

# Endogenous production of n-3 long-chain polyunsaturated fatty acids from first feeding and the influence of dietary linoleic acid and the $\alpha$ -linolenic:linoleic ratio in Atlantic salmon (*Salmo salar*)

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## **Abstract**

Atlantic salmon (*Salmo salar*) possess enzymes required for the endogenous biosynthesis of n-3 long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), from  $\alpha$ -linolenic acid (ALA). Linoleic acid (LA) competes with ALA for LC-PUFA biosynthesis enzymes leading to the production of n-6 LC-PUFA, including arachidonic acid (ARA). We aimed to quantify the endogenous production of EPA and DHA from ALA in salmon fed from first feeding on diets that contain no EPA and DHA, and to determine the influence of dietary LA and ALA:LA ratio on LC-PUFA production. Salmon were fed from first feeding for 22 weeks with three diets formulated with linseed and sunflower oils to provide ALA:LA ratios of approximately 3:1, 1:1 and 1:3. Endogenous production of n-3 LC-PUFA was 5.9, 4.4 and 2.8 mg per g fish and that of n-6 LC-PUFA was 0.2, 0.5 and 1.4 mg per g of fish in salmon fed diets with ALA:LA ratios of 3:1, 1:1 and 1:3, respectively. The ratio of n-3:n-6 LC-PUFA production decreased from 27.4 to 2.0, and DHA:EPA ratio increased and EPA:ARA and DHA:ARA ratios decreased, as dietary ALA:LA ratio decreased. In conclusion, with a dietary ALA:LA ratio of 1, salmon fry/parr produced around 28  $\mu$ g n-3 LC-PUFA per g of fish per day, with a DHA:EPA ratio of 3.4. Production of n-3 LC-PUFA exceeded that of n-6 LC-PUFA by almost 9-fold. Reducing the dietary ALA:LA ratio reduced n-3 LC-PUFA production, and EPA:ARA and DHA:ARA ratios, and increased n-6 LC-PUFA production, and DHA:EPA ratio.

## 1 Introduction

2 The omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic (EPA;  
3 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids, are recognised as being key essential nutrients  
4 in the human diet, providing a range of health benefits through their molecular, cellular and  
5 physiological actions<sup>(1,2)</sup>. While n-3 LC-PUFA have physiological functions in their own right as key  
6 components of cellular membranes<sup>(3)</sup> and regulators of gene expression<sup>(4)</sup>, many effects are dependent  
7 upon their antagonism of n-6 PUFA, especially arachidonic acid (ARA, 20:4n-6), metabolism<sup>(5)</sup>.  
8 Specifically, both EPA and DHA are precursors of eicosanoids and other highly biologically active  
9 derivatives that have important roles in blood homeostasis and the regulation of inflammation, which  
10 help to mitigate the pro-inflammatory effects of the high and imbalanced dietary n-6 PUFA content  
11 of the Western-type, industrialised diet<sup>(6-9)</sup>. Thus, dietary n-3 LC-PUFA are critical in promoting  
12 neural development and function<sup>(10-11)</sup>, and have beneficial impacts in several pathological conditions  
13 including cardiovascular disease, certain inflammatory diseases, and some cancers<sup>(12-15)</sup>.

14 Fish and seafood are the major dietary source of n-3 LC-PUFA with so called “oily” fish such as  
15 Atlantic salmon (*Salmo salar*) being one of the best in delivering a physiologically effective dose to  
16 human consumers<sup>(16-18)</sup>. This is largely due to the primary production of n-3 LC-PUFA being almost  
17 exclusively of aquatic origin and thus marine food chains are rich in EPA and DHA with fish  
18 accumulating them from their diet<sup>(19-21)</sup>. However, global fisheries are at maximum levels of  
19 exploitation, and production has stagnated for almost a quarter of a century, with the increasing  
20 demand of the burgeoning human population for fish and seafood being met by aquaculture<sup>(22)</sup>. While  
21 over 50 % of all fish and seafood is derived from aquaculture<sup>(22)</sup>, the n-3 LC-PUFA content of farmed  
22 produce could only be guaranteed by basing feeds on fishmeal (FM) and fish oil (FO), which are  
23 derived from marine fisheries that are also at sustainable limits<sup>(23)</sup>. Therefore, FO and FM are finite  
24 resources on an annual basis, and their supply would limit aquaculture growth if they were not  
25 increasingly replaced in aquafeeds by plant meals and vegetable oils (VO) derived from agriculture<sup>(24-</sup>  
26 <sup>26)</sup>. However, terrestrial plants do not produce LC-PUFA and, consequently, the use of VO in  
27 aquaculture has reduced the level of n-3 LC-PUFA in feeds with subsequent impacts on the levels of  
28 these nutrients in farmed fish<sup>(18,23)</sup>.

29 In contrast, VO can be rich sources of C<sub>18</sub> PUFA including both  $\alpha$ -linolenic acid (ALA; 18:3n-  
30 3) and linoleic acid (LA; 18:2n-6)<sup>(27)</sup>. Atlantic salmon possesses all the genes/enzymes required for  
31 the endogenous biosynthesis of both EPA and DHA from ALA<sup>(28,29)</sup>. Specifically, salmon have been  
32 shown to have *fads2* genes, coding for distinct desaturase proteins with separate  $\Delta$ 6 and  $\Delta$ 5 activities,  
33 and an *elovl5* elongase that are necessary for the production of EPA from ALA<sup>(30-32)</sup>. The  $\Delta$ 6 *fads2*  
34 desaturase along with *elovl2* and/or *elovl4* elongases that are also present enable the production of  
35 DHA from EPA<sup>(33-34)</sup>. Linoleic acid competes with ALA for the LC-PUFA biosynthesis pathway

36 enzymes leading to the production n-6 LC-PUFA, particularly ARA<sup>(19)</sup>. Although several studies have  
37 measured LC-PUFA biosynthesis in salmon fed diets with varying ALA and LA levels<sup>(35-38)</sup>, the  
38 precise impact of competing dietary LA and the dietary ALA:LA ratio on the endogenous production  
39 of EPA and/or DHA has not been quantified in Atlantic salmon. The primary aims of the present  
40 study were to quantify the endogenous production of EPA and DHA from ALA in salmon fed from  
41 first feeding on diets that contain no EPA and DHA, and to determine the influence of dietary LA  
42 and ALA:LA ratio on LC-PUFA production.

43

## 44 **Materials and Methods**

### 45 *Ethics statement*

46 All animal experimentation was conducted in compliance with the Animals Scientific Procedures Act  
47 1986 (Home Office Code of Practice. HMSO: London January 1997) and in accordance with EU  
48 regulation (EC Directive 86/609/EEC). The feeding trial was carried out in accordance with  
49 Norwegian national legislation via the Norwegian Animal Welfare Act (LOV-2015-06-09-19-65)  
50 Regulations on the Use of Animals in Experiments (FOR-2017-04-05-451) that was amended to  
51 implement the requirements contained in the European (EU) Regulations for the use of animals in  
52 scientific experimentation (Directive 2010/63/EU). Norway fully implemented the EU Directive  
53 within its legislature on 1 August 2016 via the European Economic Area Agreement. In addition, all  
54 experimentation performed by the Institute of Aquaculture, University of Stirling (UoS) is subjected  
55 to thorough ethical review carried out by the UoS Animal Welfare and Ethical Review Board  
56 (AWERB) prior to any work being approved. This involves all projects, irrespective of where they  
57 are carried out, to be submitted to AWERB for approval using detailed Ethical Approval forms that  
58 require all aspects of the experimentation to be described including all animal health and welfare  
59 issues as well as other ethical considerations. The present research was assessed by the UoS AWERB  
60 and passed the ethical review process of the University of Stirling.

61

### 62 *Fish, diets and feeding trial*

63 A triplicated feeding trial was run in Atlantic salmon (*Salmo salar*) from first-feeding (initial weight  
64 ~ 0.18 g) for 22 weeks at the Institute of Marine Research (IMR, Matre Research Station, Norway).  
65 Around 3000 Atlantic salmon eggs were sourced (Aquagen, Trondheim, Norway) and transferred to  
66 IMR, Matre, where the eggs were hatched and alevins maintained under standard rearing conditions.  
67 A series of three isoenergetic (digestible energy, DE = 18 MJ.Kg) and isoproteic (digestible protein,  
68 DP = 49 %; DP:DE, 27) diets were formulated and manufactured at the University of Stirling (Table  
69 1). The fishmeal and fish oil-free diets (A-C) contained 15 % lipid that was supplied by linseed and  
70 sunflower oils to provide three different ALA/LA ratios of approximately 3:1 (A), 1:1 (B) and 1:3

71 (C). The dry ingredients were ground, mixed with oil and water, and pelleted using a screw-press  
72 cold extruder (Dolly™, Imperia & Monferrina S.p.A, Moncalieri, Italy). Each diet was prepared as  
73 two batches of the same formulation, processed through either a 1 mm or 2 mm die. After drying  
74 overnight at 40 °C, the feeds were ground and sieved to produce pellet sizes of 0.5-0.8, 0.8-1.0, 1.0-  
75 1.5 and 1.5-2.0 mm. A further feed (REF) was included in the feeding trial as a reference to provide  
76 commercial context. The REF feed was a standard commercial feed used in the facility for rearing  
77 Atlantic salmon parr and contained primarily FM and FO. The analysed fatty acid compositions of  
78 all four feeds used in the trial are shown in Fig. 1. Analysed ALA/LA ratios were 2.61, 0.95 and 0.36  
79 in diets A, B and C, respectively. Prior to first feeding the alevins/fry were distributed into 4 tanks (1  
80 x 1 m) supplied with aerated freshwater with 750 fish per tank. In consideration of the fact that the  
81 experimental diets were fishmeal and fish oil-free, first feeding was performed in the single replicate  
82 tanks with higher fish density to ensure a good feeding response. During this initial 3-week phase,  
83 feeds were provided by hand so that feeding behaviour could be observed. At approximately 0.5 g,  
84 fish from each single tank were redistributed into 3 x 1 m diameter tanks (500L volume; 12 tanks in  
85 total) in a freshwater throughflow system with 200 alevins/fry per tank at a water temperature of 13  
86 °C and 24:0 LD. Fish were fed to excess by automatic feeders with pellet size increasing as fish size  
87 increased.

88

### 89 *Sampling*

90 At distribution of fish into tanks prior to first feeding, a triplicate sample of 50 - 60 alevins (~ 10 g  
91 wet weight) were sacrificed by anaesthetic overdose (tricaine methanesulfonate, MS-222; 400 mg.L<sup>-1</sup>  
92 in hydrogen carbonate-buffered solution) prior to being bulk weighed on an electronic top-loading  
93 balance to 0.1 g accuracy. The initial samples for compositional analyses were immediately frozen in  
94 liquid nitrogen and stored at -70 °C prior to analyses. At the end of the trial after 22 weeks feeding,  
95 40 fish per tank were sacrificed by anaesthetic overdose as above prior to being individually weighed  
96 on an electronic top-loading balance to 0.1 g accuracy. Twenty whole fish per tank were collected as  
97 two pooled samples of 10 fish and immediately frozen in liquid nitrogen and stored at -70 °C prior to  
98 analyses. Another 10 fish per tank were sampled for tissues as two pooled samples of 5 fish with  
99 samples immediately frozen in liquid nitrogen. Tissues collected were liver, intestine, flesh, gill, brain  
100 and eye. A further 10 fish per tank were sacrificed as above and liver collected into RNAlater™ as  
101 120 individual samples. These samples were stored overnight at 4 °C before freezing at -70 °C prior  
102 to RNA extraction and analysis of gene expression.

103

### 104 *Lipid and fatty acid analyses*

105 Total lipid was extracted from feeds, whole fish, and fish tissues by homogenising in  
106 chloroform/methanol (2:1, v/v) using an Ultra-Turrax tissue disrupter (Fisher Scientific,  
107 Loughborough, UK), and content determined gravimetrically<sup>(39)</sup>. Total lipids were resuspended in  
108 chloroform/methanol (2:1, v/v) at a concentration of 10 mg.ml<sup>-1</sup>. Total phospholipids (PL) and  
109 triacylglycerols (TAG) were prepared from total lipid by thin-layer chromatography on silica gel 60  
110 plates (20 × 20 cm; Merck KgaA, Darmstadt, Germany) and developed to full distance with  
111 isohexane/diethyl ether/acetic acid (85:15:1, by vol.)<sup>(40)</sup>. Lipid classes were visualised by spraying  
112 with 0.1 % (w/v) 2-7-dichlorofluorescein in 97 % aqueous methanol (v/v) and viewing under UV  
113 light at 240 nm (UVP® Mineralight® R-52G; UVP Inc., San Gabriel, California, USA). Identified  
114 classes (TAG and total PL/origin) were scraped into glass tubes and lipids eluted with  
115 isohexane/diethyl ether (1:1, v/v). Fatty acid methyl esters (FAME) were prepared from total lipid,  
116 total PL and TAG by acid-catalysed transesterification at 50 °C for 16 h<sup>(41)</sup>, and FAME extracted and  
117 purified as described previously<sup>(42)</sup>. FAME were separated and quantified by gas-liquid  
118 chromatography using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with a 30 m ×  
119 0.32 mm i.d. × 0.25 µm ZB-wax column (Phenomenex, Cheshire, UK), on-column injector and a  
120 flame ionisation detector. Hydrogen was used as carrier gas with the initial oven thermal gradient  
121 from 50 °C to 150 °C at 40 °C.min<sup>-1</sup> to a final temperature of 230 °C at 2 °C.min<sup>-1</sup>. Individual FAME  
122 were identified by comparison to known standards (Restek 20-FAME Marine Oil Standard; Thames  
123 Restek UK Ltd., Buckinghamshire, UK) and published data<sup>(42)</sup>. The data were collected and processed  
124 using Chromcard for Windows (Version 1.19; Thermoquest Italia S.p.A., Milan, Italy).

125 The within assay precision of this test is based on 6 replicates of the same sample, prepared and  
126 analysed at the same time on the same instrumentation, and found to be no greater than 5 % relative  
127 S.D. (RSD) across the 12 main fatty acids of interest. Where a difference of > 5 % RSD occurs  
128 between within assay replicates the analysis is repeated. Between assay precision is based on 6  
129 separate analyses of the same sample at separate time points and the difference across 12 fatty acids  
130 found to be no greater than 5 % RSD.

131

### 132 *Liver gene expression*

133 Total RNA was extracted from individual liver samples of 10 fish per tank by homogenising in 1 ml  
134 of TriReagent® following the producer's protocol (Sigma-Aldrich, Dorset, UK). The quantity and  
135 quality of RNA was determined by spectrophotometry (Nanodrop ND-1000; Labtech Int., East  
136 Sussex, UK) and RNA integrity assessed by agarose gel electrophoresis. cDNA was synthesised using  
137 a high capacity reverse transcription kit utilising 2 µg of total RNA and random primers in a total  
138 reaction volume of 20 µl following the manufacturer's protocol (Applied Biosystems, Warrington,

139 UK). The samples were pooled (2 samples of 5 fish per tank) to obtain n = 6 per dietary treatment. A  
140 dilution of 1:20 was applied to the resulting cDNA using milliQ water.

141 Expression levels of key genes involved in LC-PUFA biosynthesis pathways (*fads2d6*, delta-6 fatty  
142 acyl desaturase; *fads2d5*, delta-5 fatty acyl desaturase; *elovl2*, fatty acyl elongase 2; *elovl5a*, fatty  
143 acyl elongase 5 isoform a; *elovl5b*, fatty acyl elongase isoform b), lipid biosynthesis (*srebp1*, sterol  
144 regulatory element-binding protein 1; *srebp2*, sterol regulatory element-binding protein 2; *lxr*, liver  
145 X receptor; *fas*, fatty acid synthase; *hmgcr*, HMGCoA reductase) and lipid catabolism (*ppara*,  
146 peroxisome proliferator-activated receptor  $\alpha$ ; *pparg*, peroxisome proliferator-activated receptor  $\gamma$ ;  
147 *aco*, acyl Co-A oxidase; *cpt1*, carnitine palmitoyl transferase 1) were determined by real-time  
148 quantitative RTPCR in liver, as described in detail previously<sup>(43)</sup> (primers as detailed in  
149 Supplementary Table 1). Results were normalised using reference genes, hypoxanthine guanine  
150 phosphoribosyl transferase (*hpri*), elongation factor 1 alpha (*efl1a*) and TATA box binding protein  
151 (*tbp*) that were shown as the most stable according to GeNorm<sup>(44)</sup> stability number (M = 0.239 for  
152 *efl1a* and 0.213 for both *hpri* and *tbp*). Primers were designed using Primer 3 and were previously  
153 tested for efficiency, which was always over 0.80<sup>(45)</sup>. Quantitative PCR was performed using a  
154 Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in  
155 duplicate 20  $\mu$ l reaction volumes containing 10  $\mu$ l of Luminaris Color HiGreen qPCR Master Mix  
156 (Thermo Scientific), 1  $\mu$ l of primer corresponding to the analysed gene (10 pmol), 3  $\mu$ l of molecular  
157 biology grade water and 5  $\mu$ l of cDNA, with the exception of the reference genes, which were  
158 determined using 2  $\mu$ l of cDNA. In addition, amplifications were carried out with a systematic  
159 negative control (NTC-no template control) containing no cDNA. Standard amplification parameters  
160 contained an UDG pre-treatment at 50 °C for 2 min, an initial activation step at 95 °C for 10 min,  
161 followed by 35 cycles: 15 s at 95 °C, 30 s at the annealing T<sub>m</sub> and 30 s at 72 °C.

162

### 163 *Statistical analyses*

164 Based on extensive prior experience the hypothesised effect sizes of phenomic and transcriptomic  
165 responses were expected to be >1.5x SD. Using this basis, the experimental power of the design was  
166 calculated post-hoc using the “ANOVA: Fixed effects” test within the G-Power software  
167 (<http://www.gpower.hhu.de/>). Using an  $\alpha$ -value of 0.05 an average effect size of ~1.5x SD was  
168 applied for key response variables (e.g. growth, net LC-PUFA gain, and Log<sub>2</sub>FC). As an example, a  
169 power level (1- $\beta$ ) of 0.618 was determined for growth among diets A, B and C, whereas effect sizes  
170 of other parameters were larger (e.g. net LC-PUFA gain had a power level (1-  $\beta$ ) of 0.999).

171 For biochemical analyses, mean values were calculated for each tank prior to statistical analyses  
172 (n = 3). Significance of differences was determined by one-way ANOVA followed when appropriate  
173 by Tukey’s post-hoc test using GraphPad InStat, Version 3.01 32 bit for Win95/NT (GraphPad

174 Software Inc., San Diego, CA, USA). For gene expression ( $n = 6$ ), the relative expression levels (gene  
175 expression fold-change) of the target genes, normalised to the three housekeeping genes, were  
176 calculated following the method described by Pfaffl<sup>(46)</sup>. Data were tested for normality and  
177 homogeneity of variances with Levene's test prior to one-way analysis of variance (ANOVA)  
178 followed by Tukey's post-hoc test. The statistical analyses were performed using SPSS software  
179 (IBM SPSS Statistics 23; SPSS Inc., Chicago, IL, USA).

180

## 181 **Results**

182 After 22 weeks, salmon fed the diets with essentially no EPA and DHA were less than half the weight  
183 of fish fed the reference diet containing fish oil (Fig. 2). While the fish fed the highest content of LA  
184 had the lowest weight, there was no significant effect of ALA:LA ratio on growth after 22 weeks.  
185 Mortality after the initial feeding phase was very low throughout the trial and unrelated to feed.

186 The aim of the present study was to very specifically quantify endogenous production of EPA  
187 and DHA and not to quantify overall desaturation of ALA (and LA). Although 20:4n-3 satisfies our  
188 definition of LC-PUFA ( $\geq C20$  and  $\geq 3$  double bonds), it was not included in the calculations for total  
189 LC-PUFA production in the present study. Similarly, 18:3n-6 and 20:3n-6 were not included in the  
190 data for total n-6 LC-PUFA production. However, docosapentaenoic acid (DPA, 22:5n-3) was  
191 included along with EPA and DHA in total n-3 LC-PUFA, and 22:4n-6 and 22:5n-6 were included  
192 along with ARA in total n-6 PUFA (Figs. 3 & 4). Full fatty acid compositions of whole fish after 22  
193 weeks of being fed diets containing essentially no EPA and DHA are presented in Supplementary  
194 Table 2.

195 Salmon fed diets with ALA:LA ratios of 3:1, 1:1 and 1:3 contained 107, 80 and 41 mg of total n-  
196 3 LC-PUFA (EPA+DPA+DHA) per fish, almost all of which was the result of endogenous production  
197 as initial first feeding fry contained just under 1 mg total n-3 LC-PUFA per fish (Fig. 3). In the initial  
198 fry, DHA and EPA was present with a ratio of 2:1, whereas in fry fed an ALA:LA ratio of 3:1 DHA  
199 and EPA were in a ratio of over 2.6:1. This contrasted with the fish fed a standard commercial feed  
200 containing EPA and DHA, where they accumulated 757 mg of total n-3 LC-PUFA, with DHA and  
201 EPA in a ratio of 3.2:1 (Fig. 4). Fry fed the highest amount of LA (ALA:LA of 1:3) accumulated 21  
202 mg n-6 LC-PUFA, which compared to just over 28 mg in fish fed standard commercial feed, while  
203 initial fry contained only 0.07 mg of ARA (Fig. 5).

204 On a per fish basis, endogenous production of n-3 LC-PUFA was at least 5.9, 4.4 and 2.8 mg.g  
205 fish<sup>-1</sup>, and that of n-6 LC-PUFA was at least 0.2, 0.5 and 1.4 mg.g fish<sup>-1</sup> in salmon fed diets with  
206 ALA:LA ratios of 3:1, 1:1 and 1:3, respectively (Fig. 6). As daily (154 day trial) rates of production,  
207 these data corresponded to at least 38.1, 28.4 and 18.2  $\mu\text{g.g fish}^{-1}.\text{day}^{-1}$  for endogenous production of  
208 n-3 LC-PUFA, and at least 1.4, 3.4 and 9.3  $\mu\text{g.g fish}^{-1}.\text{day}^{-1}$  for n-6 LC-PUFA production in salmon

209 fed diets with ALA:LA ratios of 3:1, 1:1 and 1:3, respectively. The ratio of n-3 LC-PUFA production  
210 to n-6 LC-PUFA production decreased from 27.4 to 2.0 as dietary ALA:LA ratio decreased and,  
211 similarly, EPA:ARA and DHA:ARA ratios decreased as the dietary ratio of ALA:LA decreased. Less  
212 obviously, and more interestingly, the DHA:EPA ratio increased from 2.6 to 5.3 as the dietary ratio  
213 of ALA:LA decreased (Fig. 6).

214 The distribution of the endogenously produced LC-PUFA in specific lipid classes and tissues was  
215 also investigated. Comparing the proportion of fatty acids in total lipid and lipid classes, the  
216 percentages of n-3 LC-PUFA and n-6 PUFA were higher in total phospholipids (PL) than in  
217 triacylglycerols (TAG) (Fig. 7). To compare the distribution among tissues, the level of the particular  
218 fatty acid (DHA, EPA and ARA) in whole fish was compared with the levels in individual tissues.  
219 Setting the reference level as the amount of n-3 LC-PUFA in whole fish fed the diet containing  
220 ALA:LA at 3:1 (highest production), DHA was present at far higher levels in brain and also, to a  
221 lesser extent, liver (Fig. 8). In contrast, EPA was present in higher amounts in liver and, to a lesser  
222 extent, brain. With ARA, only liver showed a higher level than the reference, which was set as the  
223 level in whole fish fed the diet containing ALA:LA at 1:3 (highest ARA production) (Fig. 8).

224 Determination of the expression of key genes of LC-PUFA biosynthesis in liver by quantitative  
225 RTPCR showed that transcript abundances of  $\Delta 6$  (*fadsd6*) and  $\Delta 5$  (*fadsd5*) fatty acyl desaturases and  
226 elongase 2 (*elovl2*) were all significantly higher in fish fed the experimental feeds lacking LC-PUFA  
227 (Fig. 8). Although the differences were not statistically significant, expression levels of these genes  
228 were numerically lower in fish fed the diet with balanced (equal proportions) of ALA and LA. In  
229 contrast, diet did not affect the expression of *elovl5a*, and slightly reduced the expression of *elovl5b*  
230 (Fig. 9). The expression of some genes related to lipid anabolic pathways, *srebp1*, *srebp2* and *fas*,  
231 showed increasing expression with increasing dietary LA (i.e. as ALA:LA decreased), while some  
232 genes related to lipid catabolism, *ppara*, *pparg* and *aco*, showed the same pattern of expression among  
233 the three experimental feeds with the lowest expression in fish fed the diet with an ALA:LA ratio of  
234 1 (Fig. 9).

235

## 236 Discussion

237 Although the fish fed the low ALA:LA ratio had the numerically lowest final weight there were no  
238 significant differences between fish fed the experimental diets indicating that the dietary n-3 PUFA:n-  
239 6 PUFA ratio had little impact on growth. Of course, the fish fed the fishmeal- and fish oil-free feeds  
240 were significantly smaller than fish fed the REF feed, which was entirely expected. The REF feed  
241 was not a control feed in the present study, but simply a reference to place some of the data obtained  
242 into context. It has already been demonstrated that salmon post-smolts can be grown on essentially  
243 fishmeal and fish oil-free feeds without major impacts on growth using highly optimised feeds<sup>(47-48)</sup>.

244 This was not the aim of the present trial, which was not a nutritional trial in terms of growth and feed  
245 efficiency and was not testing ingredients, but was focussed entirely on nutrients and, specifically,  
246 LC-PUFA. The experimental feeds were therefore formulated to supply nutrients at currently reported  
247 requirement levels for Atlantic salmon<sup>(49)</sup>. In this respect, it is also important to clarify that the  
248 experimental feeds were not essential fatty acid (EFA)-deficient despite containing no EPA, DHA or  
249 ARA, as ALA and LA are reported to satisfy EFA requirements in salmonids (with complete LC-  
250 PUFA biosynthesis pathways) and the feeds contained ALA and LA at levels far above reported  
251 requirement levels<sup>(49)</sup>. Certainly, the fish fed the experimental feeds showed none of the classical EFA  
252 deficiency signs<sup>(50)</sup>. However, it is also known that LC-PUFA can supply EFA requirements at lower  
253 levels and so the lack of LC-PUFA may indeed be part of the reason for the lower growth  
254 obtained<sup>(51,52)</sup>. However, the commercial start feed, as with the feeds used in the fishmeal- and fish  
255 oil-free studies above, would undoubtedly be supplying many nutrients in excess of the minimal  
256 levels reported in NRC<sup>(49)</sup>. Thus, it was expected that the experimental feeds would impact growth  
257 and, hence, the reason to include the commercial feed (REF) to provide some context. Growth rate is  
258 linked to metabolic activity including intermediary metabolism and key anabolic pathways such as  
259 LC-PUFA biosynthesis and, therefore differences in growth rate could affect production rates per  
260 fish. However, growth rate is less likely to affect production per g of fish and so data presented this  
261 way should be unaffected by the differences in growth rate.

262 It had been shown previously that salmonids, Atlantic salmon and rainbow trout (*Oncorhynchus*  
263 *mykiss*), can be net producers of n-3 LC-PUFA by feeding diets with only low levels of fish oil<sup>(53,54)</sup>.  
264 While no method for assessing *in vivo* production of LC-PUFA is perfect<sup>(55)</sup>, production of EPA and  
265 DHA in these studies was calculated from total fatty acid intakes and final fatty acid contents in whole  
266 fish using “fatty acid production value, FAVP”<sup>(56)</sup> or the whole body mass balance method<sup>(57)</sup>. The  
267 present study used essentially similar methodology to these earlier studies except that the feeds were  
268 fed from first feeding and contained essentially no LC-PUFA. This simplified the calculations as,  
269 other than the very small amount of EPA, DHA and ARA in the alevins at first feeding, all the LC-  
270 PUFA present in the fish at the end of the trial was derived from endogenous biosynthesis. Feeding  
271 diets with no EPA and DHA from first feeding had been done previously with rainbow trout, but  
272 quantitative LC-PUFA production was not reported<sup>(58)</sup>.

273 The present study showed that in the trial period Atlantic salmon produced almost 6 mg n-3 LC-  
274 PUFA from ALA per g of fish ( $\sim 40 \mu\text{g}\cdot\text{g fish}^{-1}\cdot\text{day}^{-1}$ ). There are some caveats to this figure. Firstly,  
275 it should be regarded as a minimum amount, and cannot be lower, as it is highly likely that some LC-  
276 PUFA produced will be oxidised as demonstrated previously<sup>(54,57,59)</sup>. While endogenously produced  
277 LC-PUFA may be less oxidised than dietary fatty acids, there is no biochemical/physiological  
278 mechanism to prevent this oxidation<sup>(60)</sup>. Secondly, the data are limited to fry and the first half of the

279 parr (freshwater) stage and this could change over the entire life cycle. Biosynthesis of LC-PUFA  
280 from  $^{14}\text{C}$ -ALA was increased in hepatocytes of salmon undergoing parr-smolt transformation<sup>(61,62)</sup>  
281 and, therefore, this could increase the capacity for endogenous production. Therefore, existing data  
282 suggests 6 mg.g fish<sup>-1</sup> is a minimum for production during the freshwater stage that may increase  
283 prior to seawater transfer. There are few data on how the capacity for LC-PUFA biosynthesis is  
284 affected by age *per se* and, indeed, this is identified as a gap in the knowledge for humans<sup>(63)</sup>.  
285 However, there is evidence that the capacity for conversion of ALA to DHA is higher in human  
286 infants than adults<sup>(64)</sup>. In salmon, based on assays of LC-PUFA production in hepatocytes it has been  
287 speculated that activity may be lower in post-smolts in seawater<sup>(61,62)</sup>, but there are no *in vivo* studies  
288 to directly support this.

289 In humans, *in vivo* conversion of ALA to n-3 LC-PUFA has been estimated in a number of studies  
290 including stable isotope studies and is generally very modest, with some low conversion to EPA with  
291 conversion to DHA being even lower or not at all<sup>(63,65,66)</sup>. In fish, there are no previous studies that  
292 provide equivalent data with which to directly compare the present results. However, in an *in vivo*  
293 stable isotope study using 4–15 g rainbow trout fed 5 % fishmeal and 11 % vegetable oil, the rate of  
294 DHA production was reported to be low at 0.54  $\mu\text{g.g fish}^{-1}.\text{mg ALA consumed}^{-1}.\text{day}^{-1}$ <sup>(67)</sup>. This  
295 estimate is not directly comparable to the rate calculated in the present study. Extrapolating from data  
296 calculated via the whole body fatty acid mass balance method, total ALA conversion could be  
297 estimated at around 1.5  $\mu\text{g.g fish}^{-1}.\text{day}^{-1}$  when rainbow trout were fed a high ALA diet<sup>(68)</sup>. While the  
298 methodology used in the earlier trout trial was somewhat similar to that used in the present study, the  
299 data are not directly comparable as the diets were formulated with fishmeal and contained ~ 1.5 % n-  
300 3 LC-PUFA (EPA:DHA ~1), and the presence of dietary DHA would reduce  $\Delta 6$  desaturation of  
301 ALA<sup>(69,70)</sup>.

302 In humans, reducing dietary LA (increasing ALA:LA ratio) enabled increased conversion of ALA  
303 to EPA and, in some cases, also increased DHA<sup>(63,66)</sup>. The present study demonstrated how the dietary  
304 ALA:LA ratio quantitatively impacted the overall production of both n-3 and n-6 LC-PUFA in  
305 salmon. Thus, irrespective of dietary ALA:LA ratio, n-3 LC-PUFA production always exceeded n-6  
306 LC-PUFA production, being 27-fold greater when dietary ALA exceeded LA by 2.6-fold, and it was  
307 still 2-fold greater when fish were fed a diet with a 2.6-fold excess of LA. This demonstrates clearly  
308 the preferences of enzymes in the LC-PUFA biosynthesis pathway for n-3 compared to n-6 fatty acid  
309 substrates, confirming results from studies on enzyme activities determined with radiolabelled tracers  
310 in hepatocytes<sup>(61)</sup>, preferences of individual desaturase and elongase proteins in heterologous  
311 expression assays<sup>(71)</sup>, and by the whole body mass balance method<sup>(72)</sup>. While there are numerous  
312 studies investigating the effects of dietary ALA:LA ratio in fish, few have attempted to quantify  
313 impacts on LC-PUFA production. However, increasing dietary LA reduced n-3 LC-PUFA production

314 and increased n-6 LC-PUFA production in Murray cod (*Maccullochella peelii peelii*)<sup>(72)</sup>. In contrast,  
315 while increasing dietary LA had a significant impact in increasing n-6 LC-PUFA production in  
316 rainbow trout, it only marginally impacted n-3 LC-PUFA production<sup>(68)</sup>, reducing EPA production  
317 but with little effect on DHA production<sup>(73)</sup>. A further noteworthy result in the present study was that  
318 increased dietary LA (reduced ALA:LA ratio) also resulted in a reduction in overall LC-PUFA  
319 production. The overall rate of LC-PUFA production decreased from around  $\sim 39.5 \mu\text{g.g fish}^{-1}.\text{day}^{-1}$   
320 ( $38.1 \text{ n-3} + 1.4 \text{ n-6}$ ) in fish fed the high ALA:LA ratio to  $\sim 27.5 \mu\text{g.g fish}^{-1}.\text{day}^{-1}$  ( $18.2 + 9.3$ ) in fish  
321 fed the low ALA:LA ratio and, therefore, high dietary LA in effect inhibited the pathway. In contrast,  
322 this was not observed in rainbow trout, where total LC-PUFA production was largely unaffected by  
323 ALA:LA ratio<sup>(68)</sup>. Extrapolating from whole body fatty acid mass balance data,  $\sim 1.6 \mu\text{g.g fish}^{-1}.\text{day}^{-1}$   
324 ( $1.5 \text{ ALA} + 0.1 \text{ LA}$ ) to  $\sim 1.7 \mu\text{g.g fish}^{-1}.\text{day}^{-1}$  ( $1.4 + 0.3$ ) of C<sub>18</sub> PUFA were converted in fish fed  
325 diets with high and low ALA:LA ratio, respectively<sup>(68)</sup>.

326 The optimal dietary DHA:EPA ratio and its relationship to tissue DHA:EPA ratios presents a  
327 complicated situation and is poorly understood in salmon or, indeed, any species<sup>(59,74)</sup>. The present  
328 study aimed to provide some insight to the optimal dietary ratio of DHA:EPA by extrapolating from  
329 the ratio actually produced endogenously in salmon when receiving no dietary input of preformed  
330 EPA and DHA. One initial point to note is that the level of DHA in whole fish always exceeded that  
331 of EPA and so the DHA:EPA ratio was always  $>1$  in the present study. This was also the case in the  
332 earlier study in rainbow trout fed a diet completely devoid of EPA and DHA (and an ALA:LA ratio  
333 of around 1), where DHA:EPA ratio in both polar and neutral lipids in carcass, liver and intestine  
334 varied between 1.3 in carcass neutral lipid to 4.1 in liver polar lipids<sup>(58)</sup>. However, interestingly, the  
335 present study demonstrated that the DHA:EPA ratio produced through endogenous production of  
336 EPA and DHA was not fixed, but affected by the dietary ALA:LA ratio and, thus, as dietary LA  
337 increased, the DHA:EPA ratio also increased. While this is not something that had been highlighted  
338 previously, careful examination of existing literature has shown that increased dietary LA (i.e.  
339 reduced ALA:LA ratio) generally resulted in increased DHA:EPA ratios in whole fish and/or tissues  
340 of a range of freshwater and salmonid species<sup>(68,73,75-81)</sup>, although not always<sup>(72,82,83)</sup>. It must be  
341 stressed that the above results are not directly comparable with the present study as the fish in these  
342 earlier trials were all fed diets that also contained EPA and DHA, complicating interpretation of the  
343 data. However, a likely possible mechanism for the impact of dietary LA on DHA:EPA ratio is that  
344 increased LA would increase competition with ALA for the LC-PUFA pathway ( $\Delta 6$  desaturase) and  
345 this could particularly impact production of EPA, but not the production of DHA from EPA to the  
346 same extent. This explanation reflects the chemistry/biochemistry and enzyme kinetics of the  
347 biosynthesis pathways in salmon<sup>(60,71)</sup>, but an alternative explanation is that the result could possibly  
348 reflect a physiological driver with DHA being “more essential” than EPA for membrane structure

349 and function<sup>(59)</sup>. In rats, the DHA:EPA ratio is highly dependent upon tissue with EPA > DHA in  
350 blood and liver, but DHA greatly exceeding EPA in heart and brain, and dietary ALA and ALA:LA  
351 ratio (no dietary EPA and DHA) had relatively little impact on tissue DHA:EPA ratios<sup>(84)</sup>.

352 While the above discussed quantitative and semi-quantitative data were the primary focus, the  
353 present study also provided some additional insight into the fates of endogenously produced LC-  
354 PUFA. Irrespective of diet, the relative proportions of total LC-PUFA, EPA, DHA and ARA were all  
355 higher in total phospholipids than in triacylglycerols as would be expected<sup>(60)</sup>. However, this pattern  
356 was greatly enhanced in fish fed the experimental feeds compared to fish fed the reference diet, clearly  
357 indicating that endogenously produced EPA, DHA and ARA were highly preferentially incorporated  
358 into membrane phospholipids whereas greater proportions of dietary LC-PUFA are deposited in  
359 storage lipid. To discriminate tissue preferences for the deposition of endogenously produced LC-  
360 PUFA, we compared the contents of individual tissues with the content in whole fish. Thus, tissues  
361 showing higher contents per unit mass than whole fish indicates preferential deposition. This was  
362 clearly observed in the high DHA content of brain, which reflected the known importance of DHA  
363 in that tissue<sup>(60)</sup>. The only other tissue that showed higher contents of LC-PUFA was liver, likely  
364 reflecting its key metabolic role in biosynthesis<sup>(28,60)</sup>. Gene expression confirmed the active role of  
365 liver in the biosynthesis of LC-PUFA with the expression of  $\Delta 6$  and  $\Delta 5$  fatty acyl desaturases and  
366 *elovl2* elongase all being upregulated in fish fed the experimental feeds devoid of EPA and DHA,  
367 consistent with many previous studies in salmon<sup>(32,71)</sup>. However, dietary ALA:LA ratio appeared to  
368 have little effect suggesting that substrate levels were not a major driver of expression of these  
369 genes<sup>(38)</sup>. Similarly, there was no effect of dietary ALA:LA ratio on the expression of  $\Delta 6$  desaturase  
370 and *elovl5* elongase in liver of rainbow trout<sup>(68)</sup>. Dietary ALA:LA ratio affected the expression of  $\Delta 6$   
371 and  $\Delta 4$  desaturases and *elovl5* elongase in the marine rabbitfish (*Siganus canaliculatus*), which has  
372 all the genes necessary for biosynthesis of EPA and DHA, but in a variable manner with no clear  
373 pattern<sup>(81)</sup>. Interestingly, in the present study, brain showed relatively high contents of EPA in  
374 addition to DHA, which may indicate *in situ* production of DHA in brain. However, it is not possible  
375 to discriminate what proportion, if any, of brain DHA is actually the result of in-tissue production or  
376 simply deposition of DHA produced elsewhere, such as liver<sup>(28,60)</sup>. Furthermore, the fact that the high  
377 content of DHA in brain was not also reflected in ARA content of brain was surprising<sup>(60)</sup>.

378 The increased expression of the lipid anabolic genes, *srebl1*, *sreb2* and *fas*, as dietary ALA:LA  
379 decreased was also unexpected. It is worth noting that the effect of diet on gene expression can be  
380 looked at in two ways. Either that high dietary ALA and high ALA:LA ratio resulted in lower  
381 expression, or that high dietary LA and low ALA:LA ratio increased expression. Irrespective, the  
382 dietary effect does not have an obvious biochemical or physiological explanation. Similarly, while it  
383 was also interesting that genes related to lipid catabolism, *ppara*, *pparg* and *aco*, all showed a similar

384 pattern of expression among the three experimental feeds, with lowest expression at a dietary  
385 ALA:LA ratio of 1, the pattern was not readily explained. While it is perhaps noteworthy that the  
386 expression of the  $\Delta 6$  and  $\Delta 5$  fatty acyl desaturases and *elovl2* elongase also showed the same  
387 expression pattern as for the lipid catabolism genes, it does not offer an explanation. Therefore, the  
388 precise mechanism(s) underlying how dietary ALA:LA ratio affected the expression of lipid anabolic  
389 and catabolic genes was not clear and requires further study.

390 The data obtained in the present study have provided a better understanding of the capacity for  
391 LC-PUFA production in a vertebrate, Atlantic salmon, that has a complete biosynthetic pathway (via  
392 the Sprecher shunt), and how dietary ALA and LA interact and affect LC-PUFA production. Salmon  
393 farming is currently still largely dependent upon fish oil for provision of EPA and DHA, but levels  
394 of fish oil in feeds continue to decline<sup>(85)</sup> resulting in reduced levels of EPA and DHA in farmed salmon  
395 products<sup>(18)</sup>. Presently, around 70 % of oil in salmon feeds is supplied by vegetable oils that can only  
396 supply C<sub>18</sub> PUFA, LA and ALA<sup>(27)</sup> and, although there are potential new algal and GM-derived  
397 sources of EPA and DHA on the horizon, it is uncertain exactly how these will be used<sup>(86)</sup>. Currently,  
398 cost and availability restrict the use of these new sources, for the medium term at least, to being fish  
399 oil replacements to maintain EPA and DHA at current levels, and not as vegetable oil replacements  
400 and, therefore, salmon feeds will continue to contain high levels of vegetable oils. However, there are  
401 many options with oils containing differing proportions of ALA and LA, and the data from the present  
402 study enables the possibility to more precisely control dietary ALA and LA to maximise endogenous  
403 production of EPA and DHA.

404

## 405 **Conclusions**

406 With a dietary n-3/n-6 PUFA ratio of 1:1, Atlantic salmon fry/parr can produce at least 4.4 mg n-3  
407 LC-PUFA, with a DHA:EPA ratio of 3.4:1, per gram of fish. Production of n-3 LC-PUFA exceeded  
408 that of n-6 LC-PUFA by almost 9-fold. Reducing the dietary n-3/n-6 PUFA ratio reduced n-3 LC-  
409 PUFA production, and EPA:ARA and DHA:ARA ratios, and increased n-6 LC-PUFA production,  
410 and DHA:EPA ratio. The data advance nutritional science by providing insight and a clearer  
411 understanding of the quantitative capacity of the pathway in a vertebrate that has complete LC-PUFA  
412 biosynthesis pathways (via the Sprecher shunt), and how dietary ALA and LA interact and  
413 quantitatively affect LC-PUFA production.

414

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422

#### 423 **Conflict of Interest**

424 None

425

#### 426 **Authorship**

427 DRT conceived and designed the study and drafted the initial manuscript. BDG formulated and  
428 manufactured the feeds. REO and OT managed and supervised the salmon feeding trial in Norway.  
429 MBB, BDG, MS and REO performed all sampling. MS and GX carried out all the lipid and fatty acid  
430 analyses. MBB performed the liver gene expression analyses. All authors contributed to and edited  
431 the manuscript.

432

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- 658

659 **Legends to Figures**

660

661 Fig. 1. Fatty acid compositions (percentage of total fatty acids) of experimental feeds (A, B & C) and  
662 the reference feed (REF).

663

664 Fig. 2. Final weights (g) of salmon after feeding the experimental and REF diets for 22 weeks (154  
665 days). Data represent means  $\pm$  S.D. (n = 3). Different superscript letters indicate significant  
666 differences between diets as determined by ANOVA followed by Tukey's multiple comparison test.

667

668 Fig. 3. The n-3 LC-PUFA contents (mg.fish<sup>-1</sup>) of fry at first feeding/initiation of the trial (Initial) and  
669 whole fish after feeding the experimental diets for 22 weeks (Final). Data represent means  $\pm$  S.D. (n  
670 = 3). Different superscript letters above columns for each fatty acid indicate significant differences  
671 between diets as determined by ANOVA followed by Tukey's multiple comparison test.

672

673 Fig. 4. The n-3 LC-PUFA contents (mg.fish<sup>-1</sup>) of whole fish fed the experimental diets (A, B & C)  
674 in comparison with fish fed the reference diet (REF) for 22 weeks. Data represent means  $\pm$  S.D. (n =  
675 3). Different superscript letters above columns for each fatty acid indicate significant differences  
676 between diets as determined by ANOVA followed by Tukey's multiple comparison test.

677

678 Fig. 5. The n-6 LC-PUFA contents (mg.fish<sup>-1</sup>) of fry at first feeding/initiation of the trial (Initial) and  
679 whole fish after feeding the experimental diets for 22 weeks (Final). Data represent means  $\pm$  S.D. (n  
680 = 3). Different superscript letters above columns for each fatty acid indicate significant differences  
681 between diets as determined by ANOVA followed by Tukey's multiple comparison test.

682

683 Fig. 6. Production of n-3 and n-6 LC-PUFA (mg.g fish<sup>-1</sup>) and n-3 LC-PUFA:n-6 LC-PUFA,  
684 DHA:EPA, EPA:ARA and DHA:ARA ratios in whole fish after feeding the experimental diets for  
685 22 weeks. Data represent means  $\pm$  S.D. (n = 3). Different superscript letters above columns for each  
686 parameter indicates significant differences between diets as determined by ANOVA followed by  
687 Tukey's multiple comparison test. ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA,  
688 eicosapentaenoic acid; LC-PUFA, long-chain polyunsaturated fatty acids.

689

690 Fig. 7. LC-PUFA composition (percentage of total fatty acids) of whole fish total lipid (TL),  
691 phospholipids (PL) and triacylglycerol (TAG). Data represent means  $\pm$  S.D. (n = 3). Different  
692 superscript letters above columns for each parameter indicate significant differences between diets in  
693 contents in TL, PL and TAG as determined by ANOVA followed by Tukey's multiple comparison

694 test. ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LC-PUFA,  
695 long-chain polyunsaturated fatty acids; Total, total n-3 LC-PUFA (UPPER panel) and total n-6 LC-  
696 PUFA (lower panel).

697

698 Fig. 8. Tissue contents (mg fatty acid.100g of whole fish or tissue<sup>-1</sup>) of EPA, DHA and ARA. Data  
699 represent means  $\pm$  S.D. (n = 3). Different superscript letters above columns for each tissue indicate  
700 significant differences between diets as determined by ANOVA followed by Tukey's multiple  
701 comparison test. The dotted line in the upper and middle panels represents the EPA and DHA  
702 contents, respectively, of whole fish in fish fed diet A that gave highest production of EPA and DHA.  
703 Similarly, the dotted line in the lower panel represents the ARA content of whole fish in fish fed diet  
704 C that gave highest production of ARA. ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA,  
705 eicosapentaenoic acid; LC-PUFA, long-chain polyunsaturated fatty acids.

706

707 Fig. 9. Relative expression (RE) of genes of LC-PUFA biosynthesis (upper panel), lipid anabolism  
708 (middle panel) and lipid catabolism (lower panel) in liver of Atlantic salmon as determined by qPCR.  
709 Results are normalised expression ratios (means  $\pm$  SEM; n = 6). Different superscript letters above  
710 columns for each gene denote differences between diets as identified by one-way ANOVA. *aco*, acyl  
711 co-A oxidase; *cpt1*, carnitine palmitoyl transferase 1; *elovl2*, fatty acyl elongase 2; *elovl5a*, fatty acyl  
712 elongase 5 isoform a; *elovl5b*, fatty acyl elongase 5 isoform b; *fads2d5*, delta-5 fatty acyl desaturase;  
713 *fads2d6*, delta-6 fatty acyl desaturase; *fas*, fatty acid synthase; *hmgcr*, HMGCoA reductase; *lxr*, liver  
714 X receptor; *ppara*, peroxisome proliferator-activated receptor  $\alpha$ ; *pparg*, peroxisome proliferator-  
715 activated receptor  $\gamma$ ; *srebp1*, sterol regulatory element-binding protein 1; *srebp2*, sterol regulatory  
716 element-binding protein 2.

717

Table 1. Formulations and analysed proximate compositions of experimental feeds

Ingredient	A (g/kg)	B (g/kg)	C (g/kg)
Soy protein concentrate <sup>a</sup>	200.0	200.0	200.0
Soy protein isolate <sup>b</sup>	200.0	200.0	200.0
Wheat gluten <sup>a</sup>	200.0	200.0	200.0
Hydrolysate <sup>c</sup>	20.0	20.0	20.0
Casein <sup>b</sup>	17.0	17.0	17.0
Linseed oil <sup>d</sup>	134.0	90.0	44.0
Sunflower oil <sup>e</sup>	0.0	44.0	90.0
Cellulose <sup>f</sup>	77.0	77.0	77.0
Pregelised starch <sup>g</sup>	70.0	70.0	70.0
Crystalline amino acids <sup>h</sup>	26.0	26.0	26.0
Premix <sup>i</sup>	5.0	5.0	5.0
CaHPO <sub>4</sub> <sup>a</sup>	50.0	50.0	50.0
Yttrium Oxide <sup>j</sup>	1.0	1.0	1.0
<u>Proximate composition</u>	(% dry weight)	(% dry weight)	(% dry weight)
Crude protein	50.8	49.5	50.5
Digestible protein	48.9	48.9	48.9
Crude lipid	15.7	15.5	13.8
Ash	7.3	7.1	7.8
Moisture	5.6	5.7	4.0
Digestible energy	18.0	18.1	18.1

<sup>a</sup> BioMar Ltd., Grangemouth, UK; <sup>b</sup> Bulk Powders Ltd., Colchester, UK;

<sup>c</sup> HP1 from Aquativ, Elven, France; <sup>d</sup> Cold-pressed, AniForte UK Ltd., London; <sup>e</sup> Sainsbury's Supermarkets Ltd., London, UK;

<sup>f</sup> Microcrystalline cellulose, Blackburn Distributions Ltd., Nelson, UK;

<sup>g</sup> Sigma Pharmaceuticals, Gillingham, UK;

<sup>h</sup> Includes methionine (10), lysine (10), taurine (5), and also choline (1);

<sup>i</sup> OVN Salmonid from DSM Nutritional Products, Basel, Switzerland;

<sup>j</sup> Stanford Materials, Lake Forrest, CA, USA.

718

719

Fig.1

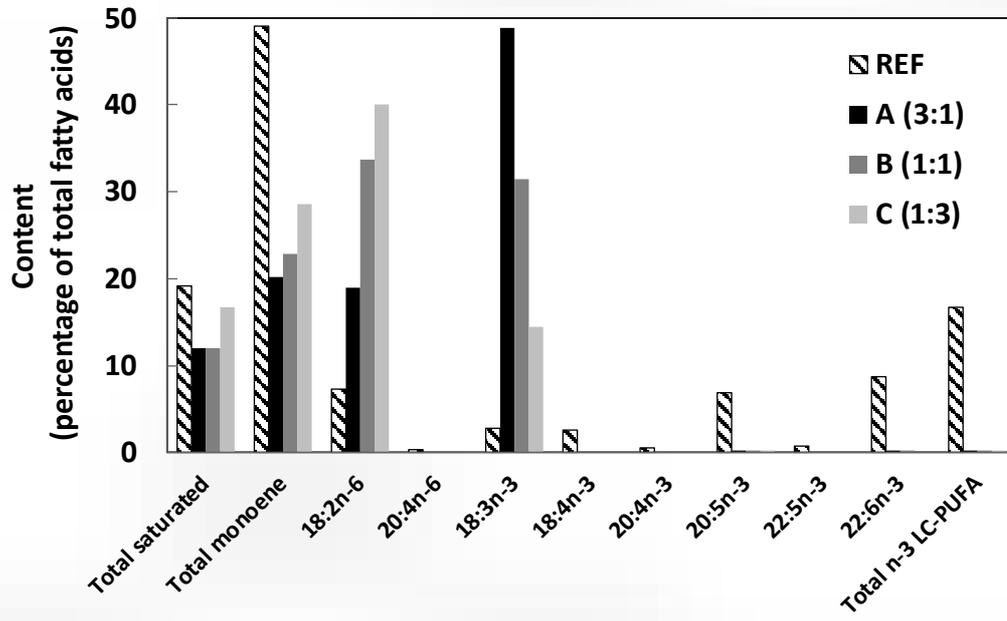
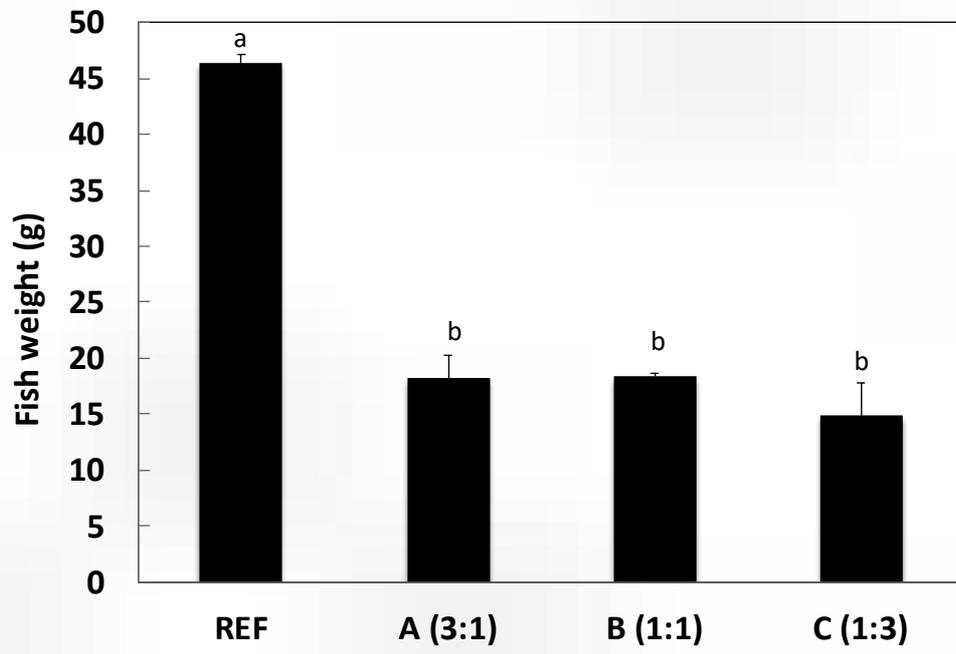
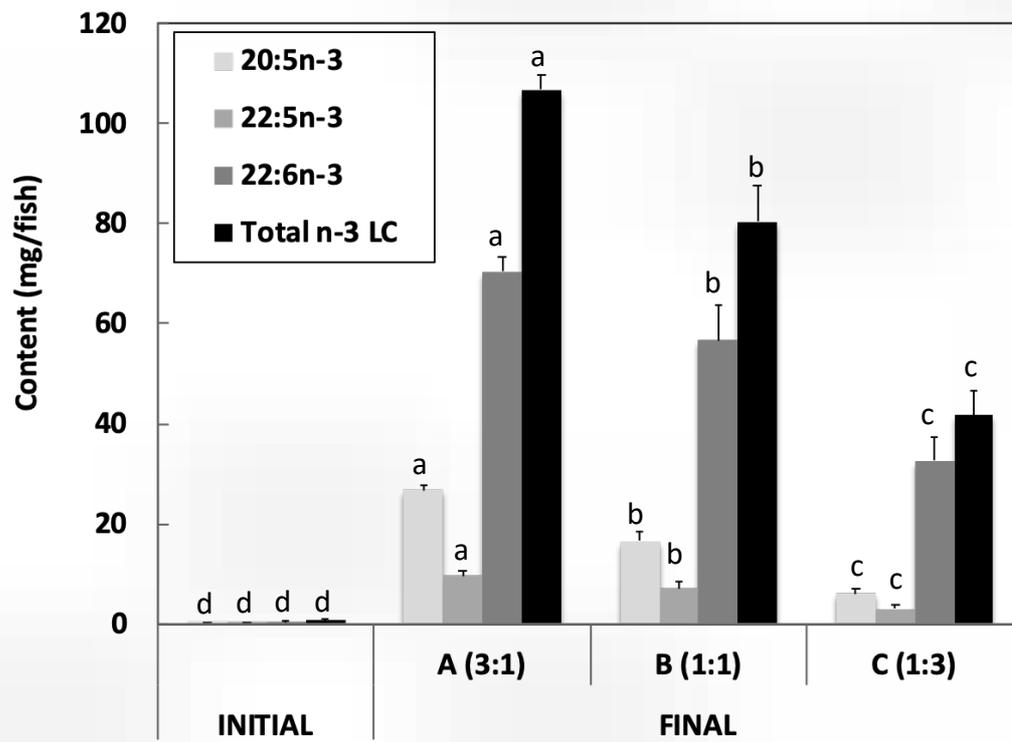


Fig. 2



721

Fig. 3



722

Fig.4

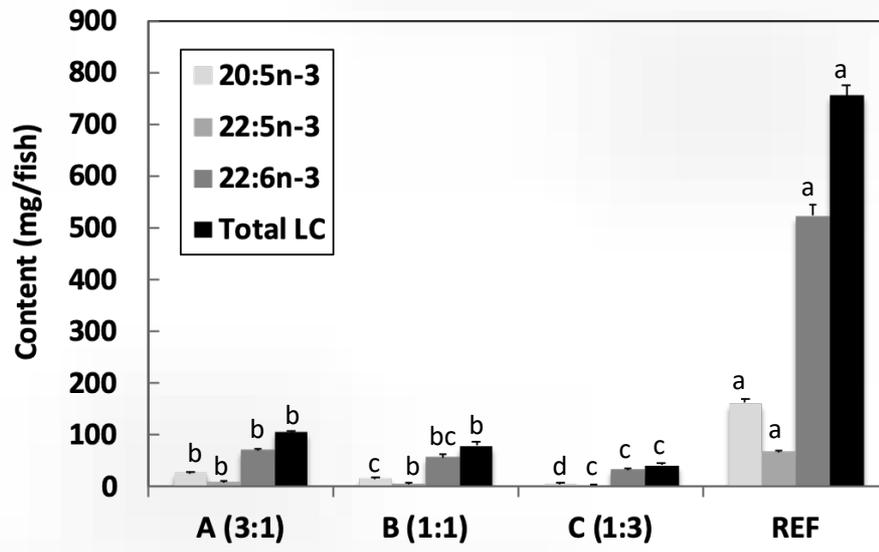


Fig. 5

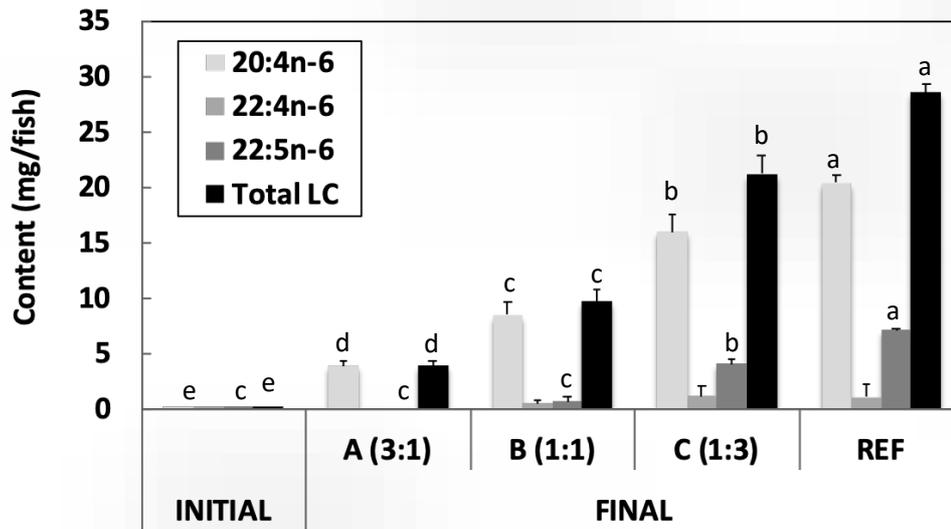
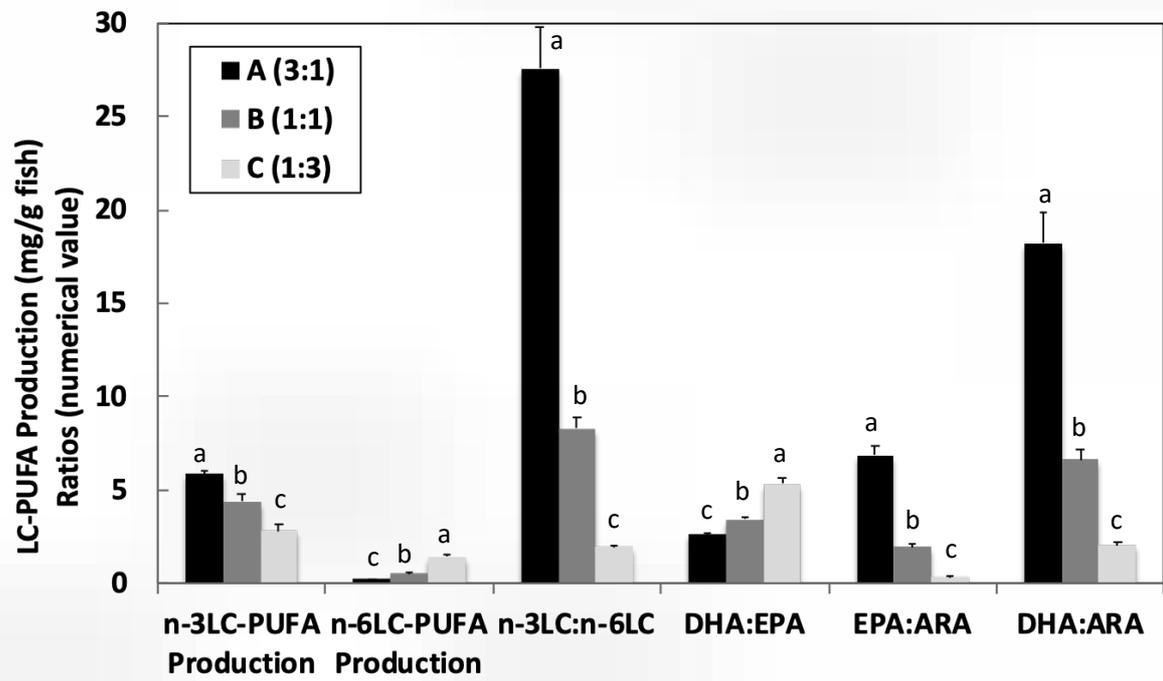
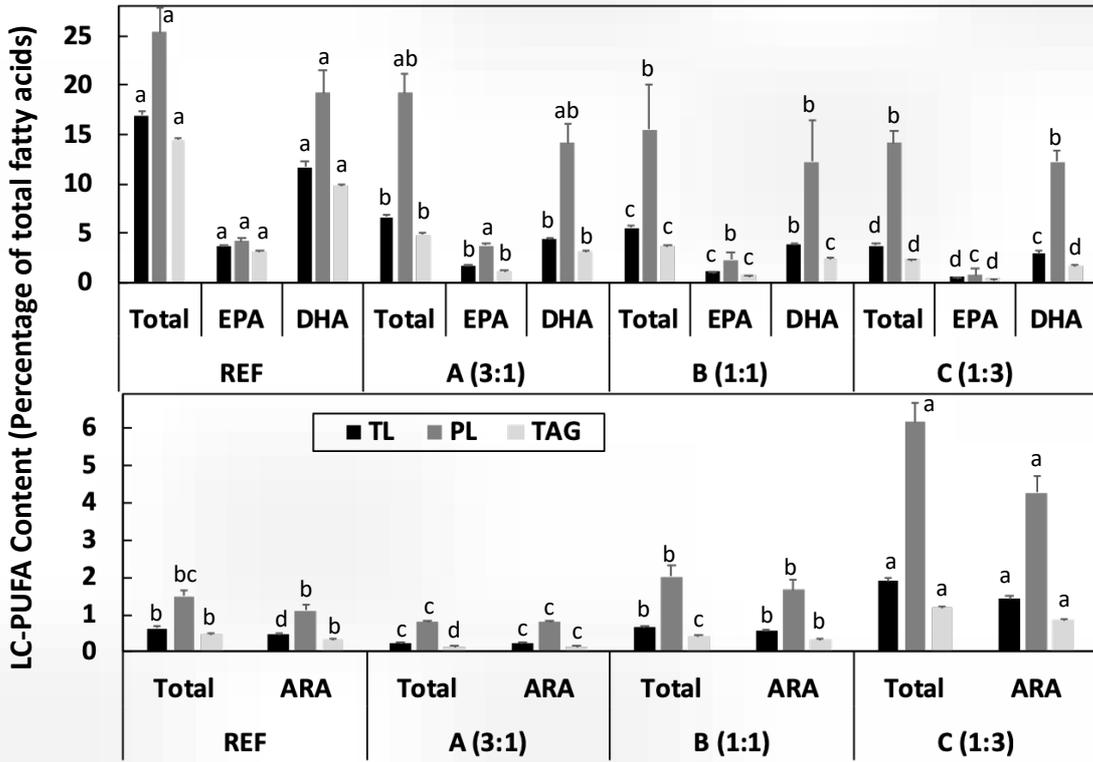


Fig. 6



725

Fig. 7



726

Fig. 8

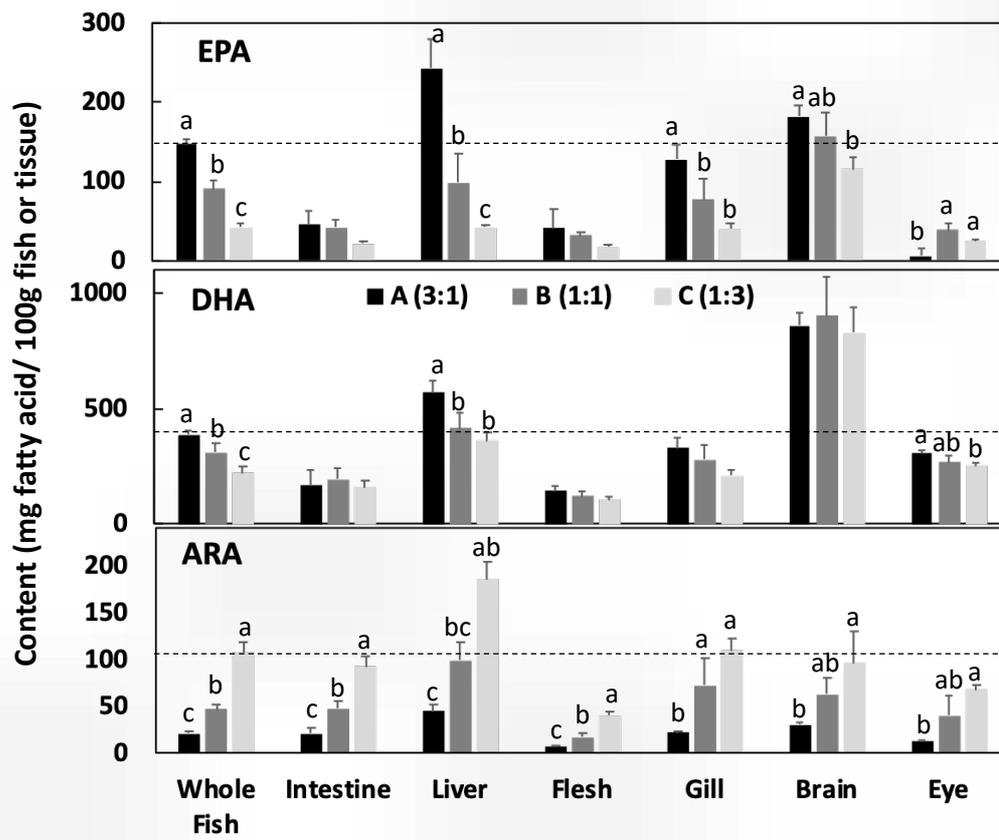
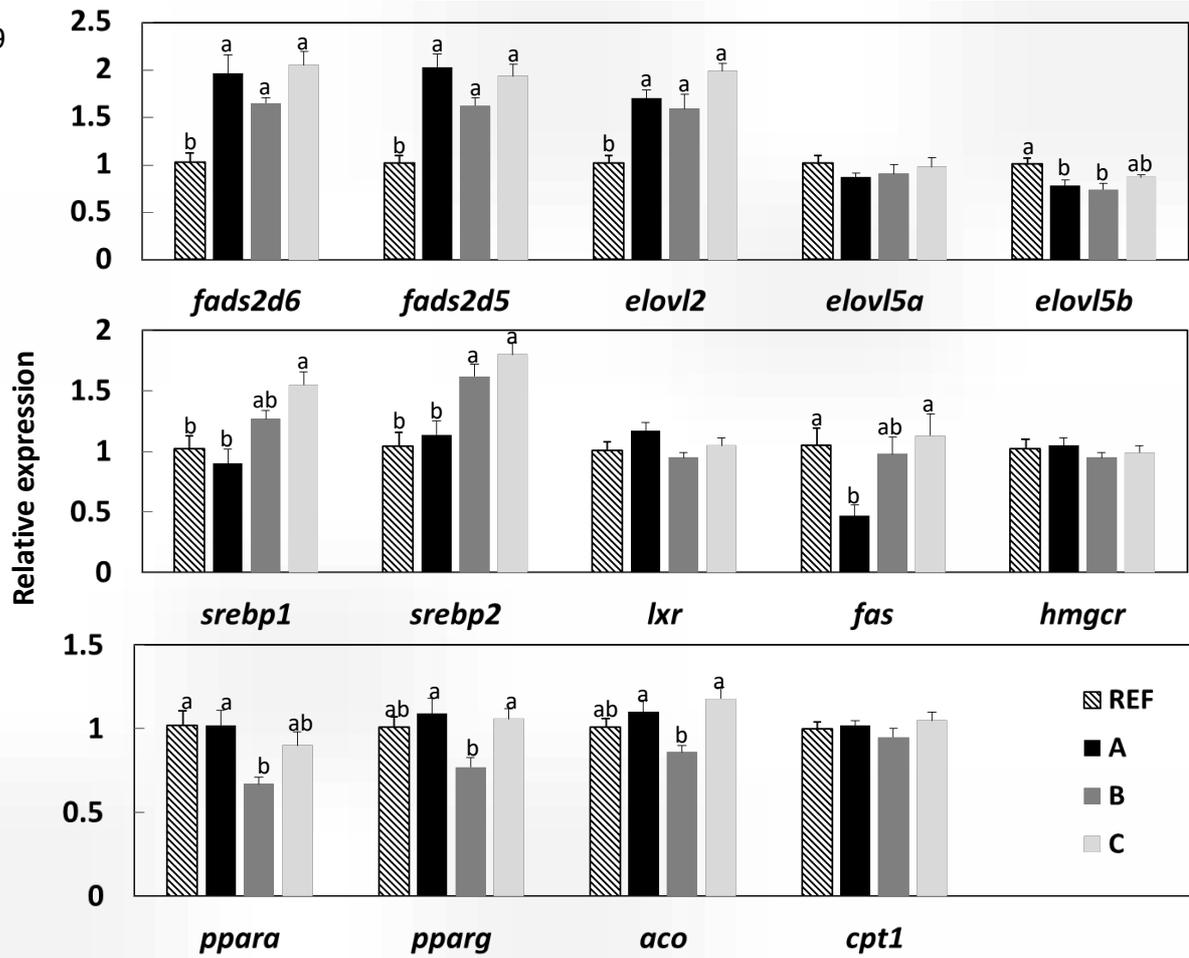


Fig. 9



728

729 Supplementary Table 1. PCR primers used in the present study for real-time quantitative PCR  
 730 (qPCR).

Transcript	Primer sequence (5'→3')	Amplicon (bp)	Ta	Accession no
<i>fads2d6</i>	F: TCCTCTGGTGCGTACTTTGT	163	59° C	NM_001123575.2 <sup>a</sup>
	R: AAATCCCGTCCAGAGTCAGG			
<i>fads2d5</i>	F: GCCACTGGTTTGTATGGGTG	148	59° C	NM_001123542.2 <sup>a</sup>
	R: TTGAGGTGTCCACTGAACCA			
<i>elovl2</i>	F: GGTGCTGTGGTGGTACTACT	190	59° C	NM_001136553.1 <sup>a</sup>
	R: ACTGTTAAGAGTCGGCCCAA			
<i>elovl5a</i>	F: TGTTGCTTCATTGAATGGCCA	150	59° C	GU238431.1 <sup>a</sup>
	R: TCCCATCTCTCCTAGCGACA			
<i>elovl5b</i>	F: CTGTGCAGTCATTTGGCCAT	192	59° C	NM_001136552.1 <sup>a</sup>
	R: GGTGTCACCCCATTTGCATG			
<i>srebp1</i>	F: GCCATGCGCAGGTTGTTTCTTCA	151	63°	TC148424 <sup>b</sup>
	R: TCTGGCCAGGACGCATCTCACACT			
<i>srebp2</i>	F: TCGCGGCCTCCTGATGATT	147	63°	TC166313 <sup>b</sup>
	R: AGGGCTAGGTGACTGTTCTGG			
<i>lxr</i>	F: GCCGCCGCTATCTGAAATCTG	210	58°	FJ470290 <sup>a</sup>
	R: CAATCCGGCAACCAATCTGTAGG			
<i>ppara</i>	F: TCCTGGTGGCCTACGGATC	111	60°	DQ294237 <sup>a</sup>
	R: CGTTGAATTTTCATGGCGAACT			
<i>ppary</i>	F: CATTGTCAGCCTGTCCAGAC	144	60°	AJ416951 <sup>a</sup>
	R: TTGCAGCCCTCACAGACATG			
<i>aco</i>	F: AAAGCCTTCACCACATGGAC	230	60°	TC49531 <sup>b</sup>
	R: TAGGACACGATGCCACTCAG			
<i>cptI</i>	F: CCTGTACCGTGGAGACCTGT	212	60°	AM230810 <sup>a</sup>
	R: CAGCACCTCTTTGAGGAAGG			
<i>hmgcr</i>	F: CCTTCAGCCATGAACTGGAT	224	60°	DW561983 <sup>a</sup>
	R: TCCTGTCCACAGGCAATGTA			
<i>fas</i>	F: ACCGCCAAGCTCAGTGTGC	212	60°	CK876943 <sup>a</sup>
	R: CAGGCCCAAAGGAGTAGC			
<i>hpert</i>	F: GATGATGAGCAGGGATATGAC	165	60°	BT125296.1
	R: GCAGAGAGCCACGATATGG			
<i>efl1a</i>	F: CTGCCCTCCAGGACGTTTACAA	175	60° C	AF321836 <sup>a</sup>
	R: CACCGGGCATAGCCGATTCC			
<i>rpl2</i>	F: CTGCCCTCCAGGACGTTTACAA	112	60° C	XM_014137227.1 <sup>a</sup>
	R: TGTTACAGCTCGTTTACCG			
<i>tbp</i>	F: TCCCAACCTGTGACGAACA	117	60° C	NM_001176374.1 <sup>a</sup>
	R: GTCTGTCCTGAGCCCCCTGA			

731  
 732 *fads2d6*, delta-6 fatty acyl desaturase; *fads2d5*, delta-5 fatty acyl desaturase; *elovl2*, fatty acyl  
 733 elongase 2; *elovl5a*, fatty acyl elongase 5 isoform a; *elovl5b*, fatty acyl elongase isoform b; *srebp1*,  
 734 sterol regulatory element binding protein 1; *srebp2*, sterol regulatory element binding protein 2; *lxr*,  
 735 liver X receptor; *ppara*, peroxisome proliferator-activated receptor alpha; *ppary*, peroxisome  
 736 proliferator-activated receptor gamma; *aco*, acyl-CoA oxidase; *cptI*, carnitine palmitoyltransferase  
 737 I; *hmgcr*, 3-hydroxy-3-methyl-glutaryl-CoA reductase; *hpert*, hypoxanthine-guanine  
 738 phosphoribosyltransferase; *efl1a*, elongation factor 1 alpha; *rpl2*, ribosomal protein L2; *tbp*, TATA  
 739 box binding protein.

740 <sup>a</sup>GenBank (<http://www.ncbi.nlm.nih.gov/>)

741 <sup>b</sup>Atlantic salmon Gene Index (<http://compbio.dfci.harvard.edu/tgi/>)

742

743

**Supplementary Table 2.** Proximate and fatty acid compositions of whole fish after feeding the diets for 22 weeks (154 days) from first feeding. Total saturated fatty acids includes 15:0, 20:0, 22:0 and 24:0 present in some samples at up to 0.4%. Total monoenes contains 16:1n-7, 20:1n-7, 20:1n-11, 22:1n-9 and 24:1n-9 present in some samples at up to 0.6%.

	REF	DIET A	DIET B	DIET C
<b>Proximate composition (% of wet weight)</b>				
Lipid	11.8 ± 0.4	10.4 ± 0.1	8.9 ± 1.0	8.9 ± 0.8
Protein	15.3 ± 0.3	14.3 ± 0.5	15.4 ± 0.3	15.0 ± 0.5
Ash	2.1 ± 0.1	2.2 ± 0.1	2.1 ± 0.2	2.3 ± 0.1
Moisture	68.9 ± 0.6	70.5 ± 0.3	70.7 ± 0.7	71.3 ± 0.5
<b>Fatty acid composition (% of total fatty acids)</b>				
14:0	4.6 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
16:0	14.7 ± 0.4	9.6 ± 0.2	10.0 ± 0.3	10.6 ± 0.3
18:0	3.5 ± 0.1	5.2 ± 0.1	5.5 ± 0.2	5.3 ± 0.2
<b>Total saturated</b>	<b>23.5 ± 0.5</b>	<b>15.4 ± 0.2</b>	<b>16.3 ± 0.4</b>	<b>16.9 ± 0.6</b>
16:1n-7	4.9 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
18:1n-9	21.7 ± 0.4	21.2 ± 0.3	22.2 ± 0.4	24.2 ± 0.3
18:1n-7	3.0 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.8 ± 0.1
20:1n-9	6.0 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	0.7 ± 0.1
22:1n-11	6.9 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
<b>Total monoenes</b>	<b>44.9 ± 0.4</b>	<b>23.5 ± 0.3</b>	<b>24.5 ± 0.4</b>	<b>26.5 ± 0.4</b>
18:2n-6	7.2 ± 0.1	15.3 ± 0.2	23.5 ± 0.4	32.2 ± 0.3
18:3n-6	0.2 ± 0.0	0.9 ± 0.1	1.7 ± 0.1	3.3 ± 0.2
20:2n-6	0.6 ± 0.0	0.7 ± 0.0	1.1 ± 0.1	1.6 ± 0.1
20:3n-6	0.2 ± 0.0	0.8 ± 0.0	1.6 ± 0.1	2.7 ± 0.1
20:4n-6	0.5 ± 0.0	0.2 ± 0.0	0.6 ± 0.0	1.4 ± 0.1
22:4n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
22:5n-6	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0
<b>Total n-6 PUFA</b>	<b>8.9 ± 0.1</b>	<b>17.9 ± 0.1</b>	<b>28.5 ± 0.5</b>	<b>41.7 ± 0.5</b>
18:3n-3	2.0 ± 0.0	25.0 ± 0.4	17.1 ± 0.2	7.1 ± 0.1
18:4n-3	1.5 ± 0.0	8.0 ± 0.4	5.6 ± 0.2	3.3 ± 0.2
20:3n-3	0.2 ± 0.0	1.2 ± 0.1	0.8 ± 0.0	0.3 ± 0.0
20:4n-3	0.9 ± 0.0	2.3 ± 0.1	1.5 ± 0.1	0.6 ± 0.0
20:5n-3	3.6 ± 0.1	1.7 ± 0.1	1.2 ± 0.0	0.5 ± 0.0
21:5n-3	0.3 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n-3	1.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.3 ± 0.0
22:6n-3	11.7 ± 0.5	4.4 ± 0.2	3.9 ± 0.1	2.9 ± 0.3
<b>Total n-3 PUFA</b>	<b>21.8 ± 0.8</b>	<b>43.2 ± 0.2</b>	<b>30.6 ± 0.4</b>	<b>15.0 ± 0.5</b>
<b>C16 PUFA</b>	<b>0.9 ± 0.0</b>	<b>0.0 ± 0.0</b>	<b>0.0 ± 0.0</b>	<b>0.0 ± 0.0</b>
<b>Total PUFA</b>	<b>31.6 ± 0.9</b>	<b>61.1 ± 0.2</b>	<b>59.1 ± 0.8</b>	<b>56.7 ± 0.9</b>
<b>n-3/n-6</b>	<b>2.4</b>	<b>2.4</b>	<b>1.1</b>	<b>0.4</b>
<b>Fatty acid composition (mg/100g fish)</b>				
14:0	445.0 ± 37.6	30.4 ± 1.9	28.1 ± 4.5	27.5 ± 5.2
16:0	1427.4 ± 144.2	843.3 ± 35.7	790.6 ± 107.6	800.8 ± 103.6
18:0	340.4 ± 37.7	459.5 ± 22.5	432.0 ± 60.3	402.3 ± 51.8
<b>Total saturated</b>	<b>2277.2 ± 224.1</b>	<b>1362.1 ± 55.9</b>	<b>1285.7 ± 175.0</b>	<b>1276.1 ± 165.1</b>
16:1n-7	478.0 ± 42.1	41.4 ± 1.2	30.4 ± 6.8	26.4 ± 5.6
18:1n-9	2101.1 ± 198.9	1869.3 ± 85.8	1747.7 ± 211.2	1833.3 ± 233.9
18:1n-7	291.9 ± 20.1	81.7 ± 2.8	70.7 ± 8.2	62.9 ± 5.7
20:1n-9	576.2 ± 48.4	44.7 ± 2.8	45.3 ± 7.7	50.3 ± 9.1
22:1n-11	666.9 ± 53.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
<b>Total monoenes</b>	<b>4342.6 ± 365.3</b>	<b>2071.6 ± 85.9</b>	<b>1928.3 ± 235.5</b>	<b>2004.1 ± 256.9</b>
18:2n-6	696.1 ± 46.6	1347.4 ± 62.3	1842.3 ± 182.2	2433.5 ± 274.5
18:3n-6	17.8 ± 1.5	76.7 ± 7.6	130.5 ± 10.5	245.9 ± 17.0
20:2n-6	60.5 ± 2.3	57.6 ± 4.7	86.8 ± 10.4	120.8 ± 19.6
20:3n-6	23.1 ± 0.7	74.2 ± 2.4	124.0 ± 16.3	205.5 ± 25.6
20:4n-6	44.0 ± 1.4	21.4 ± 2.3	46.4 ± 5.7	107.9 ± 10.2
22:4n-6	2.3 ± 2.7	0.0 ± 0.0	2.6 ± 2.1	7.9 ± 5.7
22:5n-6	15.3 ± 0.4	0.0 ± 0.0	3.7 ± 2.6	27.5 ± 2.9
<b>Total n-6 PUFA</b>	<b>859.3 ± 51.2</b>	<b>1577.2 ± 67.1</b>	<b>2236.4 ± 222.0</b>	<b>3149.0 ± 347.7</b>
18:3n-3	189.1 ± 13.4	2208.0 ± 104.4	1344.5 ± 135.6	538.6 ± 66.6
18:4n-3	144.7 ± 9.9	703.9 ± 47.5	436.2 ± 38.2	246.5 ± 23.3
20:3n-3	18.4 ± 1.2	106.1 ± 10.5	61.4 ± 6.8	21.1 ± 4.7
20:4n-3	85.4 ± 4.4	203.7 ± 14.3	119.7 ± 15.2	44.3 ± 6.8
20:5n-3	351.5 ± 12.4	146.4 ± 5.9	90.4 ± 10.4	41.3 ± 6.0
21:5n-3	31.4 ± 1.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n-3	150.3 ± 3.6	53.2 ± 5.3	38.9 ± 7.0	19.8 ± 4.8
22:6n-3	1132.6 ± 41.6	387.0 ± 15.3	308.1 ± 38.8	219.6 ± 32.2
<b>Total n-3 PUFA</b>	<b>2103.4 ± 85.7</b>	<b>3808.3 ± 152.9</b>	<b>2399.3 ± 246.4</b>	<b>1131.3 ± 137.9</b>
<b>C16 PUFA</b>	<b>87.9 ± 5.6</b>	<b>0.0 ± 0.0</b>	<b>0.0 ± 0.0</b>	<b>0.0 ± 0.0</b>
<b>Total PUFA</b>	<b>3050.6 ± 141.7</b>	<b>5385.6 ± 219.0</b>	<b>4635.7 ± 467.6</b>	<b>4280.2 ± 483.2</b>