar lipids of the liver in mg/g and % of TFA are presented in Tables A1 and A2, respectively.

Table 4. Liver fatty acid composition of polar lipids in Atlantic salmon fed diets with increasing dietary EPA + DHA (% of TFA). Three cages per diet group and 4 fish sampled per cage (n = 12). Different letters denote significant statistical differences ($p < 0.05$ one-way ANOVA with Tukey's HSD post hoc). Numbers are the mean with standard deviation.

	Diet 1.0	**Diet 1.3**	**Diet 1.6**	**Diet 3.5**
16:0	10.9 ± 0.9 ^{a}	11.0 ± 0.6 ^{a}	11.2 ± 0.7 ^{a}	12.5 ± 1.0 ^{b}
18:0	6.2 ± 0.7	6.1 ± 0.4	6.1 ± 0.4	6.3 ± 0.6
Sum SFA *	18.1 ± 1.2 ^{a}	18.1 ± 0.7 ^{a}	18.5 ± 0.8 ^{a}	20.5 ± 1.1 ^{b}
16:1n-7	0.5 ± 0.1 ^{a}	0.5 ± 0.1 ^{a}	0.5 ± 0.0 ^{a}	0.8 ± 0.1 ^{b}
18:1n-9	20.9 ± 2.7 ^{c}	19.4 ± 0.9 ^{bc}	17.9 ± 1.0 ^{ab}	12.4 ± 1.3 ^{a}
18:1n-7	2.0 ± 0.2 ^{a}	2.0 ± 0.1 ^{a}	2.0 ± 0.1 ^{a}	2.3 ± 0.1 ^{b}
20:1n-9	2.2 ± 0.3 ^{a}	2.1 ± 0.3 ^{a}	2.3 ± 0.4 ^{ab}	2.6 ± 0.6 ^{b}
Sum MUFA **	26.3 ± 2.9 ^{c}	24.6 ± 1.1 ^{bc}	23.3 ± 1.0 ^{ab}	19.3 ± 1.6 ^{a}
18:2n-6	10.9 ± 1.1 ^{c}	10.5 ± 0.7 ^{bc}	9.2 ± 0.6 ^{b}	5.3 ± 0.5 ^{a}
20:2n-6	2.1 ± 0.3 ^{ab}	2.3 ± 0.3 ^{b}	2.3 ± 0.3 ^{b}	1.8 ± 0.3 ^{a}
20:3n-6	2.5 ± 0.4 ^{d}	1.7 ± 0.3 ^{c}	1.2 ± 0.2 ^{b}	0.4 ± 0.1 ^{a}
20:4n-6 (ARA)	3.1 ± 0.4 ^{a}	3.1 ± 0.2 ^{a}	3.3 ± 0.2 ^{ab}	3.9 ± 0.3 ^{b}
Sum n-6 ***	19.2 ± 0.9 ^{d}	18.1 ± 0.7 ^{c}	16.6 ± 0.8 ^{b}	12.0 ± 0.4 ^{a}
18:3n-3	2.6 ± 0.5 ^{b}	2.6 ±0.3 ^{b}	2.5 ± 0.3 ^{b}	1.4 ± 0.4 ^{a}
20:4n-3	1.3 ± 0.2 ^{c}	1.2 ± 0.2 ^{bc}	1.1 ± 0.1 ^{b}	0.8 ± 0.1 ^{a}
20:5n-3 (EPA)	8.9 ± 0.8 ^{a}	9.5 ± 0.6 ^{ab}	10.1 ± 0.6 ^{b}	12.0 ± 0.7 ^{c}
22:5n-3	2.8 ± 0.3 ^{a}	2.8 ± 0.2 ^{a}	2.9 ± 0.2 ^{a}	3.7 ± 0.7 ^{b}
22:6n-3 (DHA)	19.2 ± 2.0 ^{a}	21.3 ± 1.1 ^{ab}	23.0 ± 1.1 ^{b}	27.5 ± 1.1 ^{c}
EPA + DHA	28.1 ± 2.6 ^{a}	30.8 ± 1.0 ^{ab}	33.1 ± 1.2 ^{bc}	39. 5 ± 1.6 ^{c}
Sum n-3 ****	35.4 ± 2.3 ^{a}	37.9 ± 1.0 ^{ab}	39.9 ± 1.1 ^{bc}	45.7 ± 0.9 ^{c}
Sum PUFA	54.6 ± 1.7 ^{a}	56.0 ± 0.7 ^{ab}	56.6 ± 1.0 ^{bc}	57.7 ± 0.9 ^{c}
n6/n3	0.6 ± 0.1 ^{c}	0.5 ± 0.0 ^{bc}	0.4 ± 0.0 ^{ab}	0.3 ± 0.0 ^{a}
TFA	21.6 ±1.6	20.4 ± 2.4	20.8 ± 2.0	21.3 ± 1.0

* Includes 14:0, 15:0, 17:0 and 20:0. ** Includes 16:1n-9, 18:1n-11, 20:1n-11, 20:1n-7, 22:1n-11, 22:1n-9 and 24:1n-9. *** Includes 22:4n-6 and 22:5n-6. **** Includes 18:4n-3, 21:5n-3, 24:5n-3 and 24:6n-3.

The FA composition of the liver neutral lipids is presented in mg/g (Table 5) due to high individual variations of total fat. However, for comparisons of diets, the FA profile is used. Although EPA + DHA increased in the liver neutral lipids with the dietary content, the amount present is lower than in the feed. The sum n-6 FA was reduced with increasing dietary EPA + DHA/decreasing sum n-6; however, the differences between the dietary groups were smaller than the differences in feed. There were higher amounts of oleic acid in liver neutral lipids with less dietary EPA + DHA, which was the same pattern as seen for oleic acid in the feed. The oleic acid content of the liver neutral lipids was higher than in the feed. SFA decreased significantly with the increasing dietary EPA + DHA and was present in lower quantities than in the diet. As in the lipid class data, the standard deviations in TFA of the neutral lipids were considerably larger in the 1.0 and 1.3 diet groups compared to the two other dietary groups.

The full FA composition of the neutral liver in mg/g and % of TFA are presented in Tables A1 and A2, respectively.

Table 5. Liver fatty acid composition of neutral lipids in Atlantic salmon fed diets with increasing dietary EPA + DHA (mg/g). Three cages per diet group and 4 fish sampled per cage ($n = 12$). Different letters denote significant statistical differences ($p < 0.05$, Kruskal–Wallis). Numbers are the mean with standard deviation.

	Diet 1.0	Diet 1.3	Diet 1.6	Diet 3.5
16:0	2.3 ± 1.5	2.3 ± 1.9	1.3 ± 0.5	0.9 ± 0.3
18:0	2.1 ± 1.6 ^{b}	1.8 ± 1.3 ^{b}	0.9 ± 0.5 ^{ab}	0.5 ± 0.2 ^{a}
Sum SFA *	5.3 ± 3.5 ^{b}	4.9 ± 3.8 ^{b}	2.7 ± 1.2 ^{ab}	1.7 ± 0.7 ^{a}
16:1n-7	0.9 ± 0.7	0.9 ± 0.8	0.5 ± 0.3	0.4 ± 0.2
18:1n-9	39.1 ± 32.4 ^{b}	34.5 ± 26.6 ^{b}	18.4 ± 9.3 ^{b}	6.5 ± 3.2 ^{a}
18:1n-7	2.5 ± 2.0 ^{b}	2.2 ± 1.6 ^{b}	1.3 ± 0.6 ^{ab}	0.7 ± 0.3 ^{a}
20:1n-9	4.0 ± 3.4 ^{b}	3.5 ± 2.7 ^{b}	2.0 ± 0.9 ^{b}	0.9 ± 0.5 ^{a}
Sum MUFA **	47.6 ± 39.2 ^{b}	42.1 ± 32.4 ^{b}	22.7 ± 11.3 ^{b}	9.1 ± 4.4 ^{a}
18:2n-6	11.0 ± 10.2 ^{b}	9.4 ± 7.1 ^{b}	5.0 ± 2.6 ^{b}	1.8 ± 0.8 ^{a}
20:2n-6	1.7 ± 1.5 ^{b}	1.6 ± 1.3 ^{b}	0.9 ± 0.5 ^{b}	0.4 ± 0.2 ^{a}
20:3n-6	0.52 ± 0.46 ^{c}	0.31 ± 0.22 ^{bc}	0.09 ± 0.15 ^{b}	0.04 ± 0.02 ^{a}
20:4n-6 (ARA)	0.5 ± 0.4 ^{ab}	0.6 ± 0.5 ^{b}	0.4 ± 0.2 ^{ab}	0.2 ± 0.1 ^{a}
Sum n-6 ***	13.9 ± 12.6 ^{b}	12.0 ± 9.2 ^{b}	6.5 ± 3.3 ^{b}	2.4 ± 1.1 ^{a}
18:3n-3	3.4 ± 2.9 ^{b}	3.3 ± 2.6 ^{b}	1.9 ± 0.9 ^{b}	0.7 ± 0.3 ^{a}
20:4n-3	0.4 ± 0.4	0.4 ± 0.4	0.2 ± 0.1	0.2 ± 0.1
20:5n-3 (EPA)	0.5 ± 0.2	0.6 ± 0.4	0.4 ± 0.1	0.6 ± 0.2
22:5n-3	0.1 ± 0.1 ^{a}	0.1 ± 0.1 ^{a}	0.1 ± 0.0 ^{a}	0.3 ± 0.1 ^{b}
22:6n-3 (DHA)	0.4 ± 0.1 ^{a}	0.5 ± 0.2 ^{a}	0.4 ± 0.1 ^{ab}	0.6 ± 0.2 ^{b}
EPA + DHA	0.9 ± 0.4	1.1 ± 0.6	0.9 ± 0.1	1.2 ± 0.3
Sum n-3 ****	5.0 ± 3.7	5.1 ± 3.8	3.2 ± 1.2	2.5 ± 0.9
Sum PUFA	19.0 ± 16.3 ^{b}	17.1 ± 13.0 ^{b}	9.7 ± 4.6 ^{ab}	4.9 ± 2.0 ^{a}
n6/n3	2.5 ± 0.6 ^{c}	2.3 ± 0.2 ^{bc}	1.9 ± 0.3 ^{ab}	1.0 ± 0.2 ^{a}
TFA	72.6 ± 59.3 ^{b}	65.0 ± 49.7 ^{b}	35.6 ± 17.3 ^{b}	16.1 ± 7.2 ^{a}

* Includes 14:0, 15:0, 17:0 and 20:0. ** Includes 16:1n-9, 18:1n-11, 20:1n-11, 20:1n-7, 22:1n-11, 22:1n-9 and 24:1n-9. *** Includes 18:3n-6 and 22:4n-6. **** Includes 18:4n-3, 21:5n-3 and 24:5n-3.

2.5. Metabolomics

2.5.1. Differences between the Groups Based on Overall Metabolite Signature

In the current metabolomics dataset, 795 biochemicals of known identity were detected. The biggest difference was observed between diet groups 1.0 vs. 3.5 (241 differentially regulated compounds), followed by diet 1.6 vs. diet 3.5 (199 differentially regulated compounds) and, finally, diet 1.0 vs. diet 1.6 (102 differentially regulated compounds). These results show that the dietary level of EPA + DHA affects the liver metabolic profile of salmon. Furthermore, they demonstrate that the metabolite profile of fish fed diet 10 and 16 are more similar, while the fish fed diet 3.5 is more distinct from the other two.

As PCA allows for the visualisation of individuals within a group based on their data-compressed principal components, it can also help in determining whether the dietary groups differ from one another based on their overall metabolite signature. As illustrated in Figure 2, PC1 vs. PC2 show that fish fed diets 1.0 and 1.6 are intermixed, whereas fish fed diet 3.5 separate more from the other two. They separate based on PC2, which explains 12.3% of the variation. Hence, a phenotypic difference in the metabolome originating from the dietary EPA + DHA is evident.

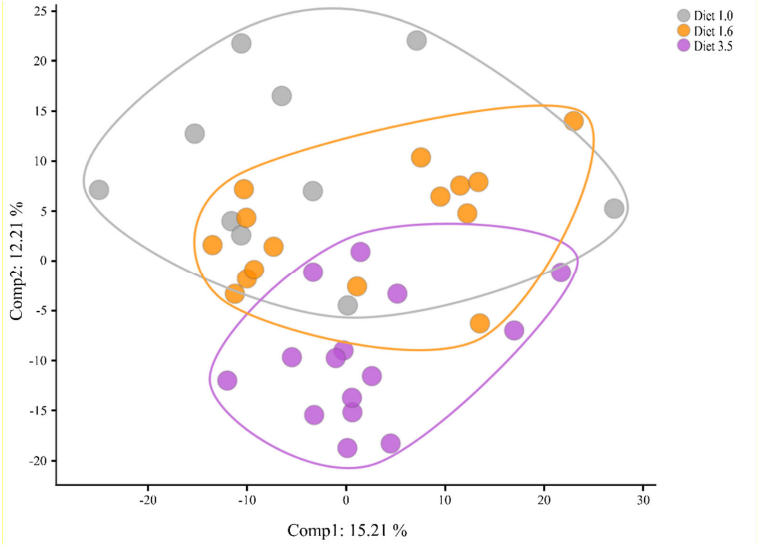

Figure 2. PCA of liver samples from Atlantic salmon fed diets with increasing dietary EPA + DHA: diet 1.0 (grey), diet 1.6 (orange) and diet 3.5 (purple).

2.5.2. Core Findings in the Metabolomics Data

The metabolomics data indicate that feeding salmon lower levels of EPA and DHA leads to an impaired energy production in the liver (overview in Table 6). Notably, it appears to compromise β-oxidation, potentially through the carnitine shuttle for transport of FA into the mitochondria or the β-oxidation reactions. This is in line with the results in other pathways that seemingly increased their activity to compensate, such as branched chain amino acid (BCAA) catabolism and the pentose phosphate pathway (PPP). The phospholipid (PL) metabolism and lysophospholipid homeostasis also appear out of balance.

Table 6. Core findings in the metabolomics data from livers of Atlantic salmon fed increasing dietary levels of EPA and DHA.

	Pathway	Metabolites	Core Finding
Energy production	Tricarboxylic acid cycle (TCA)	Decreased citrate, cis- aconitate and isocitrate and increased succinate with lower dietary EPA and DHA.	Indicates less use of FA oxidation as input into TCA cycle, and then a shift to using BCAA, PPP and creatine instead when salmon is fed a lower dietary EPA and DHA.
	Pentose Phosphate Pathway	Increased ribulose-5-phosphate (intermediate in PPP) and galactonate (feeds into the PPP) in fish fed less EPA and DHA.	
	BCAA metabolism	Increased levels of BCAA, dipeptides containing BCAA (protein degradants), and acyl carnitines involved in BCAA metabolism in fish given lower dietary EPA and DHA.	
	Creatine metabolism	Higher levels of these metabolites with lower dietary EPA and DHA	

Transport of FA into mitochondria	Carnitine metabolism	Increased levels of carnitine and deoxycarnitine in fish fed less EPA and DHA.	Suggests some dysfunction with the mitochondrial β-oxidation.
	Acyl carnitines	Higher occurrence with lower dietary EPA and DHA, particularly fish fed diet 1.6.	
Phospholipid metabolism	Phospholipid degradants	Higher content of PL degradants with lower dietary EPA and DHA.	Implies an altered PL metabolism with lowered dietary EPA and DHA. Can possibly disturb the mitochondrial membrane.
	Lysophospholipids	More of almost all lysophospholipids in liver of fish given diets low in EPA and DHA.	

2.5.3. The TCA Cycle, Pentose Phosphate Pathway, Creatine Metabolism and BCAA Metabolism Have Altered Activity When Decreasing the Dietary Content of EPA + DHA

Decreasing the dietary content of EPA and DHA resulted in changes in the metabolites involved in the tricarboxylic acid (TCA) cycle, the PPP, creatine metabolism and the BCAA metabolism (Table 7). These are all metabolic pathways that can be used for energy production. Citrate, aconitate and isocitrate were all reduced in the liver of fish given diet 1.0 compared to fish given diets 1.6 and 3.5 (Table 7). The difference was not significant between diets 1.0 and 3.5 for citrate and aconitate, possibly due to the fewer replicates in diet 1.0. Still, this result indicates a reduced input of β-oxidation products into the TCA cycle in fish fed diet 1.0 compared to the other two diet groups. Further, acetyl-CoA was numerically lower in diet groups 1.0 and 1.6 than in diet group 3.5. In the PPP, ribulose-5-phosphate was significantly increased in fish given diet 1.0 compared to the other diets. Galactonate, which feeds into the PPP, was also increased in fish given diets 1.0 and 1.6 compared to fish fed diet 3.5. This could imply an increased activity of the PPP to supply energy in the fish fed the diets with the lowest EPA and DHA contents.

Several metabolites in the BCAA metabolism were significantly higher in fish given diet 1.0 compared to fish given the diets with higher EPA + DHA contents (Table 7). This included the BCAA themselves, dipeptides containing BCAA (incomplete protein degradation products), propionylcarnitine (from isoleucine and valine catabolism) and butyrylcarnitine (from leucine and isoleucine catabolism) [36]. Some metabolites of BCAA metabolism were also significantly higher in fish fed diet 1.6 compared to diet 3.5. The BCAA are likely an alternative to FA for introducing intermediates into the TCA cycle via succinyl CoA (Figure 3) in fish fed the two lowest EPA and DHA levels investigated here. It is therefore fitting that succinate was numerically higher in fish fed diets 1.0 and 1.6 compared to fish fed diet 3.5 (Table 7). In creatine metabolism, creatine and creatinine are increased or trending higher in fish fed diets 1.0 and 1.6, while guanidinoacetate is numerically higher (Table 7). Together, these results indicate a perturbation of the energy homeostasis, possibly related to the changes in the TCA cycle, PPP and BCAA catabolism, particularly in fish fed diet 1.0.

See Figure 3 for an illustration of how the TCA cycle, PPP and BCAA catabolism interconnect.

Table 7. Metabolites included in the tricarboxylic acid cycle (TCA), pentose phosphate pathway and branched amino acid catabolism in Atlantic salmon fed diets with increasing dietary EPA + DHA. Triplicate cages for diets 1.6 and 3.5 and duplicate cages for diet 1.0 with five fish sampled per cage (n = 15 and n = 10, respectively). Data are presented as the fold change between pairwise comparisons of diet groups, e.g., for the comparison of diet 1.0/diet 3.5, a number below one would indicate less of the metabolite in diet group 1.0, while a number above one would indicated the opposite. Red indicates significantly higher and green significantly lower ($p < 0.05$), while pink indicate p-values between 0.05 and 0.1.

Pathway	Biochemical Name	Fold Change		
		Diet 1.0/Diet 3.5	Diet 1.6/Diet 3.5	Diet 1.0/Diet 1.6
TCA Cycle	citrate	0.77	1.32	0.58
	aconitate [cis or trans]	0.83	1.56	0.53
	isocitrate	0.64	1.09	0.59
	succinate	1.47	1.10	1.33
Fatty acid metabolism	acetyl-CoA	0.93	0.90	1.03
Pentose Phosphate Pathway	ribulose 5-phosphate	1.83	0.97	1.89
Fructose, Mannose and Galactose Metabolism	galactonate	1.53	1.48	1.04
Leucine, Isoleucine and Valine Metabolism	leucine	1.10	1.01	1.09
	isoleucine	1.09	1.02	1.06
	valine	1.18	1.06	1.11
	glycylisoleucine	1.56	1.05	1.48
Dipeptide	glycylleucine	1.78	1.12	1.59
	glycylvaline	2.13	1.23	1.74
	isoleucylglycine	5.03	1.21	4.14
	lysylleucine	2.73	1.23	2.23
	prolylglycine	2.00	1.31	1.53
	valylglutamine	3.75	1.31	2.86
	valylglycine	6.17	1.15	5.36
	valylleucine	3.40	1.20	2.84
	leucylglutamine	4.53	1.21	3.75
Fatty Acid Metabolism (also BCAA Metabolism)	butyrylcarnitine (C4)	1.41	1.69	0.83
	propionylcarnitine (C3)	1.48	1.33	1.12
Creatine Metabolism	guanidinoacetate	2.16	1.07	2.01
	creatine	1.06	1.20	0.89
	creatinine	1.28	1.17	1.09

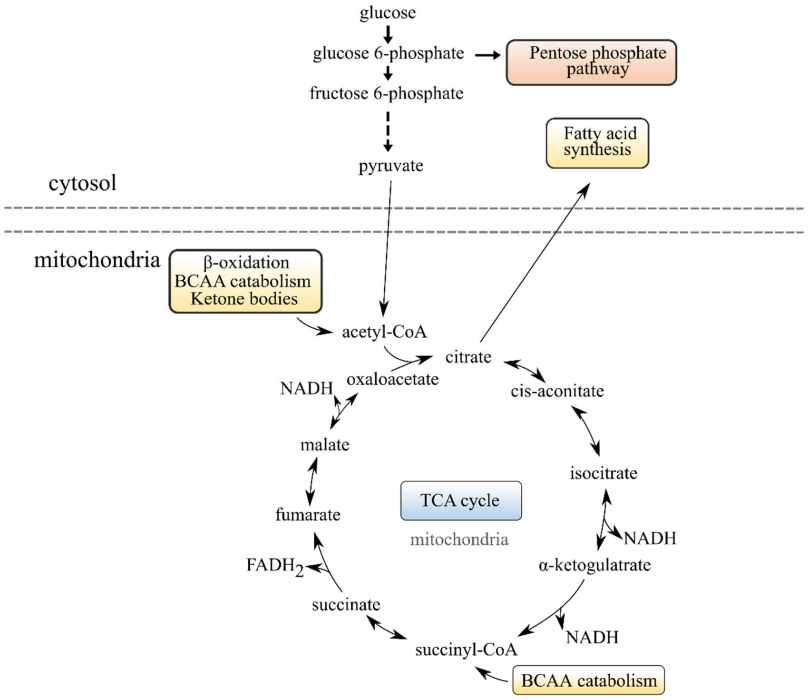

Figure 3. Illustration of how the tricarboxylic acid (TCA) cycle, pentose phosphate pathway, branched chain amino acid (BCAA) catabolism, fatty acid (FA) synthesis and β-oxidation are linked together in the cell.

2.5.4. An increased Occurrence of Free Carnitine and Acyl Carnitines in the Lower EPA + DHA Dietary Groups Point to Issues with the Mitochondrial β-Oxidation Capacity

There was significantly more free carnitine in fish fed diets 1.0 and 1.6 compared to fish fed diet 3.5. Deoxycarnitine (carnitine precursor) was also higher in these two groups, although only significant for diet 1.6 vs. diet 3.5 (Table 8). Furthermore, compared to fish given diet 3.5, fish fed diet 1.6 had significantly more acylcarnitines of various FA. Several acyl carnitines were also higher in diet 1.0 compared to diet 3.5 (Table A3), though there were fewer statistically significant differences. This could be due to a weaker statistical strength with fewer replicates in diet group 1.0 (see Section 4.5). However, the 3-hydroxyacylcarnitines were significantly higher in fish fed diets 1.0 and 1.6 compared to fish fed diet 3.5 (3-hydroxyacyl CoA are intermediates in β-oxidation; Figure 4) (Table A3). These results are indicative of an ineffective mitochondrial β-oxidation, due to either a lower transport of FA into the mitochondria, inability to reform acyl-CoA within the mitochondria or an issue with the mitochondrial β-oxidation reactions when fish are fed diets 1.0 and 1.6 compared to diet 3.5.

See Figure 4 and how it illustrates the link between FFA in the cytosol and β-oxidation in the mitochondria (transport via acyl carnitines) with its link to the TCA cycle.

Table 8. Metabolites in carnitine metabolism, ketone bodies and metabolism in the synthesis of CoA in Atlantic salmon fed diets with increasing dietary EPA + DHA. Triplicate cages for diets 1.6 and 3.5 and duplicate cages for diet 1.0 with five fish sampled per cage (n = 15 and n = 10, respectively). Data are presented as the fold change between pairwise comparisons of diet groups, e.g., for the comparison diet 1.0/diet 3.5, a number below one would indicate less of the metabolite in diet group 1.0, while a number above one would indicate the opposite. Red indicates significantly higher. (p < 0.05).

Pathway	Biochemical Name	Fold Change		
		Diet 1.0/Diet 3.5	Diet 1.6/Diet 3.5	Diet 1.0/Diet 1.6
Carnitine Metabolism	deoxycarnitine	1.13	1.37	0.83
	carnitine	1.23	1.32	0.93

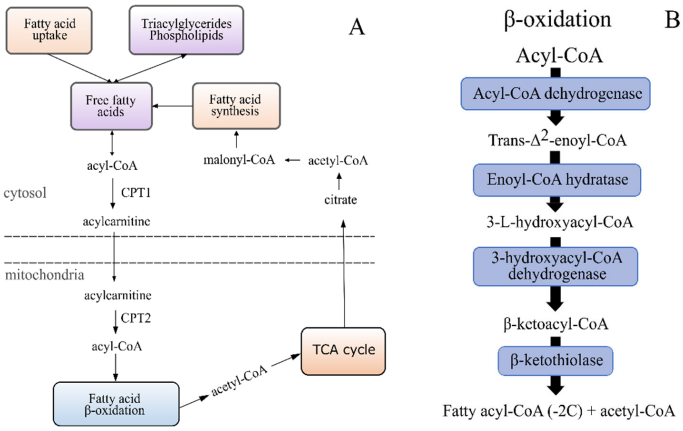

Figure 4. Illustration of the cycle of FA transport into the mitochondria, β-oxidation, influx of FA oxidation products into the TCA cycle and transport of citrate out of the mitochondria for FA synthesis (**A**). The β-oxidation reactions (**B**).

2.5.5. A Lower Dietary EPA + DHA Leads to Altered Phospholipid Metabolism, with Higher Levels of Phospholipid Degradants and Lysophospholipids

Evidence of an altered PL metabolism in the two dietary groups with the lowest EPA + DHA is seen by the increased levels of glycerophosphoethanolamine, glycerophosphoserine and glycerophosphoinositol compared to the highest EPA + DHA group. These are degradants of phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol, respectively. Furthermore, the two lower EPA + DHA groups have a higher content of almost all lysophospholipids compared to the high group. Fish fed diet 1.0 also showed significantly higher levels of many of these lysophospholipids than fish fed diet 1.6 (Table A4).

2.5.6. Alterations in Eicosanoid and Tryptophan Metabolites Levels When Changing Dietary EPA and DHA

The 5-lipoxygenase product 5-HEPE, an EPA-derived eicosanoid, was significantly higher in the fish fed diet 3.5 compared to fish fed diets 1.0 and 1.6. Fish fed the two lower EPA + DHA diets had significantly more kynurenine than fish fed the high diet, and fish fed diet 1.6 also had significantly more kynurenate than fish fed diet 3.5 (Table 9). Higher levels of kynurenine in fish fed diet 1.0 and diet 1.6 can reflect the increasing levels of inflammation compared to fish fed diet 3.5.

Table 9. Metabolites in tryptophan metabolism and eicosanoids in Atlantic salmon fed diets with increasing dietary EPA + DHA. Triplicate cages for diets 1.6 and 3.5 and duplicate cages for diet 1.0 with five fish sampled per cage (n = 15 and n = 10, respectively). Data are presented as the fold change between pairwise comparisons of diet groups, e.g., for the comparison diet 1.0/diet 3.5, a number below one would indicate less of the metabolite in diet group 1.0, while a number above one would indicate the opposite. Red indicates significantly higher and green significantly lower ($p < 0.05$).

Pathway	Biochemical Name	Fold Change		
		Diet 1.0/Diet 3.5	Diet 1.6/Diet 3.5	Diet 1.0/Diet 1.6
Eicosanoid	5-HEPE	0.29	0.33	0.88
Tryptophan metabolism	kynurenine	1.70	2.10	0.81
	kynurenate	1.03	1.90	0.54

2.5.7. Gene Expression

There were no significant differences between the dietary groups in the gene expression of *ppara*, *cpt1*, *cact*, *cpt2*, *aco* (FA oxidation), *cd36* (FA uptake), *apob100* (FA transport) or *plin2* (lipid droplet formation) (Figure A1).

3. Discussion

Hepatic lipid accumulation is considered a general sign of dietary imbalance and indicates a perturbation in energy metabolism [37]. Lowering the dietary content of EPA + DHA (≤10 g/kg) in salmon feeds has been associated with increases in the hepatic lipid level [2]. Data from the present study (HSI, lipid class and mg/g total FA) clearly illustrate an increase in liver lipids with lower dietary EPA + DHA intakes. Livers of fish fed diets 1.0, 1.3 and 1.6 had fat contents around 7–12%, while, in livers of fish fed diet 3.5, they only had 5% fat. Other trials with comparable dietary EPA + DHA to the three lowest dietary EPA + DHA of the present trial, and temperatures at ~6 °C have reported liver fat contents ranging from 7 to 17% [5,7,38]. Salmon provided >16-g EPA + DHA per kg feed are reported with liver fat levels around 6 to 7% [7,14,32], similar to this study reporting 5% liver fat I diet group 3.5. Although there is no clear threshold indicating a detrimental level of liver fat, dietary EPA + DHA above 16 g/kg results in lower, more stable liver fat contents. The variation in the lipid data within diet groups 1.0 and 1.3 was large, with some individual fish having markedly higher lipid contents than other fish fed the same diet. Liver neutral lipids from the lipid class data correlated well with both PC and PE for fish fed the two diets lowest in EPA + DHA, similar to the results of Jordal et al. [29]. Both PC and PE are needed for the formation of membranes surrounding lipid droplets. This could indicate that fish fed the lower levels of EPA + DHA in the present study formed more lipid droplets in the liver, similar to previous observations in salmon fed low EPA + DHA [3]. In accordance with this, a tendency of a decreased abundance of lipid droplets was found in the fish fed the highest dietary level of EPA + DHA in their livers in the present study [34]. Liver colour is regarded as a visual indicator of liver lipids [39], with a paler liver indicating higher fat contents and possible nutritional disorders [40]. The fish fed diet 3.5 further had marked improvement in liver colour compared to the low EPA + DHA diets [34]. Their colour was better in fish fed diet 1.6 compared to 1.0 and 1.3 but was drastically improved in fish fed diet 3.5 compared to the other diet groups. This further supports a lower lipid accumulation in diet group 3.5. Notably, there were very few outliers in diet group 1.6 and none in diet group 3.5 in the liver lipid class and FA composition data. The individual capabilities of the fish are likely crucial to the fish's ability to handle a low EPA + DHA diet, and the dietary supply must be enough to ensure the requirements of all fish are covered. Oleic acid was the major FA in the accumulated neutral fat (39.4–52.9% of TFA in neutral lipids), being higher in the neutral lipids than in the feed. This indicates that oleic acid accumulates at the expense of other FA, and it has been suggested that it is metabolised slower than other FA in salmonids [41]. Furthermore, oleic

acid-rich diets stimulate hepatic TAG accumulation in salmon to a greater degree than EPA- and DHA-rich diets [24], and higher levels of added oleic acid to salmon hepatocytes resulted in increased lipid droplet formation [42]. In HepG2 cells, oleic acid led to TAG accumulation, larger lipid droplets and a closer proximity between lipid droplets and mitochondria than when cells were treated with bovine serum albumin (control) or palmitic acid. Additionally, mitochondria from the cells added oleic acid and reduced the FA oxidation capacity compared to the control or palmitic acid-treated cells [43]. These results match well with observations from the present study and imply that increased oleic acid, which was elevated when EPA + DHA decreased, can contribute to lipid accumulation in the liver.

To further consider the possible mechanisms causing these increases in the liver lipids in our study, a metabolomic profiling of the liver was performed. The great advantage of metabolomics is its provision of a large suite of unbiased data, which can then be used to deduce the biological processes behind the observed changes [30,31]. In the present study, the global overview of the data (PCA) indicated that the metabolite signature of the fish separated based on the EPA + DHA content of their feed. Fish given diets 1.0 and 1.6 clustered together, while fish fed diet 3.5 separated slightly from the other two groups. The metabolic profile was suggestive of a disturbed energy homeostasis, with the fish seemingly unable to fully use lipids and FA for energy production when fed lower levels of EPA + DHA.

We found a significant increase in the hepatic lysophospholipid levels in fish fed diets 1.0 and 1.6 compared to diet 3.5, which, in mammals, has been found to suppress FA oxidation in the liver [44,45] by altering the mitochondrial membrane permeability [44]. Crossing the mitochondrial membrane is the first step of mitochondrial β-oxidation and is catalysed by CPT1 (formation of acylcarnitine from acyl-CoA), carnitine acyl-carnitine translocase (CACT, facilitates transport) and CPT2 (reformation of acyl-CoA within mitochondria) [46]. An increase in hepatic acylcarnitine in fish fed diets 1.0 and 1.6 compared to diet 3.5 was concurrent with the elevated lysophospholipid levels in the present study, indicating an issue with FA oxidation. A disturbance of the mitochondrial membrane causing an issue with the efficiency of CACT or CPT2 could be a possible explanation. Increased activity and gene expression of CACT have been found in rat livers [47] and increased gene expression in the hepatopancreas of grass carps [48] with higher dietary EPA + DHA. However, we did not find any significant differences between the diet groups in the mRNA levels of CACT. Similarly, studies have shown an increased gene expression of CPT2 in salmon livers [22] and CPT2 activity in brown trout livers [49] with more EPA + DHA in the feed, but no such differences were seen in our gene expression data. Hence, the issue is possibly not with the transport of FA into the mitochondria. However, a significantly higher accumulation of 3-hydroxyacylcarnitines was evident in fish fed diets 1.0 and 1.6 compared to diet 3.5 in the current study, which indicates dysfunction with 3-hydroxy-acyl-CoA-dehydrogenase (third step in mitochondrial β-oxidation) [50]. A disturbance of the mitochondrial membrane, suggested to have occurred in fish fed low EPA + DHA in the present study, could cause problems for this enzyme, as it is located on the mitochondrial membrane [51]. A general accumulation of acylcarnitines would be expected concomitant with hydroxylated acylcarnitines; however, the accumulation was less pronounced in fish fed diet 1.0 than diet 1.6. Nonetheless, the metabolic profile of the fish fed diet 1.0 distinctly demonstrated a shift away from using FA as energy. An incomplete β-oxidation cycle would lead to a deficit in acetyl-CoA for the TCA cycle. The numericreduction in acetyl-CoA, combined with reduced citrate, aconitate and iso-citrate in fish diet 1.0, support this. Although some metabolites were only numerically different, they all pointed in the same direction as other statistically significant metabolites. Altogether, this could result in the observed phenotype of accumulating fat in the livers of salmon fed less dietary EPA + DHA.

Studies on the effects of EPA + DHA in the feed on β-oxidation in salmon generally support that it increases with a higher dietary content of these FA [20,21,23,24,52], though

some studies have reported no effects (≤19.3% EPA + DHA of TFA) [28,53]. The current metabolomics data suggests that a reduced EPA + DHA in the liver membranes might hamper lipid usage through altered membrane functionality. This is in line with the results of Závorka et al. [54], who found that juvenile wild salmon fed less EPA + DHA had reduced mitochondrial efficiency in the muscle. An efficient mitochondrial activity is closely related to the growth performance in salmon [54] and brown trout [55]. In accordance with this, we found a significantly higher growth and a better functioning lipid metabolism in salmon fed the highest level of EPA + DHA. Although pathological lipidosis in the liver was not observed, the lower EPA + DHA diet groups had significantly more fat in their livers. Higher mortality rates tend to coincide with an increased frequency of fatty liver [13,14]. The mortality in these studies was suggested to be induced by a combination of low temperatures (~5 °C) and lipid nutrition (switching to a diet higher in lipids [13] or high dietary oleic acid [14]). There was also a tendency of reduced mortality caused by a single, natural outbreak of cardiomyopathy syndrome (CMS) (confirmed by a veterinarian) with increasing dietary EPA + DHA levels in the present study (reported by Lutfi et al. [34]). While this does not necessarily imply that elevated liver lipids in itself cause increased mortality, it is, at the very least, a symptom of a less robust fish. Studies have found that salmon reared at 6 °C will gain more liver fat than fish reared at 12 °C, even when provided the same dietary EPA + DHA level, demonstrating that the response to dietary EPA + DHA is influenced by temperature [5]. Insufficient dietary EPA + DHA to maintain normal membrane functionality, particularly at low temperatures, could thus result in reduced mitochondrial efficiency, reduced growth, increased liver lipids and, ultimately, a salmon less robust when exposed to additional challenges.

Changes to the dietary n-3 and n-6 FA contents in salmon feeds result in altered eicosanoid production [56,57], and we have previously linked hepatic increases in prostaglandin E_2 and prostaglandin D_2 to elevated liver TAG [56]. In the present study, the metabolomics data revealed a decrease in the EPA-derived eicosanoid 5-HEPE when lowering the dietary EPA + DHA. Wang et al. [58] discovered that 17,18-EEQ, 5-HEPE and 9-HEPE (EPA metabolites) ameliorated NAFLD induced by a high fat diet in mice. Their study indicated that these eicosanoids attenuated the inflammatory response of macrophages in adipose tissue, which further ameliorated liver lipid accumulation. As reviewed in Duan et al. [59], there are several metabolites of EPA, but also DHA, that have been demonstrated to improve hepatic steatosis in mammals. Concurrent with the reduced 5-HEPE in the livers of fish fed diets 1.0 and 1.6, a significant increase in lysophospholipids was found. The secretion of adiponectin by adipose tissue is reduced in obesity (see Reference [60]). This adipokine directly reduces the hepatic lysophospholipid levels in mammals [61]. Hence, liver lysophospholipids are increased in obesity. These data support the interplay between adipose and hepatic tissue in the development of a fatty liver-like phenotype. EPA and DHA increased the rates of β-oxidation and reduced TAG in salmon adipose tissue [62] and cultured adipocytes [63], and DHA has been demonstrated to protect salmon adipocytes against inflammation [64]. This possible connection between an inflamed adipose tissue and fatty liver-like phenotype in salmon is also something that deserves further attention.

Long-chain acylcarnitines can meditate proinflammatory responses in cultured immune cells [65], and they can prompt an expression of proinflammatory cytokines following a dose-dependent manner [66]. In addition to the accumulating acylcarnitines in the 1.0 and 1.6 diet groups, higher levels of kynurenine and kynurenate were observed. Tryptophan 2,3-dioxygenase (TDO, functioning in the liver) and indoleamine 2,3-dioxygenase (IDO, extrahepatic) are two enzymes that catalyse the first step of the kynurenic pathway, and they are both induced by inflammatory cytokines (reviewed in Reference [67]). These findings support a higher state of inflammation in fish fed these diets, possibly partly due to the accumulating acylcarnitines resulting in increased proinflammatory cytokines. Kynurenine and kynurenic acids, in opposition to other kynurenine metabolites, are anti-

inflammatory (reviewed in Reference [68]) and can serve as a brake on the immune response. Hence, although they are a sign of a higher inflammatory state, they can help alleviate inflammation.

Salmon are exposed to many challenges under farming conditions, including diseases and handling, which can lead to substantial mortalities and suboptimal performance. Whether the fishes' robustness is affected by dietary levels of EPA + DHA is of great importance, as it is an issue both of animal welfare and economic interest. This study clearly demonstrated that low dietary EPA + DHA (≤13 g/kg, diet 1.0 and 1.3) can cause elevated liver lipids and a fatty liver-like phenotype in the metabolite profile, similar to many published studies showing higher liver lipids with decreasing dietary EPA + DHA in salmon [2–4]. We found indications of a higher inflammatory status in fish fed diets 1.0 and 1.6 than in fish fed diet 3.5. Similarly, other studies have shown that a reduced dietary inclusion of EPA + DHA can result in an increased inflammatory response [69–73]. EPA + DHA at the highest level (diet 3.5) greatly ameliorated all metabolic health issues investigated in the present trial; these fish did not have hepatic lipid accumulation, had a normally functioning lipid metabolism, reduced inflammatory metabolite markers and they achieved significantly higher final weights. This is concurrent with what we observed in the metabolic profile, where fish fed diets 1.0 and 1.6 grouped together and diet group 3.5 separated slightly from the other two. Combined, these results indicated a more robust fish when fed the highest EPA + DHA levels. The substantial variation seen only in the data from diet groups 1.0 and 1.3 indicate that, while some individual fish may cope with low dietary levels of EPA + DHA, many will have reduced robustness. However, to ensure the robustness of all fish and avoid losses, it is important that sufficient levels of EPA + DHA be included in diets.

The current study indicated a dysregulation of the hepatic energy metabolism of Atlantic salmon when they were fed lower levels of EPA and DHA. Through a range of different mechanisms, the ability to use lipids for energy was reduced, and the fish needed to initiate alternative strategies for energy production. Feeding diet 1.0 was demonstrated to provide insufficient EPA + DHA for a normally functioning liver lipid metabolism. Although fish fed diet 1.6 showed some improvement compared to fish fed diet 1.0, an even further improvement was seen when going from diet 1.6 to diet 3.5. This was indicative of diet 1.6 also being suboptimal for maintaining a healthy liver metabolism. The difference in dietary EPA + DHA in diets 1.6 and 3.5 was relatively large, and further studies are therefore required to find the optimal dietary inclusion of EPA + DHA within this range for a healthy hepatic lipid metabolism.

4. Materials and Methods

4.1. Diets and Fish Trial

This trial, its experimental design and fish performances have been described in detail elsewhere [34]. Briefly, four diets with increasing levels of EPA + DHA were formulated (10, 13, 16 and 35 g of EPA + DHA/kg feed, corresponding to 2.8, 3.8, 4.7 and 10.2% of TFA, respectively). The EPA:DHA ratios were approximately 1:1 in all diets. Fish meal was included at 5% of the recipe in all diets of the two final feed batches, which are the most relevant here. To modulate the EPA + DHA content, FO replaced rapeseed oil in the diets. All diets were formulated to be isoproteic, isolipidic and isoenergetic. In order to meet the specific dietary requirements of the fish throughout the experiment, three pellet sizes (4, 6 and 9 mm) and five feed batches were produced. Dietary proximate and FA composition (from the final feed batch) are given in Table 1. More detailed information on feed formulation for all batches, proximate and FA composition for the other batches can be found by Lutfi et al. [34].

Atlantic salmon post smolts (average weight 115 g) were purchased from Mowi AS (formerly Marine Harvest Norway AS. Glomfjord, Meløy, Norway). They were acclimated to the environmental conditions at Gildeskål Research Station (GIFAS, Inndyr,

Norway) and fed a commercial diet for this size (BioMar AS, Trondheim, Norway) in advance of the trial. At the start of the trial, fish (average weight 275 g) of both sexes were haphazardly distributed into sea cages (5 × 5 × 5 m–125 m^3) with 190 individuals per cage (triplicate cages per diet). The feeding trial began in October 2017 and lasted until January 2019. The fish were fed to apparent satiation once a day during the autumn and winter and twice a day during the spring and summer. Temperature (measured at 1, 3 and 5 m in depth); salinity and oxygen (measured at a 3-m depth) were monitored daily. Average temperature overall was 7 °C, declining to 3 °C during the winter and rising to 16 °C during the summer. In September 2018, an outbreak of cardiomyopathy syndrome was diagnosed. However, few fish were found to have developed severe symptoms, and all sampled fish appeared healthy. This disease outbreak is therefore not expected to have had much of an effect on the metabolomics analysis. A CMS scoring assessment was performed by an experienced histopathologist (Dr. Øystein Evensen) from the faculty of Veterinary Medicine (Norwegian University of Life sciences, Oslo, Norway). All dead fish were analysed specifically for the CMS outbreak to assess potential differences in fish robustness due to dietary differences. Mortality was recorded throughout the experiment. The trial was conducted according to the National Guidelines for Animal Care and Welfare published by the Norwegian Ministry of Education and Research (Norwegian Food Safety Authority (FOTS), approval no. 16059).

4.2. Sampling

At the beginning of the trial (October 2017), the fish were individually weighed and lengths measured, and they were haphazardly distributed into the experimental sea cages. At the end of the trial (January 2019), a final sampling was performed. Fish were haphazardly collected and sacrificed by an anaesthetic overdose (Tricaine Pharmaq, PHARMAQ AS, Norway, 0.3 g/L). All fish were weighed and measured individually, and livers were excised and weighed. The liver was cut into two pieces in the middle, and whole vertical slices were sampled from the middle part. Pieces of liver were then taken for an analysis of lipid class (6 fish per cage), FA composition (4 fish per cage), gene expression (5 fish per cage) and metabolomics (5 fish per cage) and flash-frozen in liquid nitrogen before storage at -80 °C until analysis. Samples for each analysis were taken from the same part of the liver each time. Fish were fed until the last day before sampling.

4.3. Lipid Analyses

4.3.1. Lipid Class

Lipid classes were separated by double-development high-performance thin-layer chromatography (HPTLC) using 10 × 10-cm plates (VWR, Lutterworth, UK) based on the methods reported by Henderson and Tocher et al. [74]. Samples of the total lipids (1 to 2 μg) were applied to the plate origins and the plates resolved in a methyl acetate/isopropanol/chloroform/ methanol/0.25% KCl (25:25:25:10:9, *v*/*v*) aqueous phase to 5.2 cm. The plates were then removed from the aqueous phase, the excess solvent evaporated via air drying and vacuum desiccation and the plates then further developed to 9.5 cm using a second solvent mixture containing isohexane/diethyl ether/acetic acid (80:20:1, *v*/*v*) before termination and drying, as detailed previously. The different lipid classes were visualised following spraying the plates with a 3% (*w*/*v*) cupric acetate solution containing 8% (*v*/*v*) phosphoric acid; after which, the plates were charred at 160 °C for 20 min. Lipid classes were quantified by densitometry using a CAMAG-3 TLC Scanner (version Firmware 1.14.16; CAMAG, Muttenz, Switzerland) using winCATS software (Planar Chromatography Manager, version 1.2.3).

4.3.2. Fatty Acid Composition

Total lipids were extracted from liver samples with chloroform/methanol (2:1, Merck, Darmstadt, Germany) with roughly 20 times the sample weight and frozen overnight at

−20 °C. Neutral and polar lipids of the sample extracts were separated by solid phase extraction before analysis of the FA composition, as described by Sissener et al. [75]. Nonadecanoic acid (19:0) was added to all samples as an internal standard for quantitative determination. Briefly, extracts were filtered and evaporated, then saponified and methylated with BF_3 in methanol. FA separation was conducted using the AutoGC (Autosystem XL, Perkin Elmer Inc., Waltham, MA, USA) fitted with a flame ionisation detector. For integration, Chromeleon® version 7.2 (Thermo Scientific, Waltham, MA, USA) was used.

4.4. Gene Expression

RNA extraction, cDNA synthesis and analysis of the gene expression was performed on liver samples, as reported in Hundal et al. [56]. Melting curves were monitored in the last amplification cycle to ensure the specificity of the primers. The absorbance ratio A260/280 was 2.1 ± 0.0, A260/230 was 2.2 ± 0.0 and the RIN-value was >7.2 in all samples, hence indicating the RNA samples acceptable for RT-qPCR. 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 performed a stability analysis based on the GeNorm algorithm. Normalisation was performed using CFX Maestro. The PCR primer sequences can be found in Table 10.

The primer sequence for *cact* was created from the Atlantic salmon genome using Primer 3 Software. The specificity of the new primer *cact* was tested by a QIAGEN OneStep RT-PCR Kit. First, the primer was diluted to 0.05nmol/µL with TE buffer. Then, the master mix reagent was made according to the manufacturer's instructions. The RT reaction took place on a PCR machine (GeneAmp PCR 9700, Applied Biosystems, Waltham, MA, USA) with the following temperature program: reverse transcription for 30 min at 50 °C, PCR activation for 15 min at 95 °C, denaturation for 45 secs at 94 °C, annealing for 45 secs at 60 °C, extension for 1 min at 72 °C and a final extension for 10 min at 72 °C. The PCR product was tested on an agarose gel with Gel Red Nucleic Acid Stain as a dye by a charged voltage at 80V. After one hour, the gel was photographed by a UV light to check the results. A single band of the expected size was found in the gel.

Table 10. qPCR primer sequences, their accession number and primer efficiency.

Gene	Forward	Reverse	GenBank Accession Number	Efficiency
		Reference genes		
arp	GAAAATCATCCAATTGCTGGATG	CTTCCCACGCAAGGACAGA	AY255630	84%
b-actin	CCAAAGCCAACAGGGAGAA	AGGGACAACACTGCCTGGAT	BG933897	109%
ef1ab	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	AF321836	111%
		Target genes		
aco	CACTGCCAGGTGTGGTGGTA	GGAATTGTAC-GTTCTCCAATTTCA	DQ364432	97%
apob100	TGCAGAGAC-CTTTAAGTTCATTCA	TGTGCAGTGGTTGCCTTGAC	gi:854619	124%
cact	GTTCGCTGTCTGCTTCTTCG	TACTTCACCTCCCCTTTGGC	BT044930.1	87%
cd36	GGATGAACTCCCTGCATGTGA	TGAGGCCAAAGTACTCGTCGA	AY606034	113%
cpt1	CTTTGGGAAGGGCCTGATC	CATGGACGCCTCGTACGTTA	AM230810	92%
cpt2	TGCTCAGCTAGCGTTCCATATG	AGTGCTGCAGGACTCGTATGTG	BG934647	102%
ppara	TCTCCAGCCTGGACCTGAAC	GCCTCGTAGACGCCGTACTT	NM001123560	107%
plin2	CCACTCTGCCTCGCAAATCT	GGGTAAAAGGGACCTACCAGC	XM_014206726.1	100%

arp—acidic ribosomal protein; *ef1ab*—elongation factor 1ab; *aco*—acyl-CoA oxidase, *apob100*—apolipoprotein B 100; *cact*—carnitine acylcarnitine translocase; *cpt*—carnitine palmitoyltransferase; *ppara*—peroxisome proliferator-activated receptor α; *plin2*—perilipin 2.

4.5. Metabolomics

Analysis of metabolites was performed by Metabolon (Durham, NC, USA), as previously described [76,77], on liver samples collected from fish fed diets 1.0, 1.6 and 3.5. Due to economic constraints, only diet groups 1.0, 1.6 and 3.5 were analysed. There were 5 samples collected per cage, giving n = 15 for diet 1.6 and diet 3.5. Unfortunately, 5 replicates (all samples from one cage) were lost in the group fed diet 1.0, resulting in n = 10 in this group. For quality control purposes, several recovery standards were added prior to automated sample preparation using the MicroLab STAR® system from Hamilton Company. Protein removal was performed with methanol under vigorous shaking for 2 min using Glen Mills GenoGrinder 2000 before centrifugation. The extract was fractioned and analysed by two separate reverse-phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one by RP/UPLC-MS/MS with the negative ion mode ESI and one by HILIC/UPLC-MS/MS with the negative ion mode ESI. To remove the solvent, the samples were evaporated using TurboVap® (Zymark, Hopkinton, MA, USA), followed by drying under nitrogen and then reconstitution in solvent appropriate for each method. The solvents also contained several standards at fixed concentrations for injection and chromatographic consistency. A Waters ACQUITY ultra-performance liquid chromatography and a Thermo Scientific Q-Exactive high-resolution/accurate mass spectrometer interfaced with a heated electrospray ionisation (HESI-II) source and Orbitrap mass analyser operated at 35,000 mass resolution (further details in the abovementioned references). Compound identification was performed by comparison to a reference library containing retention time, mass charge ratio (m/z) and chromatographic data (including MS/MS spectral data) [78].

4.6. Calculations

Growth rates were calculated based on the average values per cage:

$$Specific\ growth\ rate\ (SGR) = \frac{\ln w_1 - \ln W_0}{t_1 - t_0} \cdot 100$$

$$Thermal\ growth\ coefficient\ (TGC) = \left(w_1^{1/3} - w_0^{1/3}\right) \cdot \frac{1000}{d^\circ},$$

where W_0 is the start weight (g) and W_1 is the final weight (g) at times t_0 and t_1, respectively, and d° is the sum of day degrees.

The hepatosomatic index (HSI) was calculated as follows

$$HSI = \frac{liver\ weight\ (g)}{body\ weight\ (g)} \cdot 100$$

4.7. Statistics

Statistical analyses were performed using the free software environment R (http://cran.r-project.org/, R version 3.5.3, 11 March 2019). Differences between the groups in the HSI, lipid classes, FA composition and gene expression were analysed by one-way ANOVA followed by Tukey's HSD when significant differences were found. If cage effects were found, a nested ANOVA was used (random effect = tank). A Shapiro–Wilk test and Levene's test were used to check the normal distribution and homogeneity of the variances in the data, respectively. Graphical evaluations for homogeneity of the variances were also conducted using a fitted vs. residuals plot and, for normality, a QQ plot. If the assumptions of normality or homogeneity were not met, a Kruskal–Wallis nonparametric test was used. Correlations to liver neutral lipids were tested for phosphatidylcholine and phosphatidylethanolamine. Statistical significance was set at $p < 0.05$. Results were provided as the mean ± standard deviation, unless otherwise stated.

In the metabolomics dataset, missing values were assumed to be below the limit of detection and imputed with the metabolites' minimum value (deemed as informative

blanks, minimum value imputation). The raw values from the samples for each metabolite was divided by the median of those samples to give a median of 1. The data were log-transformed using the natural log before statistical analysis in ArrayStudio and the free software environment R (http://cran.r-project.org/, R version 3.5.3, 11 March 2019). The metabolomics data were analysed using one-way ANOVA, followed by ANOVA contrasts. Multiple comparisons were accounted for by using the False Discovery Rate (q-value). Data were presented as fold changes between pairwise comparisons of diet groups, e.g., for the comparison diet 1.0/diet 3.5, a number below one would indicate less of the metabolite in diet group 1.0, while a number above one would indicate the opposite. Red indicates significantly higher and green significantly lower ($p < 0.05$), while pink and light green indicate p-values between 0.05 and 0.1

Supplementary Materials: The full metabolomics dataset can be downloaded at: www.mdpi.com/article/10.3390/metabo12020159/s1.

Author Contributions: Conceptualisation (including trial design), N.H.S., N.S.L., G.R. and T.S.; Formal analysis, B.K.H.; Investigation, B.K.H., N.H.S., E.L., G.R., T.S. and B.G.; Resources (feed formulation and provision), T.S.; Writing—original draft preparation, B.K.H.; Writing—review and editing, E.L., T.S., G.R., N.S.L., B.G. and N.H.S.; Visualisation, B.K.H.; Supervision, N.H.S. and N.S.L.; Project administration, N.H.S. and Funding acquisition, N.H.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The trial was conducted according to the National Guidelines for Animal Care and Welfare published by the Norwegian Ministry of Education and Research (Norwegian Food Safety Authority (FOTS) approval no. 16059).

Data Availability Statement: All data presented in this study are available within the paper, Appendix A or the Supplementary Materials.

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Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of the data; in the writing of the manuscript or in the decision to publish the results.

Appendix A

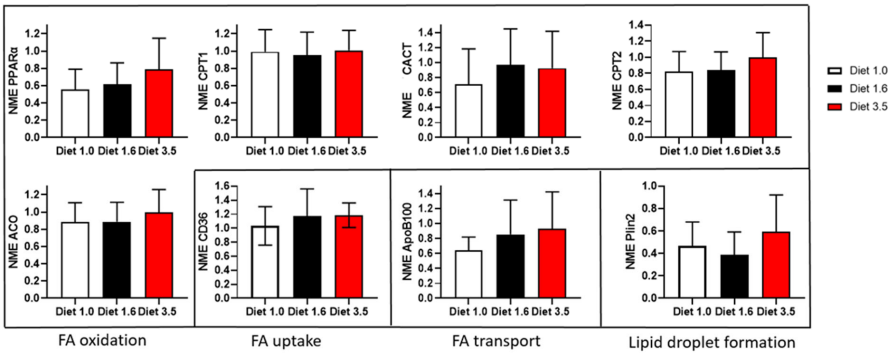

Figure A1. Normalised mean expression (NME) of selected genes involved in fatty acid (FA) usage in the liver of Atlantic salmon fed with increasing dietary EPA + DHA contents. Triplicate cages per

diet group and five fish sampled from each cage (n = 15). Analysed with one-way ANOVA with Tukey's HSD post hoc.

Table A1. Full analysed FA composition in mg/g in liver polar and neutral lipids of Atlantic salmon fed feeds with increasing dietary EPA + DHA. Triplicate cages per diet group, and 4 fish sampled per cage (n = 12). Different letters denote significant statistical differences ($p < 0.05$ one-way ANOVA with Tukey's HSD post hoc). Numbers are the mean with standard deviation.

	Polar Lipids				Neutral Lipids			
	Diet 1.0	**Diet 1.3**	**Diet 1.6**	**Diet 3.5**	**Diet 1.0**	**Diet 1.3**	**Diet 1.6**	**Diet**
14:0	0.1 ± 0.0 a	0.1 ± 0.0 a	0.1 ± 0.0 a	0.2 ± 0.0 b	0.5 ± 0.4	0.5 ± 0.4	0.3 ± 0.1	0.2 ± 0.1
16:0	2.4 ± 0.3 a	2.3 ± 0.3 a	2.3 ± 0.3 a	2.7 ± 0.2 b	2.3 ± 1.5	2.3 ± 1.9	1.3 ± 0.5	0.9 ± 0.3
18:0	1.3 ± 0.2	1.2 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	2.1 ± 1.6 b	1.8 ± 1.3 b	0.9 ± 0.5 ab	0.5 ± 0.2 a
20:0	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.05 ± 0.01	0.14 ± 0.14 b	0.11 ± 0.07 b	0.06 ± 0.03 b	0.02 ± 0.00 a
Sum SFA	3.9 ± 0.5	3.7 ± 0.4	3.8 ± 0.4	4.3 ± 0.3	5.3 ± 3.5 b	4.9 ± 3.8 b	2.7 ± 1.2 ab	1.7 ± 0.7 a
16:1n-9	0.07 ± 0.01 b	0.06 ± 0.01 b	0.06 ± 0.00 b	0.06 ± 0.00 a	0.3 ± 0.2 b	0.3 ± 0.2 b	0.2 ± 0.1 b	0.1 ± 0.0 a
16:1n-7	0.1 ± 0.0 a	0.1 ± 0.0 a	0.1 ± 0.0 a	0.2 ± 0.0 b	0.9 ± 0.7	0.9 ± 0.8	0.5 ± 0.3	0.4 ± 0.2
18:1n-11	0.02 ± 0.00 a	0.01 ± 0.00 a	0.02 ± 0.00 a	0.07 ± 0.02 b	0.03 ± 0.02 b	0.03 ± 0.01 b	0.03 ± 0.01 b	0.1 ± 0.01 a
18:1n-9	4.5 ± 0.5 b	4.0 ± 0.6 b	3.7 ± 0.4 b	2.7 ± 0.3 a	39.1 ± 32.4 b	34.5 ± 26.6 b	18.4 ± 9.3 b	6.5 ± 3.2 a
18:1n-7	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	2.5 ± 2.0 b	2.2 ± 1.6 b	1.3 ± 0.6 ab	0.7 ± 0.3 a
20:1n-11	<0.1	<0.1	<0.1	<0.1	0.05 ± 0.04	0.04 ± 0.03	0.04 ± 0.02	0.06 ± 0.04
20:1n-9	0.5 ± 0.1 ab	0.4 ± 0.1 a	0.5 ± 0.1 ab	0.6 ± 0.1 b	4.0 ± 3.4 b	3.5 ± 2.7 b	2.0 ± 0.9 b	0.9 ± 0.5 a
20:1n-7	0.01 ± 0.00 a	0.01 ± 0.00 a	0.02 ± 0.01 a	0.03 ± 0.00 b	0.10 ± 0.09 b	0.09 ± 0.07 b	0.05 ± 0.03 ab	0.03 ± 0.02 a
22:1n-11	0.01 ± 0.00 a	0.01 ± 0.00 a	0.01 ± 0.00 a	0.02 ± 0.00 b	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1
22:1n-9	<0.1	<0.1	<0.1	<0.1	0.2 ± 0.2 b	0.2 ± 0.1 b	0.1 ± 0.1 ab	0.1 ± 0.0 a
24:1n-9	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.1 ± 0.1 b	0.1 ± 0.1 b	0.1 ± 0.0 b	0.1 ± 0.0 a
Sum MUFA	5.7 ± 0.5 b	5.0 ± 0.7 ab	4.8 ± 0.5 ab	4.1 ± 0.4 a	47.6 ± 39.2 b	42.1 ± 32.4 b	22.7 ± 11.3 b	9.1 ± 4.4 a
18:2n-6	2.3 ± 0.3 b	2.2 ± 0.4 b	1.9 ± 0.3 b	1.1 ± 0.1 a	11.0 ± 10.2 b	9.4 ± 7.1 b	5.0 ± 2.6 b	1.8 ± 0.8 a
18:3n-6	<0.1	<0.1	<0.1	<0.1	0.10 ± 0.07 c	0.05 ± 0.03 bc	0.02 ± 0.01 ab	0.01 ± 0.0 a
20:2n-6	0.5 ± 0.1 ab	0.5 ± 0.1 ab	0.5 ± 0.1 b	0.4 ± 0.1 a	1.7 ± 1.5 b	1.6 ± 1.3 b	0.9 ± 0.5 b	0.4 ± 0.2 a
20:3n-6	0.5 ± 0.1 c	0.4 ± 0.1 b	0.3 ± 0.1 b	0.1 ± 0.0 a	0.52 ± 0.46 c	0.31 ± 0.22 bc	0.09 ± 0.15 b	0.04 ± 0.02 a
20:4n-6 (ARA)	0.7 ± 0.1 a	0.6 ± 0.1 a	0.7 ± 0.1 a	0.8 ± 0.1 b	0.5 ± 0.4 ab	0.6 ± 0.5 b	0.4 ± 0.2 ab	0.2 ± 0.1 a
22:5n-6	0.08 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.00	<0.1	<0.1	<0.1	<0.1
Sum n-6	4.1 ± 0.3 b	3.7 ± 0.5 b	3.4 ± 0.4 b	2.5 ± 0.1 a	13.9 ± 12.6 b	12.0 ± 9.2 b	6.5 ± 3.3 b	1.1 ± 2.4 a
18:3n-3	0.6 ± 0.1 b	0.5 ± 0.1 b	0.5 ± 0.1 b	0.3 ± 0.1 a	3.4 ± 2.9 b	3.3 ± 2.6 b	1.9 ± 0.9 b	0.7 ± 0.3 a
18:4n-3	0.03 ± 0.0 b	0.02 ± 0.0 ab	0.02 ± 0.02 b	0.01 ± 0.00 b	0.16 ± 0.11 b	0.11 ± 0.07 b	0.06 ± 0.03 ab	0.03 ± 0.01 a
20:4n-3	0.3 ± 0.0 b	0.2 ± 0.0 b	0.2 ± 0.0 ab	0.2 ± 0.0 a	0.4 ± 0.4	0.4 ± 0.4	0.2 ± 0.1	0.2 ± 0.1
20:5n-3 (EPA)	1.9 ± 0.3 a	2.0 ± 0.2 a	2.1 ± 0.2 a	2.6 ± 0.2 b	0.5 ± 0.2	0.6 ± 0.4	0.4 ± 0.1	0.6 ± 0.2
21:5n-3	0.02 ± 0.00 a	0.02 ± 0.00 a	0.02 ± 0.00 a	0.03 ± 0.00 b	0.02 ± 0.01	0.02 ± 0.02	0.02 ± 0.01	0.03 ± 0.01
22:5n-3	0.6 ± 0.1 a	0.6 ± 0.1 a	0.6 ± 0.1 a	0.8 ± 0.2 b	0.1 ± 0.1 a	0.1 ± 0.1 a	0.1 ± 0.0 a	0.3 ± 0.1 b
22:6n-3 (DHA)	4.1 ± 0.6 a	4.4 ± 0.4 a	4.8 ± 0.4 a	5.9 ± 0.3 b	0.4 ± 0.1 a	0.5 ± 0.2 a	0.4 ± 0.1 ab	0.6 ± 0.2 b
24:5n-3	0.02 ± 0.00 a	0.02 ± 0.00 a	0.02 ± 0.01 a	0.04 ± 0.04 b	0.03 ± 0.02 a	0.04 ± 0.06 ab	0.03 ± 0.02 a	0.06 ± 0.02 b
24:6n-3	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	<0.1	<0.1	<0.1	<0.1
EPA + DHA	6.1 ± 0.8 a	6.3 ± 0.6 a	6.9 ± 0.6 a	8.4 ± 0.5 b	0.9 ± 0.4	1.1 ± 0.6	0.9 ± 0.1	1.2 ± 0.3
Sum n-3	7.7 ± 0.9 a	7.7 ± 0.8 a	8.2 ± 0.8 ab	9.7 ± 0.4 b	5.0 ± 3.7	5.1 ± 3.8	3.2 ± 1.2	2.5 ± 0.9
Sum PUFA	11.8 ± 1.1	11.5 ± 1.3	11.7 ± 1.2	12.3 ± 0.5	19.0 ± 16.3 b	17.1 ± 13.0 b	9.7 ± 4.6 ab	4.9 ± 2.0 a
n6/n3	0.6 ± 0.1 c	0.5 ± 0.0 bc	0.4 ± 0.0 ab	0.3 ± 0.0 a	2.5 ± 0.6 c	2.3 ± 0.2 bc	1.9 ± 0.3 ab	1.0 ± 0.2 a
n3/n6	1.8 ± 0.2 a	2.1 ± 0.1 ab	2.4 ± 0.2 b	3.8 ± 0.2 c	0.5 ± 0.2 a	0.4 ± 0.0 a	0.6 ± 0.2 ab	1.1 ± 0.3 b
Sum fat	21.6 ± 1.6	20.5 ± 2.4	20.8 ± 2.0	21.3 ± 1.0	72.6 ± 59.3 b	65.0 ± 49.7 b	35.6 ± 17.3 b	16.1 ± 7.2 a

Table A2. Fully analysed FA composition in % of TFA in liver polar and neutral lipids of Atlantic salmon fed feeds with increasing dietary EPA + DHA. Triplicate cages per diet group and 4 fish sampled per cage (n = 12). Different letters denote significant statistical differences ($p < 0.05$ one-way ANOVA with Tukey's HSD post hoc). Numbers are the mean with standard deviation.

	Polar Lipids				Neutral Lipids			
	Diet 1.0	**Diet 1.3**	**Diet 1.6**	**Diet 3.5**	**Diet 1.0**	**Diet 1.3**	**Diet 1.6**	**Diet 3.5**
14:0	0.4 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.9 ± 0.1	0.8 ± 0.1 a	0.8 ± 0.1 a	0.9 ± 0.1 a	1.5 ± 0.2 b
16:0	10.9 ± 0.9 a	11.0 ± 0.6 a	11.2 ± 0.7 a	12.5 ± 1.0 b	3.8 ± 1.2 a	3.6 ± 0.4 a	3.8 ± 0.7 a	6.1 ± 1.1 b
18:0	6.2 ± 0.7	6.1 ± 0.4	6.1 ± 0.4	6.3 ± 0.6	3.0 ± 0.6	2.8 ± 0.4	2.6 ± 0.6	2.9 ± 0.6
20:0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0 b	0.2 ± 0.0 b	0.2 ± 0.0 b	0.1 ± 0.0 a
Sum SFA	18.1 ± 1.2 a	18.1 ± 0.7 a	18.5 ± 0.8 a	20.5 ± 1.1 b	8.0 ± 1.5 a	7.6 ± 0.7 a	7.7 ± 1.1 a	11.1 ± 1.7 b

16:1n-9	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	1.3 ± 0.3 a	1.3 ± 0.2 a	1.4 ± 0.1 a	2.6 ± 0.2 b
16:1n-7	0.5 ± 0.1 a	0.5 ± 0.1 a	0.5 ± 0.0 a	0.8 ± 0.1 b	1.3 ± 0.3 a	1.3 ± 0.2 a	1.4 ± 0.1 a	2.6 ± 0.2 b
18:1n-11	0.1 ± 0.0 a	0.1 ± 0.0 a	0.1 ± 0.0 a	0.4 ± 0.1 b	<0.1	<0.1	<0.1	0.1 ± 0.1
18:1n-9	20.9 ± 2.7 c	19.4 ± 0.9 bc	17.9 ± 1.0 ab	12.4 ± 1.3 a	52.9 ± 3.1 c	52.9 ± 0.6 bc	50.9 ± 2.6 b	39.4 ± 2.8 a
18:1n-7	2.0 ± 0.2 a	2.0 ± 0.1 a	2.0 ± 0.1 a	2.3 ± 0.1 b	3.4 ± 0.2 a	3.5 ± 0.2 a	3.6 ± 0.1 a	4.2 ± 0.2 b
20:1n-11	<LOQ	<LOQ	<LOQ	<LOQ	<0.1	<0.1	<0.1	<0.1
20:1n-9	2.2 ± 0.3 a	2.1 ± 0.3 a	2.3 ± 0.4 ab	2.6 ± 0.6 b	5.3 ± 0.7	5.4 ± 0.4	5.5 ± 0.4	5.4 ± 0.8
20:1n-7	<0.1	<0.1	<0.1	<0.1	0.1 ± 0.0 a	0.1 ± 0.0 ab	0.2 ± 0.1 ab	0.2 ± 0.0 b
22:1n-11	<0.1	<0.1	<0.1	<0.1	0.2 ± 0.1 a	0.2 ± 0.0 a	0.2 ± 0.1 a	0.8 ± 0.3 b
22:1n-9	<0.1	<0.1	<0.1	<0.1	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
24:1n-9	<0.1	<0.1	<0.1	<0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Sum MUFA	26.3 ± 2.9 c	24.6 ± 1.1 bc	23.3 ± 1.0 ab	19.3 ± 1.6 a	64.4 ± 3.6 bc	64.6 ± 0.5 b	63.1 ± 2.8 ab	55.1 ± 3.9 a
18:2n-6	10.9 ± 1.1 c	10.5 ± 0.7 bc	9.2 ± 0.6 b	5.3 ± 0.5 a	14.4 ± 1.2 b	14.4 ± 0.6 b	13.9 ± 0.8 b	10.9 ± 0.9 a
18:3n-6	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
20:2n-6	2.1 ± 0.3 ab	2.3 ± 0.3 b	2.3 ± 0.3 b	1.8 ± 0.3 a	2.3 ± 0.3	2.4 ± 0.3	2.5 ± 0.2	2.3 ± 0.2
20:3n-6	2.5 ± 0.4 d	1.7 ± 0.3 c	1.2 ± 0.2 b	0.4 ± 0.1 a	0.7 ± 0.2 c	0.5 ± 0.1 b	0.4 ± 0.1 b	0.3 ± 0.1 a
20:4n-6 (ARA)	3.1 ± 0.4 a	3.1 ± 0.2 a	3.3 ± 0.2 ab	3.9 ± 0.3 b	0.8 ± 0.1 a	0.9 ± 0.1 ab	1.1 ± 0.2 b	1.5 ± 0.2 c
22:5n-6	0.3 ± 0.1 a	0.4 ± 0.0 ab	0.4 ± 0.0 b	0.4 ± 0.0 b	<0.1	<0.1	<0.1	<0.1
Sum n-6	19.2 ± 0.9 d	18.1 ± 0.7 c	16.6 ± 0.8 b	12.0 ± 0.4 a	18.4 ± 1.4 b	18.3 ± 0.8 b	18.0 ± 0.8 b	15.1 ± 0.9 a
18:3n-3	2.6 ± 0.5 b	2.6 ±0.3 b	2.5 ± 0.3 b	1.4 ± 0.4 a	4.6 ± 0.4 ab	5.0 ± 0.4 bc	5.2 ± 0.3 c	4.2 ± 0.6 a
18:4n-3	At LOQ	At LOQ	At LOQ	At LOQ	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1
20:4n-3	1.3 ± 0.2 c	1.2 ± 0.2 ab	1.1 ± 0.1 b	0.8 ± 0.1 a	0.7 ± 0.3 a	0.6 ± 0.1 a	0.7 ± 0.1 a	1.2 ± 0.1 b
20:5n-3 (EPA)	8.9 ± 0.8 a	9.5 ± 0.6 ab	10.1 ± 0.6 b	12.0 ± 0.7 c	1.0 ± 0.8 a	1.0 ± 0.3 a	1.5 ± 0.9 ab	3.8 ± 0.8 b
21:5n-3	At LOQ	At LOQ	At LOQ	At LOQ	<0.1	<0.1	<0.1	<0.1
22:5n-3	2.8 ± 0.3 a	2.8 ± 0.2 a	2.9 ± 0.2 a	3.7 ± 0.7 b	0.2 ± 0.3 a	0.2 ± 0.0 a	0.3 ± 0.2 a	1.7 ± 0.4 b
22:6n-3 (DHA)	19.2 ± 2.0 a	21.3 ± 1.1 ab	23.0 ± 1.1 b	27.5 ± 1.1 c	1.1 ± 1.5 a	1.0 ± 0.4 a	1.6 ± 1.2 a	4.6 ± 1.8 b
24:5n-3	0.1 ± 0.0 a	0.1 ± 0.0 ab	0.1 ± 0.0 ab	0.2 ± 0.0 b	0.1 ± 0.0 a	0.1 ± 0.0 a	0.1 ± 0.0 a	0.4 ± 0.1 b
24:6n-3	0.2 ± 0.0 b	0.2 ± 0.0 b	0.1 ± 0.1 ab	0.1 ± 0.0 a	<0.1	<0.1	<0.1	<0.1
EPA + DHA	28.1 ± 2.6 a	30.8 ± 1.0 ab	33.1 ± 1.2 bc	39. 5 ± 1.6 c	2.1 ± 2.3 a	2.0 ± 0.7 a	3.1 ± 2.1 a	8.4 ± 2.5 b
Sum n-3	35.4 ± 2.3 a	37.9 ± 1.0 ab	39.9 ± 1.1 bc	45.7 ± 0.9 c	7.9 ± 2.9 a	8.1 ± 0.6 ab	9.7 ± 2.4 b	16.4 ± 2.9 bc
Sum PUFA	54.6 ± 1.7 a	56.0 ± 0.7 ab	56.6 ± 1.0 bc	57.7 ± 0.9 c	26.3 ± 2.8 a	26.5 ± 2.4 a	27.7 ± 2.4 ab	31.5 ± 2.5 b
n6/n3	0.6 ± 0.1 c	0.5 ± 0.0 bc	0.4 ± 0.0 ab	0.3 ± 0.0 a	2.5 ± 0.6 c	2.3 ± 0.2 bc	1.9 ± 0.3 ab	1.0 ± 0.2 a
n3/n6	1.8 ± 0.2 a	2.1 ± 0.1 ab	2.4 ± 0.2 b	3.8 ± 0.2 c	0.5 ± 0.2 a	0.4 ± 0.0 a	0.6 ± 0.2 ab	1.1 ± 0.3 b *
Sum fat	21.6 ±1.6	20.4 ± 2.4	20.8 ± 2.0	21.3 ± 1.0	72.6 ± 59.3 b	65.0 ± 49.7 b	35.6 ± 17.3 b	16.1 ± 7.1 a

Table A3. Various acyl carnitines in the livers of Atlantic salmon fed diets with increasing contents of EPA + DHA. Triplicate cages for diets 1.6 and 3.5, duplicate cages for diet 1.0 with five fish sampled per cage (n = 15 and n = 10, respectively). Data are presented as fold change between pairwise comparisons of diet groups, e.g., for the comparison diet 1.0/diet 3.5, a number below one would indicate less of the metabolite in diet group 1.0, while a number above one would indicate the opposite. Red indicates significantly higher and green significantly lower ($p < 0.05$), while pink and light green indicate p-values between 0.05 and 0.1.

Pathway	Biochemical Name	Fold Change		
		Diet 1.0/Diet 3.5	Diet 1.6/Diet 3.5	Diet 1.0/Diet 1.6
Fatty Acid Metabolism (Acyl Carnitine, Short Chain)	acetylcarnitine (C2)	1.25	1.43	0.87
	isocaproylcarnitine	0.53	0.83	0.64
Fatty Acid Metabolism (Acyl Carnitine, Medium Chain)	hexanoylcarnitine (C6)	1.75	1.87	0.94
	cis-3,4-methyleneheptanoylcarnitine	0.39	0.96	0.40
	decanoylcarnitine (C10)	1.65	1.52	1.09
	laurylcarnitine (C12)	1.73	1.52	1.14
	palmitoylcarnitine (C16)	0.88	1.42	0.62

Fatty Acid Metabolism (Acyl Carnitine, Long Chain Saturated)	margaroylcarnitine (C17)	0.64	1.17	0.55
	stearoylcarnitine (C18)	1.21	1.69	0.72
	arachidoylcarnitine (C20)	1.25	1.46	0.86
	behenoylcarnitine (C22)	1.47	1.39	1.06
	lignoceroylcarnitine (C24)	1.11	1.22	0.91
Acid Metabolism (Acyl Carnitine, Monounsaturated)	palmitoleoylcarnitine (C16:1)	0.62	0.98	0.63
	oleoylcarnitine (C18:1)	1.14	2.03	0.56
	eicosenoylcarnitine (C20:1)	1.18	1.89	0.62
	erucoylcarnitine (C22:1)	0.62	0.71	0.87
	ximenoylcarnitine (C26:1)	0.64	0.79	0.81
Fatty Acid Metabolism (Acyl Carnitine, Polyunsaturated)	linoleoylcarnitine (C18:2)	1.12	1.75	0.64
	linolenoylcarnitine (C18:3)	0.97	1.46	0.67
	dihomo-linoleoylcarnitine (C20:2)	1.19	1.99	0.60
	arachidonoylcarnitine (C20:4)	0.87	1.11	0.78
	dihomo-linolenoylcarnitine (C20:3n3 or 6)	2.48	2.56	0.97
	docosadienoylcarnitine (C22:2)	1.12	1.61	0.70
	docosapentaenoylcarnitine (C22:5n3)	0.64	1.01	0.63
	docosahexaenoylcarnitine (C22:6)	0.94	1.21	0.77
Fatty Acid Metabolism (Acyl Carnitine, Hydroxy)	(S)-3-hydroxybutyrylcarnitine	1.16	1.37	0.84
	3-hydroxyhexanoylcarnitine (1)	1.36	1.29	1.05
	3-hydroxyoctanoylcarnitine (1)	1.75	1.58	1.11
	3-hydroxyoctanoylcarnitine (2)	1.54	1.47	1.05
	3-hydroxydecanoylcarnitine	2.36	1.75	1.35
	3-hydroxypalmitoylcarnitine	1.54	1.47	1.05
	3-hydroxyoleoylcarnitine	1.58	1.88	0.84

Table A4. Metabolites in phospholipid metabolism and lysophospholipids in Atlantic salmon fed diets with increasing dietary EPA + DHA. Triplicate cages for diets 1.6 and 3.5 and duplicate cages for diet 1.0 with five fish sampled per cage (n = 15 and n = 10, respectively). Data are presented as the fold change between pairwise comparisons of diet groups, e.g., for the comparison diet 1.0/diet 3.5, a number below one would indicate less of the metabolite in diet group 1.0, while a number above one would indicate the opposite. Red indicates significantly higher and green significantly lower ($p < 0.05$), while pink and light green indicate p-values between 0.05 and 0.1.

Pathway	Biochemical Name	Fold Change		
		Diet 1.0/Diet 3.5	Diet 1.6/Diet 3.5	Diet 1.0/Diet 1.6
Phospholipid metabolism	glycerophosphoethanolamine	1.46	1.39	1.05
	glycerophosphoserine	1.25	1.35	0.93
	glycerophosphoinositol	1.34	1.18	1.14
Lysophospholipid	1-palmitoyl-GPC (16:0)	1.06	0.96	1.10
	2-palmitoyl-GPC (16:0)	1.62	1.03	1.57
	1-palmitoleoyl-GPC (16:1)	0.71	0.72	0.99
	2-palmitoleoyl-GPC (16:1)	0.65	0.73	0.88
	1-stearoyl-GPC (18:0)	1.41	1.31	1.08
	1-oleoyl-GPC (18:1)	1.42	1.38	1.03
	1-linoleoyl-GPC (18:2)	2.09	1.80	1.16
	1-linolenoyl-GPC (18:3)	2.01	1.91	1.05
	1-dihomo-linolenoyl-GPC (20:3n3 or 6)	3.30	1.98	1.67
	2-dihomo-linolenoyl-GPC (20:3n3 or 6)	4.62	2.85	1.62
	1-omega-arachidonoyl-GPC (20:4n3)	1.42	1.21	1.17

1-arachidonoyl-GPC (20:4n6)	0.81	0.72	1.13
1-eicosapentaenoyl-GPC (20:5)	0.70	0.76	0.93
1-lignoceroyl-GPC (24:0)	1.81	1.77	1.02
1-palmitoyl-GPE (16:0)	0.88	0.87	1.01
1-stearoyl-GPE (18:0)	1.18	1.17	1.01
2-stearoyl-GPE (18:0)	1.28	0.98	1.31
1-oleoyl-GPE (18:1)	1.19	1.17	1.02
1-linoleoyl-GPE (18:2)	1.43	1.26	1.13
2-dihomo-linolenoyl-GPE (20:3n3 or 6)	4.20	2.34	1.79
1-dihomo-linolenoyl-GPE (20:3n3 or 6)	2.36	1.67	1.41
1-omega-arachidonoyl-GPE (20:4n3)	1.24	1.06	1.17
1-arachidonoyl-GPE (20:4n6)	1.07	0.86	1.25
1-eicosapentaenoyl-GPE (20:5)	1.27	1.10	1.16
1-palmitoyl-GPS (16:0)	0.96	1.04	0.92
1-stearoyl-GPS (18:0)	1.63	2.06	0.79
1-oleoyl-GPS (18:1)	1.20	1.38	0.86
1-palmitoyl-GPG (16:0)	1.08	0.65	1.66
1-palmitoyl-GPI (16:0)	1.27	0.90	1.41
1-stearoyl-GPI (18:0)	1.42	1.05	1.35
1-oleoyl-GPI (18:1)	2.01	1.26	1.59
1-linoleoyl-GPI (18:2)	2.56	1.60	1.60
1-arachidonoyl-GPI (20:4)	1.00	0.81	1.24

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