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The level of eicosapentaenoic acid (EPA), but not docosahexaenoic acid (DHA), in blood of Atlantic salmon (*Salmo salar* L.) is related to formulation and concentration of EPA or DHA in feed

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ABSTRACT

The present study investigated how the concentration of PUFA in blood and muscle of Atlantic salmon (*Salmo salar* L.) changed when fed 4 diets containing very different amounts of LC-PUFA in fresh water for 84 days. The n-3 LC-PUFA constituted 4.4, 24.2, 18.4, and 51.5% of the total fatty acids in the diets made with rapeseed oil, anchoveta oil, a combination of rapeseed oil (60%) and anchoveta oil (40%), and EPA/DHA concentrate, respectively. No significant differences in the growth were observed in the groups during the feeding experiment. The changes in n-3 LC-PUFA in blood and muscle were however quite different in the 4 feeding groups. In the blood, DHA was mobilised to or retained at high level (30–36%) even with a very low amount of DHA in the feed. The concentration of EPA in the blood reflected to a large degree the concentration in the feed, with a rapid increase of EPA in the blood during the first 21 days of feeding. The level of EPA in the blood remained at this level for the rest of the study. However, when only small amounts (1.8%) were present in the feed the fatty acid appeared to be selectively mobilised to or retained in the blood. In the muscle, the percentage of EPA, DHA and other fatty acids mirrored the amounts in the feed suggesting increased TAG (triacylglycerides) during the feeding period.

1. Introduction

The marine fatty acids EPA and DHA are important for both human and fish health. Together with other polyunsaturated fatty acids, they serve several important biological functions. They are building blocks of all cell membranes, regulate gene expression and are precursors of a wide range of bioactive substances that regulate inflammation and physiological processes (Calder, 2015). The total n-3 PUFA (polyunsaturated fatty acids) dietary requirement of salmonids including α-linolenic acids (ALA), EPA and DHA, has been reported to range from 1 to 2.5% of the diet, depending on the species and experimental conditions (Glencross, 2009; Rosenlund et al., 2016; Ruyter et al., 2000a; Sissener et al., 2016). Studies determining the minimum requirement of n-3 PUFA have been based mainly on fish growth and survival, but fatty liver, histological changes in intestine, fin erosion, gill bleeding, deformed back bone, reduced reproduction and shock syndrome have also been reported as a consequence of low n-3 PUFA in feed (Glencross, 2009; Sargent et al., 2002).

It is known that PUFA can influence the immune system and responses in Atlantic salmon (Olsen et al., 2012). In humans, arachidonic acid (n-6), eicosapentaenoic acid (n-3) and docosahexaenoic acids (n-3), derived from membrane phospholipids in immune competent cells in the blood, are precursors of eicosanoids, resolvins and protectins which modulate leukocyte function and thereby influence the production of inflammatory cytokines and adhesion molecules (Serhan and Petasis, 2011). The eicosanoids derived from n-6 fatty acids promote pro-inflammatory responses, whereas eicosanoids and docosanoids produced from the long-chain n-3 fatty acids either lead to a reduced inflammatory response or even, in the case of resolvins and protectins, termination of the inflammatory responses (Calder, 2015; Serhan and Petasis, 2011).

Several studies have documented the fate of EPA and DHA in salmon tissues such as muscle and to a lower extent in blood and other tissues after feeding with low or moderate amounts of these fatty acids (Ruyter et al., 2006; Torstensen et al., 2000). Atlantic salmon possess all the enzymes necessary to produce DHA from α-linolenic acid (Monroig et al., 2011), but a previous study on Atlantic salmon in freshwater indicate that the requirements of salmon for EPA and DHA at the tissue level cannot be completely fulfilled by dietary α-linolenic acid (Ruyter et al., 2000a). A recent work on salmon given a diet deficient of DHA

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demonstrated a conversion of α-linolenic acid to EPA and DHA (Bou et al., 2017). Reduced level of DHA in brain and retina have been seen earlier, but only after prolonged feeding with a diet very low or deficient in DHA and EPA (Sissener et al., 2016). Knowledge about tolerable upper intake level of EPA and DHA is however scarce and in a report from the European Food Safety Authority (EFSA, 2012) it was concluded that scientific data were not available to set an upper limit for intake in humans. Long-term intake of high doses (5 g/day) of EPA and DHA in humans did not appear to increase bleeding episodes, glucose homeostasis immune functions or lipid peroxidation. Very few studies have to our knowledge been carried out on the effects of including high levels LC-PUFA in feed to fish (Brodtkorb et al., 1997). The aim of this study was therefore to increase the knowledge of the dynamics and relationship of EPA and DHA in blood and muscle tissue of Atlantic salmon after feeding 4 different diet levels of EPA and DHA for 84 days. The concentration of EPA and DHA combined in the diets ranged from 4.2 to 48.4% of the total fatty acids in the experimental diets. The effects of different feeding regimes on fish growth were recorded together with histopathological evaluation of the mid intestine.

2. Materials and methods

2.1. Materials

Unless otherwise is stated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyunsaturated methyl esters (PUFA1, PUFA2 and PUFA3) standards were obtained from Supelco Analytical (Bellefonte, PA, USA) and GLC411 from Nu-Chek Prep, Inc. (Elysan, MN, USA). Hematoxylin and Shandon Instant Eosin from Thermo Scientific (Osterode, Tyskland), Histo-Clear from National Diagnostics (Atlanta, Georgia, USA) and Histo-Wax from HistoLab (Gothenburg, Sweden). Benzoak VET. (ACD Pharmaceuticals, Leknes, Norway). Rapeseed oil, anchoveta oil and palm oil were obtained from Nofima BioLab (Fyllingsdalen, Bergen, Norway). Fish oil concentrate (EPAX TG6000N) containing minimum 300 mg/g EPA and 200 mg/g DHAwas a gift from Epax Norway (Ålesund, Norway).

2.2. Fish

Atlantic salmon (*Salmo salar* L.) (Aqua gen Q-TRL strain) reared on a commercial diet to an average weight of 50 g were provided by Havbruksstasjonen AS in Tromsø. The fish were kept in circular 300 l tanks at Havbruksstasjonen with continuous flow of fresh water. The water temperature was about 4 °C ten days prior to the start, and was gradually increased to and kept at 10 °C throughout the experiment. The fish were kept under constant 24:0 h light:dark conditions and fed ad libitum for 6 h per day. Water temperature, fish appetite, behavior and mortality were checked daily, while O_2 saturation was checked weekly. The fish displayed no sign of disease or mortality at the start of the experiment. Prior to the start of the experiment a total of 610 fish were sorted and divided in four different groups of 150 fish in each except for group 1 that had 160 fish. At day zero 10 fish were sacrificed from group 1 for registration of mean length and weight. The experiment was approved by the Norwegian Animal Research Authority (NARA), under ID number 8498. (https://www.etikkom.no/en/ethical-guidelines-for-research/ethical-guidelines-for-the-use-of-animals-in-research/).

2.3. Diets

Non-coated experimental pellets of 3 mm were formulated and manufactured by BioMar AS, Trondheim, Norway (Table 1). Fat coating of the pellets was done by Nofima AS, Bergen. Four experimental diets were made by adding different oils or mixtures of oils with different fatty acid composition to the pellets. The following oils were added to the feed: rapeseed oil (RO), anchoveta oil (AO), a mixture of rapeseed and anchoveta oil 60:40 (RO/AO) and an omega 3 concentrate in TG form (EPA/DHA). To avoid oxidation the oils contained 200 ppm of butylated hydroxytoluene (BHT). One percent palm oil was added to the oils before the coating process to reduce the potential of oil leaking from the pellets during storage. The amount of oil coated on the pellet was kept the same for all diets and the total fat content of the formulated pellets became approximately 20%. To ensure appetite and similar taste of pellets with different fatty acid composition, the pellets contained 2% krill meal.

Table 1
Ingredients of the experimental dry pellet (3 mm) as fed, BioMar AS, Trondheim, Norge.

Formulation	%
Fishmeal low temperature	18.4
Fishmeal south American	22.5
Krill meal (56% protein, Aker BioMarine AS)	2.5
Soy protein concentrate (non GMO)	15.0
Sunflower expeller meal	3.5
Wheat gluten	15.0
Whole wheat	4.9
Wheat meal	15.9
Mono sodium phosphate	1.2
Amino acids	1.9
Vitamins and minerals[a]	0.9
Astaxanthin (Lucantin Pink, BASF)	0.004
Chemical composition	
Water content	6.8
Gross energy (MJ/kg)	26.0
Protein	50.4
Fat	5.9
Ash	8.8
Phosphorus	1.4

[a] Vitamin and mineral composition is based on NRC 2011 and Hemre et al. (2016).

2.4. Feeding trial

Automatic feeders provided the feed. The feeding levels were aiming on overfeeding to obtain maximum voluntary feed intake in each tank. The fish were fed the experimental diets for a period of 84 days.

2.5. Sampling and sample preparation

The total weight of the fish biomass was registered at day −10 and day 60. Parallel series of 10 fish were randomly collected at day 0, day 21, day 42, day 63 and day 84 after start of the feeding trial. The fish were anesthetized with Benzoak VET (20–40 mg/l) and individual body weight and length were recorded. The fish were then killed by an overdose of Benzoak VET (200–400 mg/l) and a sharp blow to the head, immediately followed by blood sampling. Peripheral blood was collected from *Vena caudalis* into clot activator tubes (BD Biosciences, CA, USA) and kept on ice until freezing at -80 °C about 2 h after sampling. Samples of fillet were frozen at -80 °C and stored for later analysis of fatty acid composition. Samples of hindgut were cut in sizes suitable for histological analyses and fixed in 10% formalin.

2.6. Fatty acid composition analyses

Total lipids were extracted from feed pellets following the Folch's method (Folch et al., 1957). A 1 ml sample from the chloroform-methanol phase of the fat extracted from the different pellets was used for analysis of FA composition of total lipids using the method of methylation described (Stoffel et al., 1959). Analysis of the fatty acid composition of blood and fillet was done by a method of direct methylation (Viga and Grahlnielsen, 1990) and modified by Dulavik et al. (1998). In

short, 4 ml 2 M HCl/MeOH with 0.05% BHT per 100 mg muscle tissue was added in airtight Kimax tubes and heated for 2 h at 110 °C. The samples were then "dried" by flushing with N_2 and then 2 ml H_2O and 10 ml of heptane per 100 mg starting sample was added and centrifuged (Multifuge 1 S-R, Hereaus, Germany) at 1000*g* for 5 min. Four ml of the heptane/lipid phase from each sample was then transferred to new tubes, dried with N_2 and solubilized in 50 µl heptane. Direct methylation of blood was done by adding 1 ml of 0.5 M HCL in MeOH to 50 µl of blood and transferring to airtight tubes then heated for 1 h at 70 °C. The samples were mixed with 1 ml H_2O, 1 ml KCL and 2 ml heptane before centrifugation at 1000 × *g* for 5 min. The heptane/lipid phase of each sample was transferred to Eppendorf tubes and centrifuged at 25000 × *g* for 5 min (Eppendorf Centrifuge, 5417R, Hamburg, Germany) to remove insoluble matters. The samples were then transferred to new tubes, dried with N_2 and solubilized in 50 µl of heptane. The methylated fatty acids from muscle and blood were analyzed using gas chromatography (Agilent 6890 N, Agilent Technologies, Santa Clara, CA, USA) with a GC capillary column (CP7419, 50 m × 25 m, Varian Inc., Middelburg, Netherlands). The methylated fatty acids were separated with helium as a carrier gas and identified by using known standards.

2.7. Histology

Histopathological evaluation was performed on mid intestine from all treatment groups at all sampling times. Paraffin-embedded samples were cut with a Leica microtome (Erns Leitz Wetzlar GmBH) and stained with standard hematoxylin-eosin (Merck, KGeA). Stained slides were examined using a Light microscope (Zeiss AXIO, Germany). Images were captured by a Moticam 5 camera and Motic Images pluss 2.0 software (Motic Incorporatipn Ltd., Hong Kong). Samples were subjected to blind histopathological evaluation followed by a second evaluation after decoding of the samples.

2.8. Statistical analysis

All collected data were treated and analyzed statistically by using the software Prism version 7.02 (GraphPad Software Inc., USA). Group means were compared by using two-way ANOVA with Tukey Multiple Comparison test, and differences were considered significant with a *p*-value less than 0.05 ($p < .05$).

3. Results

3.1. Fatty acids composition of the diets

The fatty acids composition of the experimental diets reflected, as expected, to a large degree the composition of the oils added to the feed (Table 2). The n-3 LC-PUFA constituted 4.4, 24.2, 18.4 and 51.5% of the total fatty acids in the diets made with RO, AO, RO/AO and EPA/DHA concentrate, respectively. Elevated levels of 18:2 n-6 and 18:3 n-3 were present in the feed containing rapeseed oil. The small amount of EPA and DHA found in the diet added only rapeseed oil originate from the fishmeal and krill meal included in the production process. Fish oil usually contain only 1–2% linoleic acid (McGill and Moffat, 1992). Linoleic acid (18:2 n-6) were also found in the AO and EPA/DHA diets, 4.4–4.5% respectively, and is probably due to the presence of some lipids included in the plant meals and/or present in the commercial feed given prior to the start of the experimental feeding (Jobling et al., 2002)

3.2. Growth and survival

Mortality was monitored daily during the course of the experiment, and no mortalities were registered in any of the experimental groups.

The total biomass of all fish in all groups was measured at day −10 ($n = 600$) and at day 60 ($n = 440$) of the experiment. The total biomass demonstrated an increase in the total weight of all feeding groups, from an average of 6 kg at day −10 to 14 kg at day 60. The average weight per fish, calculated from the measured biomass, was 45 g at day −10. At day 60 the average weight per fish was 130 g in the RO-diet and RO/AO-diet groups, and 117 g in the EPA/DHA diet group (results not shown). The specific growth rate (SGR) from day −10 to day 60 was calculated to 1.49 in the RO diet group, 1.34 in the AO diet and EPA/DHA diet groups, and 1.44 in the RO/AO diet group. There were no statistically significant differences in weight gain between the different feeding groups ($p > .05$). The body weight was registered for all fish sampled at all time points ($n = 10$) during the course of the study. At day 84 the fish fed a diet containing anchoveta oil had the lowest average mean weight of 150.3 ± 21.7 g, while the fish fed a diet with rapeseed oil had the highest average mean weight of 168.1 ± 36 g (Fig. 1). However, there were no statistically significant differences in mean weight between the different feed groups at day 84 ($p > .05$).

3.3. Histology

The intestine of the sampled fish was investigated at all time points and histopathological slides were made from the midsection of the distal gut (Fig. 2). Diarrhea was observed in the fish fed the EPA/DHA diet. At day 84, several macroscopically visible white spots could be observed in the mucosa of the distal gut in fish from all four diets groups (results not shown). In the group fed the diet containing EPA/DHA concentrate all 10 sampled fish had white spots in the intestine at day 84. In the group fed the rapeseed oil diet 8 of 10, in the group fed anchoveta oil diet 6 of 10 and in the group fed the RO/AO diet 10 of 10. Histopathological examination of tissue slides from the affected area demonstrated no or mild inflammation with recruitment of inflammatory cells in the *lamina propria* (Fig. 2), but no severe pathology.

3.4. Blood lipids

The fatty acid composition was investigated in whole blood. During the first three weeks of the study, there was a rapid increase in the proportion of EPA (20:5 n-3) in the blood of the groups fed the diets with EPA/DHA concentrate and anchoveta oil (Fig. 3A). The percentage of EPA in blood increased significantly from 6.4 ± 0.4% at day 0 to 19.5 ± 0.8% at day 21 in the group fed with the diet containing EPA/DHA concentrate ($p < .0001$), and to 12.4 ± 0.5% in the group fed the AO diet ($p < 0{,}0001$), respectively. EPA remained at approximately these levels for the rest of the study. There was no significant change in the EPA level in the blood of fish fed the rapeseed oil diet during the study, while a small increase, was observed in the group fed the RO/AO diet. In the group fed the rapeseed oil diet, there was a significant increase in the level of LA (18:2 n-6) in the blood, from 3.9 ± 0.2% at day 0 to 8.9 ± 0.3% at day 21 ($p < .0001$), with no further increase observed for the rest of the feeding period (Fig. 4A). There was no significant increase in the blood level of LA in the blood of fish fed the diet with a mixture of rapeseed oil and anchoveta oil. The level of oleic acid (18:1 n-9) increased significantly in the blood, from 15% at day 0 to 25% at day 21 in the group fed rapeseed oil diet ($p < .0001$) (Fig. 4C). In the group fed the EPA/DHA diet, oleic fatty acid decreased significantly ($p < .0001$), from 15% at day 0 to 7.5% at day 21. No further reduction was observed. The level of ALA (18:3 n-3) remained low in blood of all groups during the experiment (Fig. 5C). However, an exception was noted on day 21 for the groups fed the RO/AO and EPA/DHA diets, where the blood levels were high for some of the individual fish.

The levels of DHA (22:6 n-3) in the blood of the AO, RO/AO and EPA/DHA diet groups increased slightly, but not significantly ($p > .05$), from 32 to 33% at day 0 to 36–37% after 42 days, while a low and not significant decrease was observed in the RO diet group at day 42, 63 and 84. However, at day 21 there is a slight and transient

Table 2
Fatty acid composition (% of total FA) in the 3 mm experimental diets (n = 3).

Fatty acid	Diet 0[a]	RO	AO	RO/AO	EPA/DHA
14:0	5.3 ± 0.1	2.9 ± 0.8	9.3 ± 0.2	4.7 ± 0.3	3.5 ± 0.1
16:0	14.8 ± 0.2	9.7 ± 0.3	24.3 ± 0.7	17.1 ± 0.2	10.8 ± 0.4
18:0	3.0 ± 0.0	2.1 ± 0.0	4.7 ± 0.1	3.4 ± 0.2	4.9 ± 0.2
ΣSFA	**23.1**	**14.7**	**38.3**	**25.2**	**19.1**
16:1 n-7	5.8 ± 0.0	1.4 ± 0.1	7.0 ± 0.0	4.8 ± 0.0	2.4 ± 0.0
18:1 n-7	3.1 ± 0.0	3.0 ± 0.1	2.6 ± 0.1	3.0 ± 0.1	2.9 ± 0.2
18:1 n-9	31.7 ± 0.1	45.1 ± 1.3	13.8 ± 0.8	29.1 ± 0.6	11.9 ± 0.3
22:1 n-9	0.9 ± 0.0	0.8 ± 0.2	0.7 ± 0.0	0.4 ± 0.0	1.3 ± 0.0
ΣMUFA	**41.5**	**50.3**	**24.1**	**37.4**	**18.4**
18:2 n-6	11.0 ± 0.0	17.3 ± 0.6	4.5 ± 0.2	10.8 ± 0.1	4.4 ± 0.1
20:4 n-6	0.9 ± 0.0	2.4 ± 0.7	2.0 ± 0.2	0.6 ± 0.2	1.4 ± 0.2
Σn-6	**11.4**	**19.7**	**6.5**	**11.4**	**5.8**
18:3 n-3	4.6 ± 0.0	7.9 ± 0.2	1.0 ± 0.1	4.2 ± 0.1	0.9 ± 0.1
18:4 n-3	2.3 ± 0.0	1,4 ± 0.1	1.4 ± 0.1	1.0 ± 0.0	1.4 ± 0.0
20:5 n-3	7.5 ± 0.1	1.8 ± 0.2	13.5 ± 0.1	10.0 ± 0.3	27.3 ± 0.3
22:5 n-3	1.3 ± 0.0	0.2 ± 0.0	1.6 ± 0.0	1.2 ± 0.0	3.1 ± 0.0
22:6 n-3	6.0 ± 0.0	2.4 ± 0.3	9.1 ± 0.0	7.3 ± 0.1	21.1 ± 0.2
Σn-3	**21.7**	**13.7**	**26.6**	**23.7**	**53.8**
Σn-6/Σn-3	**0.52**	**1.43**	**0.24**	**0.48**	**0.1**
EPA + DHA	**8.8**	**4.2**	**22.6**	**17.2**	**48.4**
ΣLc PUFA	**14.8**	**4.4**	**24.2**	**18.4**	**51.5**

[a] Diet 0; the diet the fish were fed prior to one week before the start of the feed trial. The dietary groups are named according to their oil content: RO (rapeseed oil), AO (anchoveta oil), RO/AO (a 60/40 mixture of rapeseed oil and anchoveta oil) and EPA/DHA (Epax 6000, a TG 3322 omega 3 concentrate).

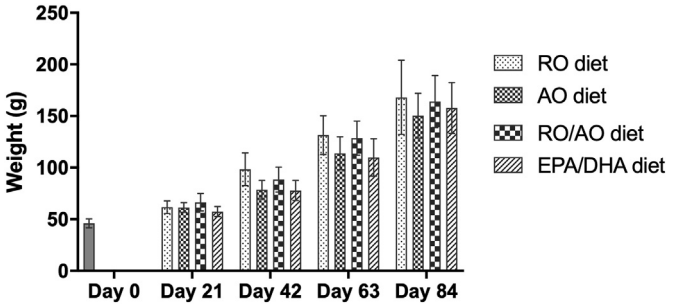

Fig. 1. Average weight gain (g) in Atlantic salmon fed diets containing either rapeseed oil (RO diet), anchovy oil (AO diet), a mixture of rapeseed oil and anchovy oil (RO/AO diet), or fish oil concentrate rich in eicosapentaenoic acid and docosahexaenoic acid (EPA/DHA diet) for a duration of 84 days. Each column represent mean ± SD (n = 10). There was no significant differences among the groups p < .05 within each timepoint.

significant decrease (p = .0133) (Fig. 3C). The levels of arachidonic acid (20:4n-6) in the blood were not affected by the diets, and remained low at between 2 and 3% for all groups throughout the experiment (Fig. 5A).

In Table 3, we compared the total fatty acid composition in the blood of the salmon from all four diet groups at day 84. There was a significant difference with p values between 0.0001 and 0.0014 in the levels of oleic acid (18:1 n-9), LA (18:2 n-6) and EPA (20:5 n-3) in the blood between the groups as expected from the fatty acid compositions of the different diets the fish groups were fed (diets presented in Table 2). The level of DHA in the blood at day 84 was not significantly different in the groups fed AO diet, RO/AO diet or EPA/DHA diet, even if the level of DHA given in the diet was different. The total level of DHA in the diet ranging from 7.3% in the RO/AO diet to 21.1% in the EPA/DHA diet (Table 2). The group fed a diet with pure rape seed oil had a significantly lower level of DHA in the blood compared to the other groups (p < .0001). The total level of the DHA in the RO diet was 2.4% (Table 2).

3.5. Muscle lipids

In the salmon fed EPA/DHA and anchoveta oil diets, an increase in the proportion of EPA in the muscle tissue was observed throughout the experiment, from 4.8% at day 0, to 9.3% at day 21, and up to 18.5% at day 84 in the former group (p < .0001). In the group fed the AO diet, EPA increased from 4.8% at day 0 to 9.1% at day 84 (Fig. 3B). In the group fed the rapeseed oil diet the proportion of EPA in the muscle decreased, from 4.8% at day 0 to 2.0% at day 84 (p = .0002). The proportion of DHA in the muscle tissue was stable in the group fed the EPA/DHA diet and remained at approximately 28% throughout the feed trial, while it was reduced to approximately 22% for the AO diet group at the end on day 84 (Fig. 3D). The percentage of DHA in the muscle tissue of the RO and the RO/AO diet groups decreased to 10 and 15%, respectively, at day 84. The level of LA increased in the muscle tissue of the group fed the rapeseed oil diet, from 8% at day 0 to 15% at day 84 (p < .0001). In the group fed the RO/AO diet, the level of LA in the muscle tissue remained at 8% throughout the experiment (Fig. 4B). There was a slight decrease in the level of LA in the muscle tissues of the groups fed the anchoveta oil and EPA/DHA diets. The level of oleic acid increased in the muscle tissues of the group fed the diet with rapeseed oil, remained stable in the group feed RO/AO diet and decreased in the groups feed EPA/DHA and anchoveta oil diets (Fig. 4D). The level of ALA (18:3 n-3) in the muscle tissue increased in the group fed rapeseed oil and RO/AO (Fig. 5D). The level of ALA decreased in the muscle tissues of the groups fed the anchoveta oil and EPA/DHA diets. The level of AA (20:4 n-6) in the muscle tissue was not affected by the diet, and remained at about 1% for all groups at all time points (Fig. 5B).

4. Discussion

In the present study, Atlantic salmon in freshwater phase were fed four diets with different fatty acid composition for a period of almost 3 months. The total EPA/DHA content in the oil fraction of the different diets ranged from 4.2 in the rapeseed oil diet formulation to 48.4% in the EPA/DHA diet concentrate formulation while the total omega-3 content in the oils, including ALA (18:3 n-3) and DPA (22:5 n-3) were from 13.9% to 53.8%. The RO/AO diet was selected and especially prepared to mimic a commercial diet with a 60/40 combination of rapeseed oil and anchoveta oil (Trygve Sigholt, BioMar AS, pers. comm) while the AO diet resembled the feed given to Atlantic salmon around two decades ago (Ytrestoyl et al., 2015). The diets containing omega-3 concentrate or pure rapeseed oil were included to investigate the effects of feeding extreme high and low levels respectively of EPA and DHA to the salmon. The quantitative requirements of EPA and DHA in Atlantic

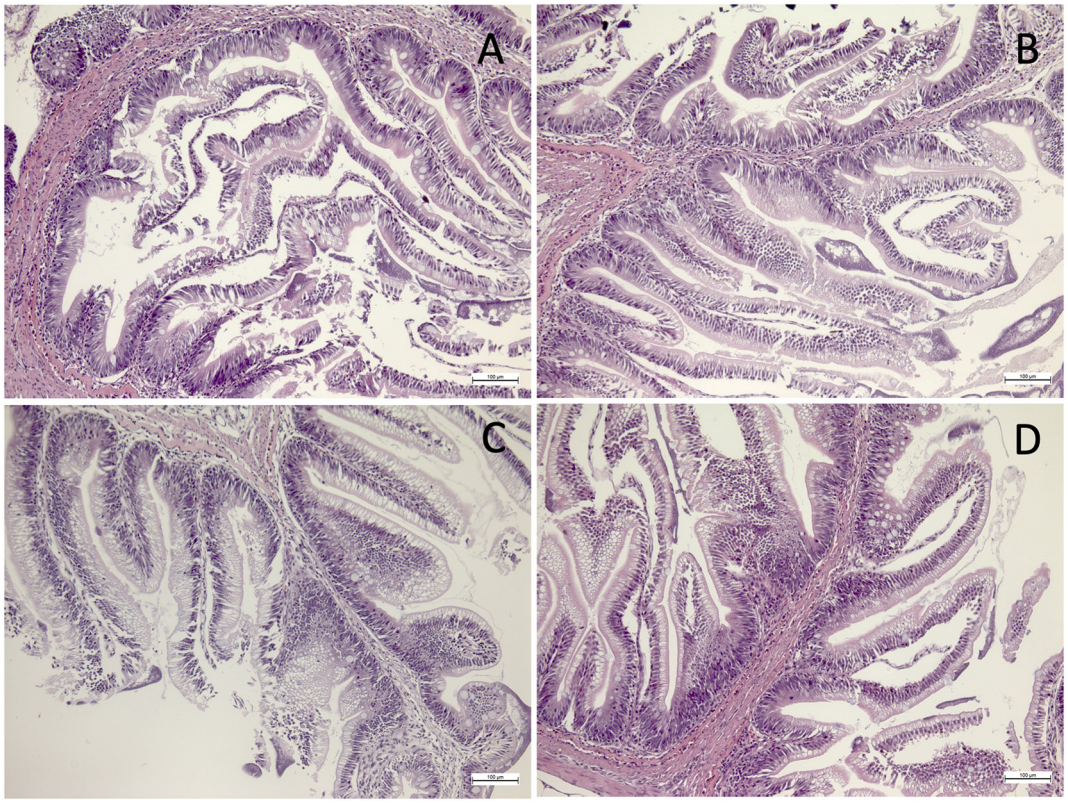

Fig. 2. Histological appearance of formaldehyde-fixed distal intestine from Atlantic salmon fed diets added 18% of either rapeseed oil (RO diet) (A), Anchovy oil (AO diet) (B), a mixture of rapeseed oil and anchovy oil (60:40) (RO/AO diet) (C), or fish oil concentrate rich in eicosapentaenoic acid and docosahexaenoic acid (EPA/DHA diet) (D). (H&E, x100; No or mild pathological changes in the form of inflammation were observed after 84 days of feeding).

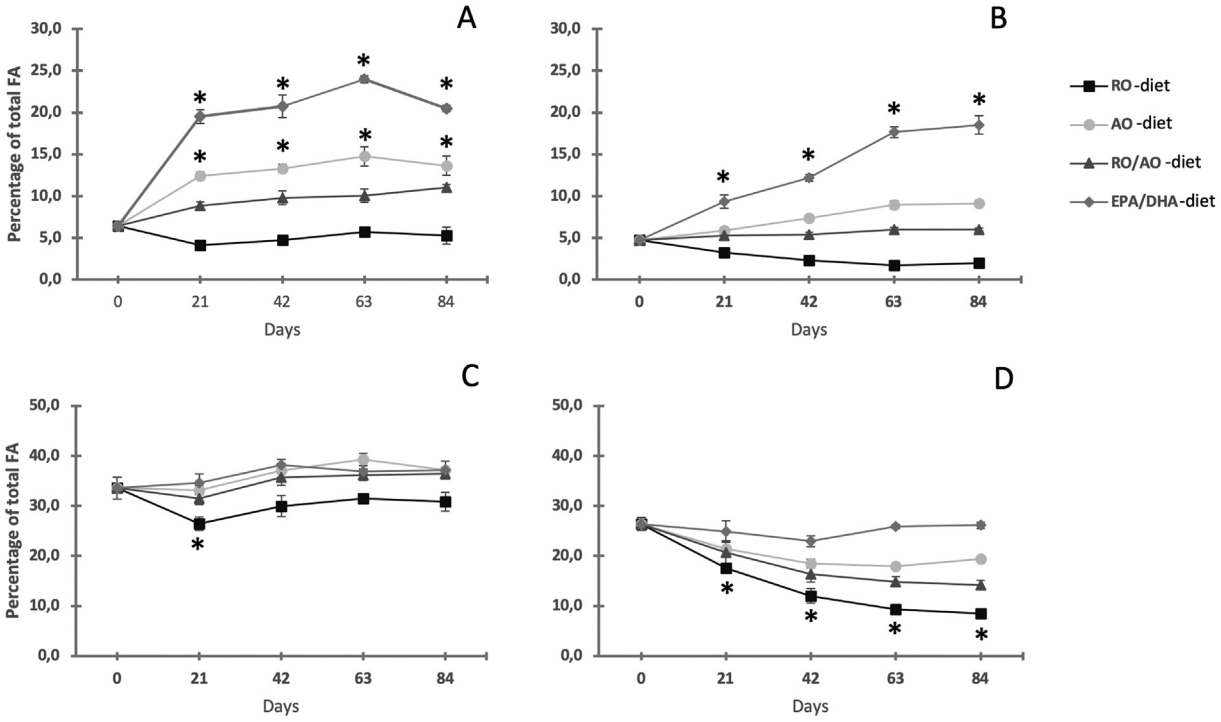

Fig. 3. Levels (area %) of EPA (20:5n-3) and DHA (22:6n-3) in whole blood and muscle of Atlantic salmon fed diets containing either rapeseed oil (RO diet), anchovy oil (AO diet), a mixture of rapeseed oil and anchoveta oil (RO/AO diet) or fish oil concentrate enriched with EPA and DHA (EPA/DHA diet), for a duration of 84 days. EPA in blood (A), EPA in muscle (B), DHA in blood (C) and DHA in muscle (D). Each point represents mean ± SD (n = 8). * Significant difference ($p < .0001$) between measured value at given timepoint and day 0.

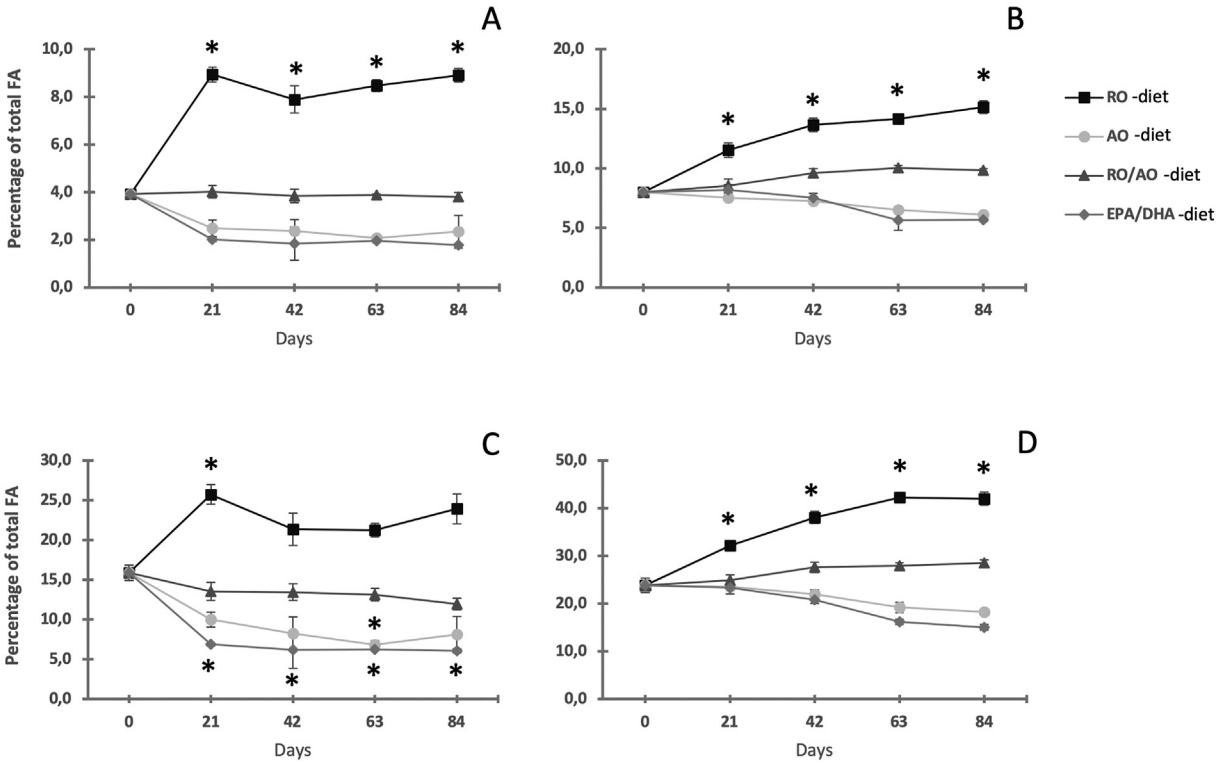

Fig. 4. Levels (area %) of LA (18:2n-6) and oleic acid (18:1n-9) in whole blood and muscle of Atlantic salmon fed diets containing either rapeseed oil (RO diet), anchovy oil (AO diet), a mixture of rapeseed oil and anchoveta oil (RO/AO diet) or fish oil concentrate enriched with EPA and DHA (EPA/DHA diet for a duration of 84 days. LA in blood (A), LA in muscle (B), oleic acid in blood (C) and oleic acid in muscle (D). Each point represents mean ± SD ($n = 8$). * Significant difference ($p < .0001$) between measured value at given timepoint and day 0.

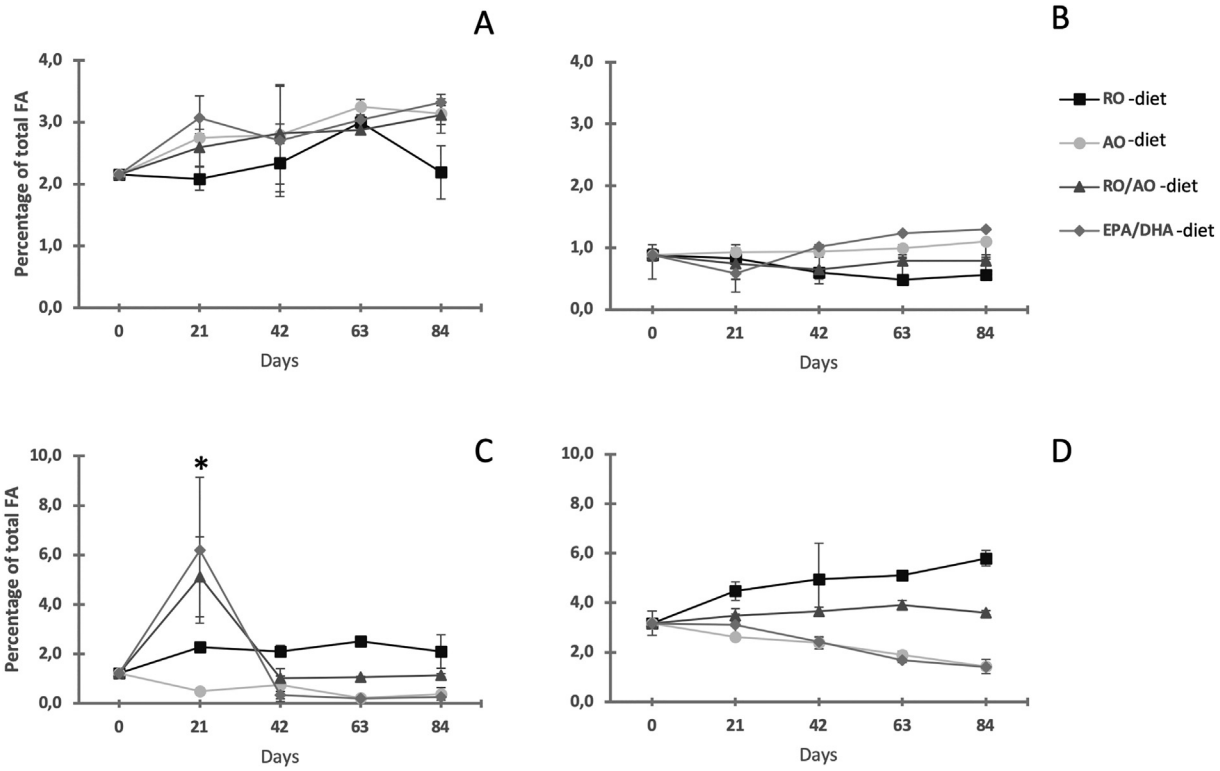

Fig. 5. Levels (area %) of AA (20:4n-6) and ALA (18:3n-3) in whole blood and muscle of Atlantic salmon fed diets containing either rapeseed oil (RO diet), anchovy oil (AO diet) rapeseed oil anchoveta mix (RO/AO diet) or fish oil concentrate enriched with EPA and DHA (EPA/DHA diet) for a duration of 84 days. AA in blood (A), AA in muscle (B), ALA in blood (C) and ALA in muscle (D). Each point represent mean ± SD (n = 8). * Significant difference ($p < .0001$) between measured value at given timepoint and day 0.

Table 3
Fatty acid composition (area % / % of total FA) in blood of Atlantic salmon, fed four experimental diets for 84 days added 18% of either rapeseed oil (RO), anchovy oil (AO), a mixture of rapeseed oil and anchovy oil (60:40) (RO/AO), or a fish oil concentrate EPA/DHA (EPAX 6000TGN) rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Presented as mean ± SD.

Day 84	RO	AO	RO/AO	EPA/DHA
Fatty acid	(n = 8)	(*n* = 8)	(n = 8)	(n = 8)
14:00	0.5 ± 0.4	1.9 ± 0.2	1.3 ± 0.1	0.6 ± 0.0
16:00	17.6 ± 0.7	19.0 ± 0.7	17.9 ± 0.5	14.9 ± 0.3
18:00	4.0 ± 0.9	4.5 ± 0.1	4.2 ± 0.2	5.5 ± 0.1
ΣSFA	**22.2**	**25.4**	**23.4**	**21**
16:1 n-7	1.4 ± 0.2	3.1 ± 0.3	1.8 ± 0.1	1.2 ± 0.1
18:1 n-9	23.9 ± 1.9	8.2 ± 2.2	12.0 ± 0.7	6.1 ± 0.3
18:1 n-7	0.3 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.7 ± 0.0
20:1 n-9	0.8 ± 0.2	0.5 ± 0.2	0.9 ± 0.1	0.4 ± 0.0
22:1 n-9	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.6 ± 0.0
ΣMUFA	**27.5**	**13.9**	**17.0**	**10.1**
18:2n-6	8.9 ± 0.3	2.3 ± 0.7	3.8 ± 0.2	1.8 ± 0.1
20:4n-6	2,2 ± 0,4	3.1 ± 0.3	3.1 ± 0.2	3.3 ± 0.1
Σn-6	**11.1**	**5.5**	**6.9**	**5.1**
18:3 n-3	2.1 ± 0.7	0.4 ± 0.3	1.1 ± 0.1	0.3 ± 0.0
18:4 n-3	0.7 ± 0.3	0.6 ± 0.1	nd	nd
20:5 n-3	5.3 ± 1.0	13.6 ± 1.2	11.0 ± 0.4	20.5 ± 0.4
22:5 n-3	0.5 ± 0.4	2.8 ± 0.2	2.2 ± 0.1	3.4 ± 0.2
22:6 n-3	30.9 ± 2.3	37.1 ± 1.8	36.4 ± 0.9	37.1 ± 0.6
Σn-3	**38.8**	**53.9**	**50.7**	**61.3**
Σn-6/Σn-3	**0.29**	**0.10**	**0.14**	**0.08**
EPA + DHA	**36.2**	**50.7**	**47.4**	**57.6**
ΣLc PUFA	**36.0**	**49.7**	**49.6**	**61.0**

nd; not detectable
Bold indicates the sum of the different fatty acids to highlight these numbers.

salmon has been determined and a concentration of EPA and DHA less than approximately 1% per kg feed is not recommended (Glencross, 2009; Rosenlund et al., 2016; Ruyter et al., 2000a; Sissener et al., 2016). The diets contained 20% fat and assuming that the fatty acids account for 60 to 65% of the lipids (Jensen et al., 2012) in the diet. The rapeseed oil diet (RO) contained less than 0.5% EPA and DHA per kg feed. However, the level of ALA (18:3 n-3) in this formulation was high. It is known that Atlantic salmon possess all enzymes necessary to convert 18:3 n-3 to EPA and DHA (Liland et al., 2013; Monroig et al., 2011; Thanuthong et al., 2011). Delta 6-desaturase, the rate limiting enzyme in conversion of C-18 PUFA to C-20-22 PUFA, is more expressed in Atlantic salmon in fresh water than in the saltwater phase and it is also higher in salmon on diets where fish oil is substituted with vegetable oil (Zheng et al., 2005). The fish in all our groups through the experiment showed no mortality and good growth. There were no significant differences in weight gain among the fish in the different diet groups demonstrating that the appetite was good and that the different oil combination in the feed did not affect the eating behavior of the fish. Menoyo et al. (2003) reported that high concentrations of LC-PUFA (25.2%) in the feed may inhibit growth of large Atlantic salmon in salt water. We did not however observe significant reduced growth when feeding a diet containing EPA/DHA concentrate and this may be due to the small size of the fish (50 to 150 g). This is in line with the results reported by Brodtkorb et al. (1997) who included 41.8% LC-PUFA in diets to juvenile salmon. The histology did show only minor or no sign of inflammation or other types of damage to the intestinal tissues in the hind gut demonstrating that the different oil blends in the feed was well tolerated by the salmon in our experiment. A study by Moldal et al. (2014) showed shorter folds in the mid intestine of salmon fed vegetable oil compared to a group fed fish oil. This finding might be associated with reduced intestinal surface and impaired nutrition absorption and growth, but their results did not demonstrate any major negative impact on the intestinal health of the salmon.

The differences in fatty acid composition in the blood and muscle are caused by the fatty acid composition of the feed and the relative amounts of PL and TAG present in the tissues. It is well established that the fatty acids composition of triacylglycerol reflects the fatty acid in composition present in the feed (Ruyter et al., 2006; Turchini et al., 2009). The high concentrations of DHA in the blood throughout the experimental period in all feeding groups independent of the concentration in the feed confirm that the main lipid class is phospholipids. These results are in line with several previously published works (Bell et al., 1993; Ruyter et al., 2000b; Thomassen et al., 2017) and demonstrate that this fatty acid is selectively retained in phospholipids even when only minor amounts are present in the feed. The amount of EPA on the other hand appeared much less regulated. When a relatively high concentration was present in the feed such as in RO/AO diet group (10%), AO diet group (13,5%) and EPA/DHA diet group (27%), the blood level seemed to reflect the level in the feed. However, when a small amount is in the feed as it is in the RO diet group (1.8%), blood concentration is mobilised to or retained at about 5% during the experimental period. A different saturation level of EPA in the blood cells dependent on feed formulation may be of great importance for the biological functions of EPA in blood cells of fish. In humans and mammals, low levels of EPA and DHA in the blood are a well-known a risk factor for development of heart disease (Harris, 2007). The reduced risk of heart disease and other disease in humans with high level of EPA/DHA in blood is very much related to production of eicosanoids and pro inflammatory signals from fatty acids present in the membrane of immunologically active blood cells (Conklin et al., 2007; Harris, 2007; von Schacky and Harris, 2007). It was therefore interesting to observe when very low levels of EPA was present in the feed this fatty acid was or mobilised to or retained at a higher level in the blood of the salmon.

We did not determine the fat content of the muscle, but it has been reported that fillets of juvenile farmed salmon of similar size fed a low fat diet contained 3–5% lipid suggesting that 70–80% of this is TAG (Bell et al., 2001; Jobling et al., 2002). However, we found that DHA was present in about 27% at the start of the experiment (day 0) although the content in Diet 0 was only 6%. This suggests that phospholipids are the main lipid class in the muscle at this point. During the feeding experiment, the concentration of DHA changed almost accordingly to level in the feed indicating that the amount of TAG increases in the muscle during the feeding experiment. A similar conclusion can be drawn from the changes in 20:5n-3, 18:2n-6, 18:n-3 and 18:1n-9 in the muscle during the feeding period reflecting that the fatty acid composition in TAG is determined by the fatty acid composition in the feed.

Of interest, the salmon in our experiment fed a diet with a mixture of 60% rapeseed oil and 40% anchoveta oil did not get a similar increase in LA as EPA in blood, despite high levels of LA in the feed. The diets containing AO and EPA/DHA had around 4.5% LA but this fatty acid decreased in the blood to 2–2.5% during the feeding experiments. This may indicate that LA is less mobilised to or retained in the phospholipids than EPA. It is known from studies in vegans that humans consuming high amounts of ALA or LA need at least 8 times as much EPA/DHA in the diet to reach the same level of EPA and DHA in the blood as a non-vegan (De Meester et al., 2013). We may see a similar tendency in the results presented in this paper or more simple that compared to the EPA, the LA is not incorporated in the red blood cell membrane.

To conclude, our study confirms that the amount of DHA is mobilised to or retained at a high level in the blood even when only small amounts are included in the feed. The level of EPA in the blood on the other hand reflected to a large degree the amount present in the diet. However, when only minor amounts were found in the feed it appeared that EPA is concentrated in the blood. The amounts of DHA and EPA in the muscle tissue during the experimental period reflected the concentrations found in the diets suggesting deposition of TAG in the muscle.

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.

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