

**Diet × genotype interactions in hepatic cholesterol and lipoprotein metabolism in Atlantic salmon (*Salmo salar*) in response to replacement of dietary fish oil with vegetable oil**

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## 26    **Abstract**

27    This study investigates effects of genotype on responses to alternative feeds in Atlantic salmon.  
28    Microarray analysis of the liver transcriptome of two family groups, Lean or Fat, fed a diet  
29    containing either fish oil (FO) or a vegetable oil (VO) blend indicated that pathways of cholesterol  
30    and lipoprotein metabolism might be differentially affected by diet depending on the genetic  
31    background of the fish, and this was further investigated by RT-qPCR, plasma and lipoprotein  
32    biochemical analysis. Results indicate a reduction in VLDL and LDL levels, with no changes in  
33    HDL, when FO is replaced by VO in the Lean family group, whereas in Fat fish fed FO levels of  
34    apoB-containing lipoproteins were low and comparable to those fed VO in both family groups.  
35    Significantly lower levels of plasma triacylglycerol (TAG) and LDL-TAG were measured in the Fat  
36    group, that were independent of diet, whereas plasma cholesterol was significantly higher in fish fed  
37    the FO diet in both groups. Hepatic expression of genes involved in cholesterol homeostasis,  $\beta$ -  
38    oxidation and lipoprotein metabolism showed relatively subtle changes. Significantly lower  
39    expression of genes considered anti-atherogenic in mammals (ABCA1, apoAI, SR-BI, LPLb and  
40    LPLc) was found in Lean fish, compared with Fat, when fed VO. Furthermore, the Lean family  
41    group appeared to show a greater response to diet composition in the cholesterol biosynthesis  
42    pathway, mediated by SREBP2. Finally, the presence of 3 different transcripts for lipoprotein lipase  
43    (LPL), with differential patterns of nutritional regulation, was demonstrated.

44

45

46    As worldwide demand for seafood continues to grow and traditional fisheries are at best stable or  
47    in decline, aquaculture production will need to bridge the gap. An inevitable outcome of growing  
48    marine aquaculture production, associated with reduced availability of raw materials from wild  
49    fisheries, has been the need to look for more sustainable alternatives to replace fish oil (FO) and fish  
50    meal (FM) in aquafeed formulations. Recent estimates suggest that 88.5% of global production of  
51    FO is currently used by the aquaculture sector, with salmonid culture taking the largest share (56%

52 of total FO production)<sup>(1)</sup>. Insufficient FM and FO supply may seriously limit aquaculture growth  
53 and so future activity depends on reduced dependency on FO and its replacement with alternative  
54 oils, while maintaining fish welfare and health benefits for the human consumer. Extensive studies  
55 have shown that VO<sub>s</sub> can replace up to 100% of FO in salmonid diets without compromising fish  
56 growth or condition, but above 50% of FO replacement a significant reduction is observed in tissue  
57 levels of n-3 long-chain polyunsaturated fatty acids (LC-PUFA), namely eicosapentaenoic acid  
58 (EPA) and docosahexaenoic acid (DHA), diminishing the beneficial, health-promoting, nutritional  
59 profile for human consumption<sup>(2-4)</sup>. There is now evidence that flesh n-3 LC-PUFA level is a  
60 heritable trait in Atlantic salmon<sup>(5)</sup>. This being the case, combining genetic selection with changes  
61 in commercial diet formulations (i.e., high levels of FM and FO replacement) might be a viable  
62 strategy to meet worldwide growing demands for aquaculture products. Therefore, in order to  
63 investigate the feasibility of this approach, large-scale studies exploring diet formulation × genotype  
64 interactions are essential. This was the overarching objective of the present study, which  
65 investigated the effect of genotype on responses to alternative feeds where FO was replaced by VO  
66 in Atlantic salmon.

67 Early studies on dietary FO replacement in salmon suggested that high inclusion levels of certain  
68 VO might negatively affect fish health and resistance to stress by changing cardiac membrane fatty  
69 acid composition, and diets containing sunflower oil were reported to result in considerable  
70 cardiomyopathy, extensive thinning and necrosis of the ventricular muscle wall<sup>(6,7)</sup>. Other studies  
71 could not directly show an involvement of dietary fatty acid composition on the development of  
72 arteriosclerotic changes in Atlantic salmon but could not exclude it either<sup>(8)</sup>. This is therefore an  
73 area that is still open for discussion. On the other hand, a relationship between nutritional factors,  
74 especially dietary level of n-3 LC-PUFA, and risk of developing atherosclerosis has been well  
75 demonstrated in mammals and, furthermore, genetic polymorphisms/variants have been identified  
76 in several genes involved in cholesterol and lipoprotein metabolism that can explain different  
77 susceptibilities and responses to diet<sup>(9-12)</sup>. No such associations have been reported in fish, where

knowledge is still quite fragmentary. Therefore, the specific aim of the current study was to further explore the potential influence of dietary oil source on cholesterol and lipoprotein metabolism, which may ultimately affect propensity to develop cardiac lesions, in Lean and Fat family groups of Atlantic salmon, differing in flesh adiposity<sup>(13)</sup> by quantifying the gene expression of key genes informed by a microarray analysis of the hepatic transcriptome.

## Methods

### *Feeding trial and sampling*

A trial was conducted using two genetically characterised and contrasting groups of Atlantic salmon (*Salmo salar*) post-smolts comprising full-sib families selected from the Landcatch Natural Selection Ltd (LNS) breeding program (Argyll, Scotland). Choice was based on estimated breeding values (EBVs) of the parents for high or low flesh adiposity, assessed by Torry Fatmeter (Distell Industries, West Lothian, UK), a trait with heritability ranging from 0.17 to 0.39 in this dataset. The two groups were created from four unrelated full-sib families; two families from the extreme lower end of the EBV distribution for flesh lipid content (“Lean”) and two families from the extreme upper end of the distribution (“Fat”). The average EBV for the lipid content of the two Fat families was 2.00 percentage units higher than that of the two selected Lean families, representing a standardised selection differential of 2.33 standard deviations (sds).

Two thousand fish of each group were stocked into eight 12 x 5m<sup>3</sup> net pens at the Ardnish Fish Trials Unit (Marine Harvest Scotland, Lochailort, Scotland; 500 fish pen<sup>-1</sup>). Each group was fed one of two experimental diets (Skretting ARC, Stavanger, Norway) formulated to fully satisfy the nutritional requirements of salmonid fish for 55 weeks until reaching ~3 kg. Duplicate pens of each group were fed a similar basal diet containing 25-32% fish meal and 40-45% plant meals, and 27.5-30% oil supplied either as northern fish oil (FO) or as a vegetable oil (VO) blend comprising rapeseed, palm and *Camelina* oils in a ratio of 5:3:2. Diets contained similar levels of PUFA

104 (around 31%) but different n-3 and n-6 contents, 25.3% and 4.6% in the FO diet and 13.4% and  
105 17.1% in the VO diet of PUFA, respectively. Further details of the trial including diet formulations,  
106 and proximate and fatty acid compositions of the feeds can be found in Bell *et al.* <sup>(13)</sup>.

107 At 55 weeks, 25 fish were sampled per pen, killed by a blow to the head following anaesthesia  
108 using MS222. Samples of liver were collected for molecular analyses and stored at -80°C.  
109 Additionally, ten samples of liver and flesh (Norwegian Quality Cut) were collected per pen and  
110 stored at -20°C pending biochemical analysis. Four pools comprising 5 fish/pool were prepared for  
111 flesh and liver lipid analyses from the duplicate pens per family and diet <sup>(13)</sup>. Blood was collected  
112 from the caudal vein using EDTA vacutainers from 5 fish per pen and centrifuged at 3000 × g for  
113 10 min to obtain plasma fractions, which were then pooled for lipoprotein analysis. Experimental  
114 procedures complied with the UK Home Office code of practice for the care and use of animals for  
115 scientific purposes and all protocols were approved by the Institute of Aquaculture and University  
116 of Stirling ethics committees. There were no aspects of this trial that would cause aggravated or  
117 unnecessary harm or stress to the fish.

118

#### 119 *RNA extraction and purification*

120 Liver tissue (0.2 g) from six individuals per group was homogenised in 2mL of TRI Reagent  
121 (Ambion, Applied Biosystems, Warrington, U.K.). Total RNA was isolated following  
122 manufacturer's instructions, 100µg were further purified by mini spin-column (RNeasy Mini Kit,  
123 Qiagen, Crawly, UK), and RNA quality and quantity assessed by gel electrophoresis and  
124 spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, U.S.A.).

125

#### 126 *Microarray hybridisations and analysis*

127 The TRAITS/SGP (v.2.1) salmon 17k cDNA microarray was used in this experiment  
128 (ArrayExpress accession: A-MEXP-1930) <sup>(14)</sup>. A dual-labelled experimental design was employed  
129 for the microarray hybridisations. Each experimental sample was competitively hybridised against a

130 common pooled-reference, obtained with equal amounts of all samples, which permits valid  
131 statistical comparisons across all treatments to be made. The entire experiment comprised 24  
132 hybridisations - 2 lipid phenotype groups (Lean/Fat)  $\times$  2 diets (FO/VO)  $\times$  6 biological replicates.

133 Antisense amplified RNA (aRNA) was produced from each total RNA isolation using the Amino  
134 Allyl MessageAmp<sup>TM</sup> II aRNA Amplification Kit (Ambion, Applied Biosystems), following the  
135 manufacturer's methodology, followed by Cy3 or Cy5 fluor incorporation through a dye-coupling  
136 reaction. Briefly, 500 ng of total RNA were amplified and column-purified according to  
137 manufacturer's instructions including a 17 h transcription step, and aRNA quantified and quality  
138 assessed as above. Cy dye suspensions (Cy3 & Cy5) in sufficient quantity for all labelling reactions  
139 were prepared by adding 36  $\mu$ L high purity dimethyl sulphoxide (Stratagene, Hogehilweg, The  
140 Netherlands) to each tube of Cy dye (PA23001 or PA25001, GE HealthCare, Little Chalfont, UK).  
141 To attach the Cy dyes, 3  $\mu$ g each aRNA sample was suspended in 6  $\mu$ L nuclease-free H<sub>2</sub>O and  
142 heated to 70 °C for 2 min. When cooled to room temperature, 2  $\mu$ L of coupling buffer (0.5 M  
143 NaHCO<sub>3</sub>; pH 9.2) and 2  $\mu$ L of Cy3 dye suspension stock was added and then incubated for 1 h at  
144 25°C in the dark. For labelling the common pooled reference sample with Cy5, a scaled-up reaction  
145 was similarly performed. Unincorporated dye was removed by column purification (Illustra  
146 AutoSeq G-50 spin columns; GE Healthcare). Dye incorporation and aRNA yield were quantified  
147 by spectrophotometry (NanoDrop ND-1000) and quality controlled by separating 0.4  $\mu$ L on a thin  
148 mini-agarose gel and visualising products on a fluorescence scanner (Typhoon Trio, GE  
149 Healthcare).

150 Microarray hybridisations were performed in a Lucidea semi-automated system (GE Healthcare),  
151 without a pre-hybridisation step. For hybridisation of each array, each labelled biological replicate  
152 and corresponding pooled reference (40 pmol each dye, c. 150 ng aRNA) were combined and  
153 volume made up to 25  $\mu$ L with nuclease-free water. After heating the aRNA at 95 °C for 3 min in a  
154 thermocycler, 225  $\mu$ L of pre-heated (60 °C) hybridisation solution, comprising 185  $\mu$ L 0.7X  
155 UltraHyb buffer (Ambion), 20  $\mu$ L poly(A) at 10 mg/mL (Sigma-Aldrich, Dorset, UK), 10  $\mu$ L

156 herring sperm at c. 10 mg/mL (Sigma-Aldrich) and 10  $\mu$ L ultra pure BSA at 10 mg/mL (Sigma-  
157 Aldrich), was added and the mixture kept at 60 °C in the dark until being applied to the microarray.  
158 After loading the slides and hybridisation solution into the Lucidea chambers (heated at 60 °C),  
159 chamber temperature was raised to 70 °C for 10 min and then lowered to 42 °C, at which  
160 temperature hybridisation was continued for 17 h with pulse mixing every 15 min. Two post-  
161 hybridisation automatic washes (800  $\mu$ L per slide at 8  $\mu$ L/s) were performed with 1.0 $\times$  SSC; 0.1%  
162 SDS (wash 1) and 0.3 $\times$  SSC; 0.2% SDS (wash 2), after which temperature was lowered to 40 °C.  
163 Slides were then manually washed using the EasyDip<sup>TM</sup> Slide staining system (Canemco Inc.,  
164 Quebec, Canada): 2 times with wash 2 solution for 3 min each (125 rpm; 45 °C), followed by 3  
165 times with 0.2 $\times$  SSC for 2 min each (125 rpm; 45 °C) and a final 20 s dip (room temperature) in  
166 0.1 $\times$  SSC. Slides were then dried by centrifugation (500  $\times$ g for 5 min) and kept in a desiccator, in  
167 the dark, before scanning.

168 Scanning was performed at 10  $\mu$ m resolution using an Axon GenePix 4200AL Scanner (MDS  
169 Analytical Technologies, Wokingham, Berkshire, U.K.). Laser power was kept constant (80%) and  
170 the “auto PMT” function within the acquisition software (v.4) was enabled to adjust PMT for each  
171 channel such that less than 0.1% of features were saturated and that the mean intensity ratio of the  
172 Cy3 and Cy5 signals was close to one. BlueFuse software (BlueGnome, Cambridge, U.K.) was used  
173 to identify features and extract fluorescence intensity values from the resultant TIF images.  
174 Following a manual spot removal procedure and fusion of duplicate spot data (BlueFuse proprietary  
175 algorithm), the resulting fluorescence intensity data and quality annotations for the 17,102 gene  
176 features were exported into the GeneSpring GX version 10.0.2 analysis platform (Agilent  
177 Technologies, Wokingham, Berkshire, U.K.) after undergoing a block Lowess normalisation. All  
178 control features were excluded from subsequent analyses. Data transformation and quality filtering  
179 were as follows: (a) all intensity values  $<1$  were set to 1 and (b) data were filtered using a Blue-Fuse  
180 spot confidence value  $>0.3$  in at least 75 % of the values in any 2 out of 4 conditions and BlueFuse  
181 spot quality of  $\geq 0.5$  in at least 75 % of the values in any 2 out of 4 conditions. This gave a list of

14,772 genes eligible for statistical analysis. Experimental annotations complied fully with minimum information about a microarray experiment (MIAME) guidelines<sup>(15)</sup>. The experimental hybridisations are archived on the EBI ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress/>) under accession number E-TABM-1089. No multiple test correction was employed as previous analyses, confirmed by RT-qPCR, indicate that such corrections are over-conservative for this type of data<sup>(16)</sup>. Hybridisation data were analysed by two-way ANOVA, which examined the explanatory power of the variables ‘diet’ and ‘family’ and the interaction between the two, at a significance level of 0.05. In the present study we focussed on lipid metabolism genes whose expression was differentially affected by diet (FO replacement by VO) depending on fish leanness/fatness and thus only data from the significant interaction list is presented.

#### RT-qPCR

Expression of selected genes showing a significant diet × family interaction in the microarray analysis, and other genes relevant to lipid metabolic pathways, was studied by reverse transcription quantitative real time PCR (RT-qPCR). Primers were either found in literature or designed from EST sequences using Primer3 software ([http://biotools.umassmed.edu/bioapps/primer3\\_www.cgi](http://biotools.umassmed.edu/bioapps/primer3_www.cgi)) (Table 1). Amplification of three potential reference genes, *cofilin-2*, elongation factor-1 $\alpha$  (*elf-1 $\alpha$* ) and  *$\beta$ -actin*, was performed but only *cofilin-2* expression proved to be stable across treatments.

For RT-qPCR, one  $\mu$ g of column-purified total RNA per sample was reverse transcribed into cDNA using the VersoTM cDNA kit (ABgene, Surrey, U.K.), following manufacturer’s instructions, using a mixture of random hexamers and anchored oligo-dT (3:1, v/v). Negative controls (no enzyme) were performed to check for genomic DNA contamination. cDNA was then diluted 20-fold with water, after a similar amount of cDNA was pooled from all samples. RT-qPCR analysis used relative quantification with the amplification efficiency of the primer pairs assessed by serial dilutions of the cDNA pool. qPCR amplifications were carried out in duplicate (Quanta, Techne, Cambridge, U.K.) in a final volume of 20  $\mu$ L containing either 5  $\mu$ L (for most genes) or 2



208  $\mu\text{L}$  (for the reference genes and other highly expressed genes) diluted (1/20) cDNA, 0.5  $\mu\text{M}$  of each  
209 primer and 10  $\mu\text{L}$  Absolute<sup>TM</sup> QPCR SYBR<sup>®</sup> Green mix (ABgene). Amplifications were carried  
210 out with a systematic negative control (NTC). The qPCR profiles contained an initial activation step  
211 at 95 °C for 15 min, followed by 30 to 40 cycles: 15 s at 95 °C, 15 s at the specific primer pair  
212 annealing  $T_m$  (Table 1) and 15 s at 72 °C. After the amplification phase, a melt curve of 0.5 °C  
213 increments from 75 °C to 90 °C was performed, confirming amplification of single products. RT-  
214 qPCR product sizes were checked by agarose gel electrophoresis and identities confirmed by  
215 sequencing. Non-occurrence of primer-dimer formation in the NTC was also verified. Results were  
216 analysed using the relative expression software tool (REST 2008, [http://www.gene-](http://www.gene-quantification.info/)  
217 [quantification.info/](http://www.gene-quantification.info/)), which employs a pair wise fixed reallocation randomisation test (10,000  
218 randomisations) with efficiency correction <sup>(17)</sup>, to determine the statistical significance of expression  
219 ratios (or gene expression fold-changes) between two treatments.

220

#### 221 *Plasma and lipoprotein lipid analysis*

222 Plasma and lipoprotein lipids were analysed by means of a clinical bioanalyser (Maxmat PL  
223 analyzer, Montpellier, France). Very low density lipoprotein (VLDL), low density lipoprotein  
224 (LDL) and high density lipoprotein (HDL) in plasma were obtained by sequential centrifugal  
225 flotation <sup>(18,19)</sup> as described by Lie *et al.* <sup>(20)</sup> at 197 600  $\times g$  and 4°C (Beckman Optima<sup>TM</sup>XL-100K  
226 Ultracentrifuge and SW41Ti rotor). Density intervals were obtained by addition of solid KBr <sup>(21)</sup>,  
227 and run time for separation of lipoproteins was: VLDL,  $d < 1.015 \text{ g/mL}$  for 20 h; LDL,  $1.015 \text{ g/mL}$   
228  $< d < 1.085 \text{ g/mL}$  for 20 h and HDL,  $1.085 \text{ g/mL} < d < 1.21 \text{ g/mL}$  for 44 h.

229

#### 230 *Lipid class analyses*

231 Total lipids were extracted from flesh or liver according to Folch *et al.* <sup>(22)</sup> and tissue lipid class  
232 compositions determined by single-dimension double-development high-performance thin-layer  
233 chromatography (HPTLC) and densitometry as described previously <sup>(23)</sup>.

234

## 235 *Statistical analysis*

236 Differences in lipid class composition in liver and flesh and in levels of cholesterol and  
237 triacylglycerol (TAG) in plasma and lipoproteins were assessed by two-way analysis of variance  
238 (ANOVA), at a significance level of  $p < 0.05$ . The RT-qPCR data were analysed both using the  
239  $\Delta\Delta C_t$  method with efficiency correction in REST and by two-way ANOVA of normalised gene  
240 expression values obtained from the standard curve performed with cDNA serial dilutions.

241

## 242 **Results**

243

### 244 *Microarray data*

245 In order to identify genes involved in lipid metabolic processes whose expression is dependent  
246 on the combined effects of both diet and family, i.e., for which the effect of diet depends on family,  
247 the two-way ANOVA interaction list obtained from the analysis of the microarray data was  
248 examined. This list contained 529 features that were significantly differentially regulated of which  
249 17 features (corresponding to 15 genes) were related to lipid metabolism (Table 2). The top 100  
250 genes, sorted by p-value, were categorised according to function and the lipid metabolism category  
251 corresponded to 15 % of the total annotated genes (and excluding genes of miscellaneous function).  
252 The lipid metabolism genes found in the interaction list can be broadly described as being involved  
253 in the following processes: cholesterol/ isoprenoid biosynthesis (IPI-isopentenyl-diphosphate  
254 isomerise, SQLE-squalene monooxygenase/epoxidase and ACAT2-acetyl-CoA acetyltransferase 2),  
255 cholesterol transport/cellular efflux (ABCA1-ATP-binding cassette transporter A1), lipoprotein  
256 metabolism (Angptl4-angiopoietin-like 4, LPL-lipoprotein lipase, EL-endothelial lipase),  $\beta$ -  
257 oxidation (CRAT-carnitine O-acetyltransferase, ECH1-delta3,5-delta2,4-dienoyl-CoA isomerase  
258 and ECHS1-enoyl Coenzyme A hydratase 1), fatty acid synthesis ( $\Delta 5$  and  $\Delta 6$  fatty acyl desaturase,  
259 Fad) and transport (ACBP-acyl-Coenzyme A-binding protein), glycerophospholipid/

260 phosphatidylcholine (PC) biosynthesis (PEMT-phosphatidylethanolamine N-methyltransferase) and  
261 regulation of energy metabolism through switch on/off of multiple catabolic/anabolic pathways  
262 (AMPK-5'-AMP-activated protein kinase subunit gamma-3).

263

#### 264 *RT-qPCR analysis of gene expression*

265 Relative gene expression of a series of genes involved in some of the preponderant lipid  
266 metabolism pathways mentioned above, mostly associated to cholesterol biosynthesis and its  
267 regulation and transport, fatty acid  $\beta$ -oxidation and lipoprotein metabolism, was determined by RT-  
268 qPCR (Table 3). This included some genes found in the significant interaction list from the  
269 microarray analysis (IPI, ABCA1, EL and LPL). Although the fold-changes obtained by both  
270 methods (microarray and RT-qPCR) differed, the general trend was similar for both IPI and  
271 ABCA1. In the case of IPI, although differences were not statistically significant there was a clear  
272 trend for it to be up-regulated when VO replaced FO in the diet in the Lean fish and down-regulated  
273 in the Fat group. This difference appears to result from a lower expression of IPI in Lean fish,  
274 compared to Fat, when they are fed the FO-diet. In contrast, the opposite was observed with  
275 ABCA1, with a trend for down-regulation in Lean fish fed VO compared to FO and an up-  
276 regulation in the Fat fish. In this case, gene expression was significantly lower in the Lean group  
277 compared to the Fat, when fed the VO diet. Agreement between the RT-qPCR and microarray  
278 results initially proved problematic for EL and LPL but, on closer examination, multiple transcripts  
279 for both genes were identified. In the case of EL, an EST was identified in the GenBank database  
280 (DY694576) that is 86% identical, in the aligned area, to the Atlantic salmon EL reference sequence  
281 (NM\_001140535), and this is likely to have resulted in cross-hybridisation in the microarray.  
282 Indeed, an initially tested primer pair showed very similar fold changes as those obtained in the  
283 microarray experiment and was later found to have amplified both sequences. When RT-qPCR was  
284 repeated using primers specific for NM\_001140535, quite different results were obtained, with  
285 significant up-regulation being observed in both Lean and Fat families fed VO compared to FO. On

the other hand, determination of LPL expression was initially performed using a primer pair available in the literature (here termed LPLa) but this resulted in a pattern of expression not corresponding to the microarray results. Further investigation revealed that the three LPL clones in the microarray significant interaction list correspond to two different clusters (DFCI-The Gene Index Project; <http://compbio.dfci.harvard.edu/tgi/tgipage.html>), TC67836 and TC84899, which are 85 % identical to each other in the aligned region (90 % query coverage), and which we here named LPLb and LPLc, respectively. The published LPLa primers amplify a sequence corresponding to TC91040, which does not align with TC sequences from LPLb or LPLc. The primers designed for LPLb and LPLc gave comparable results to the microarray experiment, and broadly similar to each other, with a trend for down-regulation in Lean fish when fed the VO diet instead of FO and an up-regulation (significant for LPLc) in Fat fish fed the VO diet. In both cases, this was associated with a significantly lower expression of these transcripts in Lean, compared to the Fat group, when fish were fed the VO diet.

From the RT-qPCR analysis of other genes involved in cholesterol biosynthesis, only mevalonate kinase (MEV) showed a pattern of expression broadly similar (in terms of up/down-regulation) to IPI (Table 3). The expression of the regulatory transcript sterol-responsive element-binding protein 2 (SREBP2) also showed the same general trends observed in IPI, MEV (Table 3) and SQLE (Table 2), with a pronounced up-regulation in the Lean fish when VO replaced FO in the diet, coupled with lower expression in Lean salmon, compared to Fat, when fed FO.

The microarray experiment had indicated potential differential regulation of fatty acid  $\beta$ -oxidation in Lean and Fat families, as suggested by the presence of three genes in the significant interaction list, although with marginal fold changes. To verify this, we assayed the relative levels of expression of two genes involved in the  $\beta$ -oxidation pathway, including acyl-CoA oxidase (ACO), and carnitine palmitoyl transferase 1 (CPT1), responsible for facilitating the transfer of long chain fatty acids into the mitochondria and thus a common indicator of  $\beta$ -oxidation<sup>(16)</sup>. However, no significant changes were observed for these genes.

312 To further analyse physiological mechanisms related to lipoprotein metabolism, quantification of  
313 apolipoprotein genes (ApoAI, ApoCII and ApoB) and lipoprotein receptors (SR-BI-scavenger  
314 receptor class B type 1 and LDLR-low density lipoprotein receptor) was performed. In general,  
315 dietary FO replacement by VO tends to increase the expression of the three apolipoproteins and to  
316 reduce that of the two lipoprotein receptors assayed in both experimental fish groups. However, few  
317 statistically significant differences were observed, apart from significantly lower expression of  
318 ApoAI and SR-BI in the Lean group, compared to Fat, when fed VO.

319 To fully ascertain the effects of the factors “diet” and “family” on gene expression, data were  
320 also expressed as normalised values that could be analysed by two-way ANOVA (Fig. 1). A  
321 significant dietary effect was observed in the expression of ApoAI, EL and LPLc, with the VO diet  
322 inducing a higher level of expression of these genes (Fig. 1G, L and O). In addition, a significant  
323 family effect was also observed in ApoAI and LPLc expression, with higher levels of transcripts  
324 being measured in Fat fish compared to Lean. In both cases, and particularly for LPLc, the Fat-VO  
325 group presented the highest up-regulation, and thus had the greatest influence. Finally, LPLb  
326 showed significant interaction as a result of the lowest and highest levels of expression being  
327 measured in Lean-VO and Fat-VO, respectively (Fig. 1N).

328

#### 329 *Lipid biochemical composition of plasma, lipoprotein classes, liver and flesh*

330 To assess possible biochemical consequences of altered gene expression, total lipid levels and  
331 lipid class composition of liver and flesh, and cholesterol and TAG in plasma and lipoproteins  
332 (VLDL, LDL and HDL) 24 hours after the last meal were analysed (Tables 4 and 5). Plasma  
333 cholesterol was significantly affected by diet, with higher levels found in fish fed FO independent  
334 of family (Table 4). Cholesterol in VLDL showed significant interaction, due to nearly doubling in  
335 Lean fish fed FO, with no difference between the other groups. A significant family effect was  
336 measured for plasma and LDL-TAG, with the Lean group showing significantly higher levels of  
337 TAG than the Fat group (Table 4). In liver, significantly lower proportions of TAG, and

338 correspondingly higher percentages of phospholipids and sterols, were found in fish fed FO  
339 independent of family (Table 5). Significantly higher relative phospholipid level was also found in  
340 flesh of fish fed FO but, contrary to liver, the relative level of sterols was significantly higher in  
341 both family groups fed VO. In addition, a significant family effect was observed in flesh  
342 phospholipids, with higher levels in the Fat group.

343

## 344 **Discussion**

345

346 Microarray analysis of the liver transcriptome of Atlantic salmon from two family groups, Lean  
347 or Fat, fed diets containing either FO or VO, returned a high number of genes involved in lipid and  
348 lipoprotein pathways showing significant interaction between genotype and diet. Considering the  
349 roles of some of these transcripts and the possibility for functional relationships, we hypothesise  
350 that some of the expression changes are interrelated. This prompted further investigation of the  
351 expression of genes involved in cholesterol homeostasis, including cholesterol biosynthesis and  
352 cellular efflux and in the regulation of these pathways, as well as some implicated in fatty acid  $\beta$ -  
353 oxidation and lipoprotein metabolism, including apolipoproteins, membrane lipoprotein receptors  
354 and lipases. The gene expression data are discussed in relation to plasma, lipoprotein, liver and flesh  
355 compositions, advancing our knowledge on how dietary VO, with altered PUFA and cholesterol  
356 content, may alter lipid metabolism and transport and how these effects may depend on genetic  
357 background.

358

### 359 *Cholesterol metabolism*

360 Replacing FO with VO reduced plasma cholesterol in both family groups, which can be  
361 explained by the typically lower level of cholesterol in VO compared to FO. In addition, some VO  
362 are naturally rich in phytosterols, which reduce plasma cholesterol, LDL-cholesterol and LDL-TAG  
363 in brook trout (*Salvelinus fontinalis*) <sup>(24)</sup>, and LDL-cholesterol in humans, by inhibiting intestinal

364 cholesterol absorption <sup>(25)</sup>. Similarly, the lipid composition of liver, with higher levels of sterol in  
365 fish fed FO, probably also reflects dietary cholesterol intake.

366 Previously in Atlantic salmon, up-regulation of SREBP2 and genes involved in cholesterol  
367 biosynthesis was observed and attributed to lower dietary cholesterol supply by VO diets <sup>(16)</sup>. In that  
368 study, apart for HMG-CoA, which was not significantly regulated, cholesterol biosynthesis genes  
369 and SREBP2 were all over 2-fold up-regulated in VO in relation to FO. In the present study a clear  
370 response in terms of cholesterol biosynthesis genes was not observed and fold-changes were lower.  
371 Although not determined, dietary cholesterol levels likely varied in the diets in the two studies and  
372 the differential in supply between FO and VO diets might have been larger in the previous study <sup>(16)</sup>.  
373 On the other hand, a blend of VO, formulated to resemble more closely FO in terms of fatty acid  
374 composition <sup>(13)</sup>, rather than single VOs as previously <sup>(16)</sup>, may have resulted in a dietary fatty acid  
375 composition with less effect on cholesterol biosynthesis. Nonetheless, gene expression data indicate  
376 that cholesterol biosynthesis may be up-regulated in the Lean family group when VO replaces FO,  
377 whereas this pathway does not appear to be affected in Fat fish. Consistent with this, the expression  
378 of SREBP2, which in mammals is positively correlated and induces the expression of all 12  
379 enzymes of the cholesterol biosynthetic pathway <sup>(26)</sup>, shows a greater increase in the Lean fish,  
380 compared to the Fat group, when VO replaces FO. This reinforces the hypothesis that, similar to  
381 mammals, regulation of cholesterol biosynthesis in fish is at least partly mediated by SREBP2 <sup>(16)</sup>.  
382 In addition, these differences between family groups seem to arise mostly when feeding the more  
383 “natural” FO diet containing higher cholesterol, with the Lean fish showing a tendency for a greater  
384 down-regulation of cholesterol biosynthesis genes. On the other hand, low dietary supply of  
385 cholesterol has been reported to activate SREBP2 that down-regulates ABCA1 transcription and  
386 cholesterol efflux in mice liver and human vascular endothelial cells <sup>(11,27,28)</sup>. This was not obvious  
387 in salmon but circumstantial evidence from the gene expression data indicates that, if such a  
388 response exists, it may only occur in the Lean family group, since a trend for an inverse relationship  
389 between SREBP2 and ABCA1 expression when VO replaces FO was only observed in this group

390 and, furthermore, a significant down-regulation of the ABCA1 transporter was measured in the  
391 Lean family, compared to Fat fish, when these were fed VO. Therefore, these data suggest that Lean  
392 fish might be more responsive to dietary cholesterol and adjust the level of expression of genes  
393 involved in cholesterol metabolism and transport more tightly than the Fat family.

394

#### 395 *Lipoprotein synthesis and hepatic TAG metabolism*

396 Previous studies in rainbow trout and Atlantic salmon found a significant reduction in plasma  
397 cholesterol and LDL levels, as well as a trend towards lower VLDL levels, when VO replaced  
398 dietary FO <sup>(4,29)</sup>. In the present study, VLDL-cholesterol levels showed a diet × family interaction  
399 with a reduction observed when VO replaced dietary FO, but only in the Lean group. In contrast, no  
400 effects were observed in LDL-cholesterol, while LDL-TAG was affected by family, with higher  
401 levels in the Lean group. However, a dietary trend was observed with lower levels of LDL  
402 associated with the VO diet. Together, these results indicate a tendency towards reduced levels of  
403 plasma cholesterol, VLDL and LDL as a result of the replacement of FO by VO in salmonids, as a  
404 result of differences in cholesterol levels and relative levels of n-3/n-6 PUFA in these oils.

405 Salmon in the Fat group had lower plasma TAG and LDL-TAG compared to the Lean family  
406 irrespective of diet. Reduced levels of VLDL and LDL-TAG in mammals can be caused by several  
407 complex and interrelated factors <sup>(30)</sup>. Analogies have been established between teleost and  
408 mammalian lipoprotein metabolism <sup>(31,32)</sup> but we can only speculate that regulatory mechanisms are  
409 equivalent. On one hand, decreased circulating TAG may be related to decreased hepatic VLDL  
410 synthesis and secretion to the circulation that may be a consequence of lower availability of  
411 precursor TAG. However, in the present study, liver lipid composition was affected by diet but not  
412 family, suggesting that differences between the families in circulating TAG might be influenced by  
413 differences in uptake by peripheral tissues rather than hepatic lipid metabolism. The  
414 hypotriglyceridemic effect of dietary FO has been established in mammals and is believed to result  
415 from a coordinated effect of n-3 LC-PUFA (particularly EPA) in suppressing hepatic lipogenesis



416 and enhancing fatty acid oxidation in liver and muscle through inhibition of SREBP-1c and  
417 peroxisome proliferator-activated receptor (PPAR) activation, respectively <sup>(33-35)</sup>. As previously  
418 observed in salmon <sup>(29,36)</sup>, PL/TAG ratios were affected by diet with FO inducing lower TAG and  
419 correspondingly higher PL, which is attributed to similar hypotriglyceridemic mechanisms of n-3  
420 LC-PUFA in FO as those described in mammals. Consistent with this, lower expression of fatty  
421 acid synthase (FAS), which plays a key role in lipogenesis, was measured in fish fed FO,  
422 independent of family (S Morais, unpublished results). On the other hand, microarray data  
423 suggested an interaction between diet and family affecting hepatic  $\beta$ -oxidation (CRAT, ECH1 and  
424 ECHS1) and the expression of AMPK, a metabolic “sensor” responsible for regulating energy  
425 homeostasis <sup>(37)</sup>. However, fold changes were marginal and when expression of  $\beta$ -oxidation genes  
426 ACO and CPT1 was assessed by qPCR there were no differences between diet or family groups.

427

#### 428 *Lipoprotein uptake and reverse cholesterol transport*

429 Another possible mechanism to affect circulating levels of TAG and lipoproteins is through  
430 lipoprotein uptake by liver and peripheral tissues. Gene expression was only assessed in liver,  
431 which does not enable assessment of uptake by peripheral tissues, given that some genes are  
432 regulated in a tissue-specific manner (e.g., mammalian and fish LPL; <sup>38-41</sup>). A likely explanation for  
433 the observed differences in circulating TAG in the two family groups might be related to their lipid  
434 storage phenotype. Hence, decreased circulating lipids 24 h after the last meal in the Fat family  
435 group may be due to more efficient uptake of lipids by muscle and viscera, as indicated by higher  
436 lipid contents in these tissues <sup>(13)</sup>. However, this might only partly explain the results as diet  $\times$   
437 family interaction was observed, and the family phenotype (Fat-Lean) was only maintained at the  
438 end of the trial in fish fed FO. On the other hand, a higher level of sterols was found in flesh of fish  
439 from both groups fed VO, which could be explained by higher uptake of LDL. Another possibility  
440 could be a decreased rate of reverse cholesterol transport from peripheral tissues to liver when fish  
441 are fed VO that could be linked to decreased dietary cholesterol levels and induced hepatic

442 cholesterol synthesis <sup>(16)</sup>. Nonetheless, neither diet nor genetic background affected HDL  
443 composition in the present study, as was also reported previously <sup>(4,29)</sup>. Dietary FO has been  
444 associated with enhanced reverse cholesterol transport in mammals but Davidson <sup>(35)</sup> hypothesised  
445 that, since n-3 LC-PUFA can stimulate simultaneously four metabolic nuclear receptors, the net  
446 effect may result in only minimal changes in HDL levels.

447 A key step in VLDL and LDL clearance is lipoprotein-TAG lipolysis ahead of receptor-mediated  
448 endocytosis. The microarray data indicated a possible interaction between diet and family in the  
449 regulation of LPL and EL. Noteworthy was the change in expression of an angiopoietin-like 4  
450 cDNA that has been found to inhibit LPL in mammals <sup>(42)</sup> and thus a similar relationship might exist  
451 in fish. The qPCR analysis revealed a trend for up-regulation of LPLb and LPLc when salmon were  
452 fed VO but only in the Fat group. In Lean salmon, where differences in VLDL content related to  
453 diet were observed, LPL was either not affected (LPLa and LPLc) or down-regulated (LPLb) by the  
454 VO diet. LPL is believed to be regulated at the transcriptional level and therefore these results are  
455 likely to reflect enzyme activity <sup>(38)</sup>. Conversely, expression of EL was up-regulated in both family  
456 groups fed the VO diet. This enzyme has mainly a PL-hydrolysing activity in mammals and higher  
457 activity towards HDL although it hydrolyses all classes of lipoproteins <sup>(43,44)</sup>. Taken together, these  
458 mechanisms may result in higher levels of circulating VLDL and LDL in fish fed FO with more  
459 marked effect in Fat fish, opposite to what was observed.

460 The expression of ABCA1 also responded differently to diet depending on the genetic  
461 background of the fish with a trend for lower expression in the Lean fish fed VO, compared to FO.  
462 ABCA1 is a membrane transporter with roles in HDL synthesis and reverse cholesterol transport  
463 and thus this result may be related to either HDL metabolism or cholesterol biosynthesis <sup>(27)</sup>.  
464 However, its involvement in VLDL and LDL metabolism has recently been shown with deletion of  
465 the ABCA1 gene leading to increased VLDL production and elevated plasma TAG accompanied by  
466 enhanced LDL clearance through overexpression of hepatic LDLR <sup>(45)</sup>. Although we cannot exclude  
467 the possibility of increased clearance rate of LDL in Lean salmon fed VO, this does not appear to

involve hepatic LDLR as the expression of this gene was not affected. Another lipoprotein receptor implicated in the metabolism of apoB-containing lipoproteins is SR-BI. Although mainly known for selective uptake of HDL cholesterol, SR-BI has been shown to affect VLDL secretion in mice, even if effects are not consistent <sup>(46,47)</sup>. In the present study we did not detect changes in SR-BI expression in salmon when examining the effect of diet in both family groups.

An interesting observation was that differences in gene expression between family groups were more apparent in fish fed VO. In particular, there was a different response to dietary VO inclusion in HDL metabolism, as several genes implicated in HDL synthesis and uptake (ABCA1, ApoAI and SR-BI) had lower expression in the Lean group compared to Fat salmon fed VO. Expression of apoAI was affected by both factors, diet (VO>FO) and family (Fat>Lean). ABCA1 initiates the formation of mature HDL by facilitating cellular efflux of PL and cholesterol for lipidation of apoAI and apoE, and its overexpression in transgenic mice can result in an anti-atherogenic plasma profile <sup>(48)</sup>. In addition, it increases flux of cholesterol to the liver through enhanced reverse transport from peripheral tissues <sup>(27)</sup>. SR-BI also stimulates reverse cholesterol transport by mediating the selective cellular uptake of cholesteryl esters from HDL, transport of HDL-cholesterol into bile for excretion, and recycling of apolipoproteins, particularly in hepatic and steroidogenic cells <sup>(49)</sup>. Finally, LPL and EL can both influence lipoprotein metabolism by catalysing the hydrolysis of TAG and PL, respectively, thus facilitating lipoprotein catabolism and clearance <sup>(39,43)</sup>. Again, Lean fish fed the VO diet showed lower LPLa and LPLb expression, and a trend for reduced EL expression, than the corresponding Fat family group. This correlates well with the expression of SR-BI and also LDLR, which might be expected to be similarly regulated to catabolise the delipidated HDL and LDL particles after the action of LPL and EL <sup>(44)</sup>. The gene expression results thus suggest that the Fat family group might have faster lipoprotein turnover when fed VO but the physiological and health effects of this, including the development of arteriosclerotic changes when VO replaces FO, requires elucidation <sup>(6,7)</sup>.

494 *Lipoprotein lipase transcripts are differentially regulated in liver in response to diet*

495 This work has also emphasised the need for caution in future studies when assaying expression  
496 of LPL (and possibly EL), as several transcripts may exist, with different patterns of nutritional  
497 regulation. Whereas the expression of LPLa, corresponding to the gene assayed previously <sup>(50)</sup>, was  
498 not affected by either diet or family, a strong diet  $\times$  family interaction was found for LPLb and  
499 LPLc, with LPLc expression also affected by both diet and family. In mammals, LPL is only  
500 expressed in extrahepatic tissues <sup>(39)</sup>, whereas fish also show relatively strong expression in liver  
501 <sup>(38,41,51)</sup>. Hepatic LPL expression was investigated in red sea bream, *Pagrus major*, where it was  
502 shown that dietary fatty acids exert a regulatory effect on mRNA expression, although the effect  
503 depended on feeding status and could not be solely linked to fatty acid unsaturation <sup>(51)</sup>. In the  
504 present study the VO diet, containing higher oleic and linolenic acid levels <sup>(13)</sup>, induced a similar  
505 response as in red sea bream <sup>(51)</sup> only in the Fat group and for LPLb and LPLc. It therefore appears  
506 that the fatty acid composition of the diet may regulate LPL expression but this may depend on  
507 genetic background. Two LPL genes were reported in red sea bream <sup>(41)</sup>. The existence of more than  
508 one LPL gene in salmon was therefore not surprising, particularly in a species that has undergone a  
509 whole genome duplication event <sup>(52)</sup>. More interesting is that the LPL transcripts appear to be  
510 differentially regulated in liver, even if expressed at broadly comparable levels. Apart from its role  
511 in lipid uptake and lipoprotein catabolism, LPL activity has an important function in providing non-  
512 esterified fatty acids and 2-monoacylglycerols for tissue utilisation, either storage or oxidation,  
513 depending on tissue and nutritional state <sup>(39)</sup>. Consequently, LPL is subject to tissue-specific  
514 regulation, with reciprocal changes often being measured in response to diet composition and  
515 physiological changes, both in mammals and fish <sup>(38-41,51)</sup>. Thus, different transcripts may have  
516 evolved to respond to particular nutritional conditions in tissues with specific metabolic functions  
517 and demands.

518

519 *Conclusions*

520 The present study suggests that FO replacement by VO in salmon feeds can be accomplished  
521 without major detrimental changes in cholesterol and lipoprotein metabolism. A potential effect,  
522 associated with changes in dietary levels of n-3 LC-PUFA and cholesterol, may be a reduction in  
523 circulating apoB-containing lipoproteins, although mechanisms remain elusive. However, the  
524 genetic background of the fish may affect the physiological response to VO diets, although  
525 differences in gene expression were often quite subtle. Therefore, other mechanisms of regulation in  
526 addition to transcriptional, and affected by genetic factors (e.g., genetic variants inducing  
527 modifications of protein activity or specificity), may be responsible for the observed differences in  
528 tissue, plasma and lipoprotein lipid composition. In general, however, we can conclude that when  
529 salmon were fed VO the expression of genes considered anti-atherogenic in mammals was higher in  
530 the Fat fish, compared with the Lean fish. This was associated with significantly lower levels of  
531 plasma TAG and LDL-TAG in the Fat group, independent of diet, whereas plasma cholesterol  
532 likely reflected dietary intake in both family groups. In contrast, differences in VLDL and LDL  
533 between FO and VO fed fish were only obvious in Lean fish, given that in Fat fish levels of apoB-  
534 containing lipoproteins were low and comparable to those fed VO in both groups. Lean fish also  
535 showed a stronger response in the cholesterol biosynthesis pathway, mediated by SREBP2, to  
536 dietary lipid composition.

537

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549

550

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Table 1. Primers used for RT-qPCR.

Transcript	Primer name	Primer sequence	Amplicon	Tm	Accession No.	Source
HMG-CoA	HMG-1F	5'-CCTTCAGCCATGAACCTGGAT-3'	224 bp	60°C	TC102374 <sup>2</sup>	Leaver et al. (2008)
	HMG-1R	5'-TCCTGTCCACAGGCAATGTA-3'				
MEV	MEV-1F	5'-CCCTTAATCAGGGTCCCAAT-3'	247 bp	60°C	DW005667 <sup>1</sup>	Leaver et al. (2008)
	MEV-1R	5'-GGTGCTGGTTGATGTCAATG-3'				
IPI	23-3p jbt1F	5'-ACAGCCCTATGGTTATGTGTCATCTC-3'	230 bp	60°C	CK875291 <sup>1</sup>	Leaver et al. (2008)
	23-3p jbt1R	5'-CAAGGTGAGGCGAATGTTTGAAC-3'				
DHCR7	7DCHR-1F	5'-CTTCTGGAATGAGGCATGGT-3'	230 bp	60°C	TC99602 <sup>2</sup>	Leaver et al. (2008)
	7DCHR-1R	5'-ACAGGTCTCTTCTGGTGGTTG-3'				
SREBP2	SREBP2-1F	5'-GACAGGCACAACAAGGTG-3'	215 bp	60°C	DY733476 <sup>1</sup>	Leaver et al. (2008)
	SREBP2-1R	5'-CAGCAGGGGTAAGGGTAGGT-3'				
ABCA1	ABCA1-UTR-F2	5'-GGACGAACCCTGTGTCTGTT-3'	203 bp	60°C	EG836783 <sup>1</sup>	New design
	ABCA1-UTR-R2	5'-ATTTGCATTGCGTTTCAGTG-3'				
CPT1	CPT1-1F	5'-CCTGTACCGTGGAGACCTGT-3'	212 bp	60°C	AM230810 <sup>1</sup>	Leaver et al. (2008)
	CPT1-1R	5'-CAGCACCTCTTTGAGGAAGG-3'				
ACO	ACO-2F	5'-AAAGCCTTCACCACATGGAC-3'	230 bp	60°C	TC49531 <sup>2</sup>	Leaver et al. (2008)
	ACO-2R	5'-TAGGACACGATGCCACTCAG-3'				
apoA1	SsApoA1-F1	5'-CCATCAGCCAGGCCATAAA-3'	73 bp	60°C	CB506105 <sup>1</sup>	Kleveland et al. (2006)
	SsApoA1-R1	5'-TGAGTGAGAAGGGAGGGAGAGA-3'				
apoCII	SsApoCII-F1	5'-GGAACCAAGTCGCAGATGTTGA-3'	145 bp	60°C	DN047858 <sup>1</sup>	Kleveland et al. (2006)
	SsApoCII-R1	5'-TGAGGACATTCTGGCCCTTC-3'				
apoB100	SsApoBfQ	5'-AGCCTTCGATGCTGTCGGCCA-3'	153 bp	60°C	TC79364 <sup>2*</sup>	New design
	SsApoBrQ	5'-AGGAGCACAGGCAGGGTGGTT-3'				
SR-BI	SsSRBI-F1	5'-AACTCAGAGAAGAGGCCAACTTG-3'	204 bp	60°C	DQ266043 <sup>1</sup>	Kleveland et al. (2006)
	SsSRBI-R1	5'-TGCGGCGGTGATGATG-3'				
LDLR	SsLDLR-F1	5'-GCATGAACCTTTGACAATCCAGTGTAC-3'	78 bp	60°C	AJ003118 <sup>1</sup>	Kleveland et al. (2006)
	SsLDLR-R1	5'-TGGAGGAGTGCCTGCTGATAT-3'				
EL	SsEL-F4	5'-CCGGTGTCTGCTGGAGGAAGC-3'	378 bp	60°C	NM_001140535 <sup>1</sup>	New design
	SsEL-R5	5'-CGACATGCAGGTCATCGGT-3'				
LPLa	SsLPL-F1	5'-TGCTGGTAGCGGAGAAAGACAT-3'	114 bp	60°C	BI468076 <sup>1**</sup>	Kleveland et al. (2006)
	SsLPL-R1	5'-CTGACCACCAGGAAGACACCAT-3'				
LPLb	SsLPL-F4	5'-GGCAGCCCTACATGATAACC-3'	172 bp	60°C	TC67836 <sup>2***</sup>	New design
	SsLPL-R4	5'-TCTGTCCAAAGCCACTCACA-3'				
LPLc	SsLPL-F6	5'-AGGGCGTTAATCCATGTCAG-3'	223 bp	60°C	TC84899 <sup>2</sup>	New design
	SsLPL-R6	5'-GACCTTTCAAAGGGCATGA-3'				
Reference genes:						
Elf-1α	ELF-1A jbt2	5'-CTGCCCCCTCCAGGACGTTTACAA-3'	175 bp	60°C	AF321836 <sup>1</sup>	Leaver et al. (2008)
	ELF-1A jbt2	5'-CACCGGGCATAGCCGATTCC-3'				
β-actin	BACT-F	5'-ACATCAAGGAGAAGCTGTGC-3'	141 bp	56°C	AF012125 <sup>1</sup>	Leaver et al. (2008)
	BACT-R	5'-GACAACGGAACCTCTCGTTA-3'				
Cofilin-2	B2F	5'-AGCCTATGACCAACCCACTG-3'	224 bp	60°C	TC63899 <sup>2</sup>	Leaver et al. (2008)
	B2R	5'-TGTTACAGCTCGTTTACCG-3'				

<sup>1</sup> GenBank (<http://www.ncbi.nlm.nih.gov/>)<sup>2</sup> Atlantic salmon Gene Index (<http://compbio.dfci.harvard.edu/tgi/>)

\* Primer was designed in the region of the sequence corresponding only to the C terminal half of ApoB-100 (i.e., not containing the N-terminal region which is common to ApoB-48)

\*\* Corresponding to TC91040 (Atlantic salmon Gene Index), which does not align with TC sequences from LPLb or LPLc.

\*\*\* TC67836 (LPLb) and TC84899 (LPLc) have 85% identity in the aligned region (90% query coverage).

Table 2. Genes involved in lipid metabolism whose expression in the liver transcriptome showed a significant diet  $\times$  family interaction (identified by two way-ANOVA), revealing transcripts whose level of expression is dependent on the combined effects of both factors. Indicated are also the accession numbers for each clone; the expression ratios between fish fed VO and those fed FO, for each one of the families, and between Lean and Fat fish fed either FO or VO; the p-value; and the position of the feature in the interaction list (n= 529 total features) ordered by ascending p-value.

Accession no	Gene	VO/FO		Lean/Fat		p-value	Position in Sig. list
		Lean	Fat	FO	VO		
BM413891	Angiopoietin-like 4	1.4	- 1.1	- 1.2	1.3	0.0010	13
CK890036	Lipoprotein lipase	- 1.3	1.6	1.4	- 1.5	0.0026	20
CO470568	Lipoprotein lipase	- 1.1	1.3	1.2	- 1.2	0.0033	28
CO472476	Lipoprotein lipase	- 1.2	1.7	1.2	- 1.7	0.0045	38
BI468033	ATP-binding cassette sub-family A member 1	- 2.0	1.1	- 1.0	- 2.2	0.0051	45
CK883097	5'-AMP-activated protein kinase (AMPK) subunit gamma-3	- 1.0	1.8	1.1	- 1.6	0.0053	46
CK875291	Isopentenyl-diphosphate isomerase	2.0	- 1.2	- 1.9	1.3	0.0055	49
CK894278	Carnitine O-acetyltransferase	1.2	- 1.2	- 1.2	1.2	0.0056	50
EG648040	Acyl-Coenzyme A-binding protein	1.7	1.0	- 1.5	1.1	0.0103	89
CK880279	Delta3,5-delta2,4-dienoyl-CoA isomerase	1.2	- 1.1	- 1.3	1.1	0.0128	114
BM414066	Endothelial lipase precursor	- 1.2	1.2	- 1.1	- 1.6	0.0166	145
BM414094	Phosphatidylethanolamine N-methyltransferase	1.2	- 1.4	- 1.5	1.1	0.0167	147
GU294485	Delta-5 fatty acyl desaturase	2.1	1.2	- 1.7	1.0	0.0179	159
CK879648	Squalene monooxygenase (Squalene epoxidase)	1.9	1.1	- 1.9	- 1.1	0.0224	215
AJ425698	Acetyl-CoA acetyltransferase 2	1.1	- 1.1	- 1.1	1.1	0.0244	238
AY736067	Delta-6 fatty acyl desaturase	2.1	1.4	- 1.3	1.1	0.0317	320
BM413811	Enoyl Coenzyme A hydratase 1	- 1.1	1.3	1.3	- 1.1	0.0403	420

Table 3. Relative analysis of gene expression (REST2008) of genes involved in cholesterol biosynthesis and its regulation, cholesterol transport/cellular efflux,  $\beta$ -oxidation and lipoprotein metabolism, assayed by RT-qPCR in liver of two groups of Atlantic salmon (Lean and Fat families), after a year of feeding diets containing either 100% FO or 100% VO. Values are normalised (by *cofilin-2*) gene expression ratios (up-regulation if >1 and down-regulation if <1) and p-values, when each group is fed either a 100% FO or 100% VO diet or when comparing each one of the groups fed either one of the diets.

Genes	VO/FO				Lean/Fat			
	Lean		Fat		FO		VO	
	Ratio	p-value	Ratio	p-value	Ratio	p-value	Ratio	p-value
Cholesterol biosynthesis, regulation and transport								
HMG-CoA	0.78	0.642	0.90	0.697	1.02	0.947	0.89	0.795
MEV	1.88	0.109	0.91	0.757	0.53	0.116	1.09	0.697
IPI	1.41	0.612	0.49	0.236	0.38	0.280	1.10	0.741
DHCR7	1.27	0.405	1.06	0.826	0.65	0.190	0.78	0.304
SREBP2	2.06	0.134	1.36	0.575	0.65	0.474	0.99	0.964
ABCA1	0.78	0.504	1.67	0.111	1.12	0.841	0.52	0.016
$\beta$ -oxidation								
ACO	0.93	0.809	1.28	0.369	1.46	0.278	1.06	0.786
CPT1	0.86	0.381	0.75	0.101	1.02	0.890	1.17	0.330
Lipoprotein metabolism								
ApoAI	1.67	0.195	2.39	0.071	0.85	0.753	0.59	0.039
ApoCII	1.28	0.527	1.70	0.065	1.23	0.589	0.93	0.646
ApoB	1.40	0.443	1.84	0.152	0.87	0.791	0.66	0.076
SR-BI	0.66	0.075	0.96	0.859	0.92	0.687	0.63	0.049
LDLR	0.59	0.234	0.67	0.319	0.68	0.401	0.60	0.059
EL	3.52	0.034	8.57	0.002	1.38	0.494	0.57	0.115
LPLa	0.87	0.631	0.89	0.789	0.76	0.573	0.75	0.119
LPLb	0.43	0.053	2.75	0.067	1.57	0.375	0.24	0.010
LPLc	0.95	0.762	2.09	0.011	0.98	0.929	0.45	0.002

HMG-CoA: 3-hydroxy-3-methyl-glutaryl-CoA reductase; MEV: Mevalonate kinase; IPI: Isopentenyl diphosphate isomerase; DHCR7: D- 7-dehydrocholesterol reductase; SREBP2: Sterol-responsive element-binding protein 2; ABCA1: ATP-binding cassette, sub-family A, member 1; ACO: acyl-CoA oxidase; CPT1: carnitine palmitoyltransferase I; ApoAI: Apolipoprotein AI; ApoCII: Apolipoprotein CII; ApoB100: Apolipoprotein B100; SR-BI: Scavenger receptor class B type 1; LDLR: Low density lipoprotein receptor; EL: Endothelial lipase; LPLa: Lipoprotein lipase , TC91040; LPLb: Lipoprotein lipase , TC67836; LPLc: Lipoprotein lipase, TC84899.

Table 4. Levels of circulating plasma (mM) or lipoprotein (VLDL, LDL and HDL;  $\mu\text{mol/mL}$  plasma) cholesterol and triacylglycerols (TAG) in Atlantic salmon Lean and Fat families, determined after a year of feeding diets containing either 100% FO or 100% VO. Significance levels of two-way ANOVA are indicated for the factors “diet”, “family” and interaction “diet  $\times$  family”.

	Lean				Fat				ANOVA p-value		
	FO		VO		FO		VO		Diet	Family	D×F
	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
<i>Cholesterol</i>											
Plasma	8.87	0.66	6.92	0.15	8.10	0.80	6.87	0.23	0.014	0.340	0.396
VLDL	0.11	0.01	0.06	0.00	0.06	0.02	0.06	0.01	0.062	0.075	0.046
LDL	0.96	0.10	0.65	0.10	0.64	0.17	0.55	0.12	0.089	0.080	0.278
HDL	5.91	0.45	6.11	0.94	6.05	2.45	5.33	0.67	0.808	0.760	0.661
<i>TAG</i>											
Plasma	2.03	0.08	2.09	0.12	1.89	0.01	1.87	0.07	0.742	0.037	0.519
VLDL	0.22	0.05	0.16	0.00	0.16	0.01	0.18	0.02	0.371	0.287	0.107
LDL	0.45	0.01	0.40	0.02	0.39	0.01	0.37	0.02	0.068	0.018	0.265
HDL	2.63	0.15	2.59	0.29	2.48	0.96	2.28	0.22	0.766	0.562	0.845

Table 5. Liver and flesh total lipids (g/100g of wet weight) and lipid class composition (% total lipid) in Atlantic salmon Lean and Fat families, determined by thin-layer lipid chromatography (TLC), after a year of feeding diets containing either FO or VO. Steryl esters were not detected in flesh.

	Lean				Fat				ANOVA p-value		
	FO		VO		FO		VO		Diet	Family	D×F
	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
<i>Liver</i>											
Total lipids	4.0	0.3	4.1	0.1	3.4	0.1	4.8	0.4	0.015	0.799	0.024
Phospholipids	44.3	0.6	38.3	0.6	46.7	2.0	36.9	2.9	0.000	0.608	0.057
Triacylglycerols	29.8	0.8	37.6	2.2	26.7	3.1	39.8	3.8	0.000	0.776	0.072
Free fatty acids	2.0	0.3	1.8	1.0	2.9	0.9	1.7	0.4	0.082	0.298	0.204
Sterols	15.3	0.2	12.8	0.5	14.4	0.5	12.4	1.0	0.000	0.058	0.420
Steryl esters	7.1	1.2	7.2	1.7	8.5	1.5	6.9	2.4	0.408	0.574	0.348
<i>Flesh</i>											
Total lipids	11.6	0.2	12.8	0.3	13.2	0.2	12.9	0.2	0.050	0.006	0.010
Phospholipids	13.3	0.2	11.3	0.9	14.4	1.1	12.4	0.7	0.000	0.016	0.942
Triacylglycerols	74.1	1.4	74.6	1.1	72.5	2.8	73.7	0.5	0.326	0.153	0.683
Free fatty acids	5.6	1.0	5.0	0.8	5.7	1.0	5.7	0.5	0.438	0.402	0.485
Sterols	7.0	0.5	9.1	0.5	7.4	1.0	8.2	0.5	0.001	0.482	0.090

## Figure legends

**Fig. 1.** Normalised gene expression levels (obtained by dividing the number of copies of the target gene by the number of copies of *cofilin-2*) of genes involved in cholesterol biosynthesis and its regulation, cholesterol transport/cellular efflux and lipoprotein metabolism, determined by RT-qPCR in liver of two groups of Atlantic salmon (Lean and Fat families), after a year of feeding diets containing either 100% FO or 100% VO. A- 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA); B- Mevalonate kinase (MEV); C- Isopentenyl diphosphate isomerase (IPI); D- 7-dehydrocholesterol reductase (DHCR7); E- Sterol-responsive element-binding protein 2 (SREBP2); F- ATP-binding cassette, sub-family A, member 1 (ABCA1); G- Apolipoprotein AI (apoAI); H- Apolipoprotein CII (apoCII); I- Apolipoprotein B100 (apoB100); J- Scavenger receptor class B type 1 (SR-BI); K- Low density lipoprotein receptor (LDLR); L- Endothelial lipase (EL); M- Lipoprotein lipase, TC91040 (LPLa); N- Lipoprotein lipase, TC67836 (LPLb); O- Lipoprotein lipase, TC84899 (LPLc). Significance levels of two-way ANOVA are indicated for the factors “diet”, “family” and interaction “diet x family”, when  $p < 0.05$  (ns, not significant).



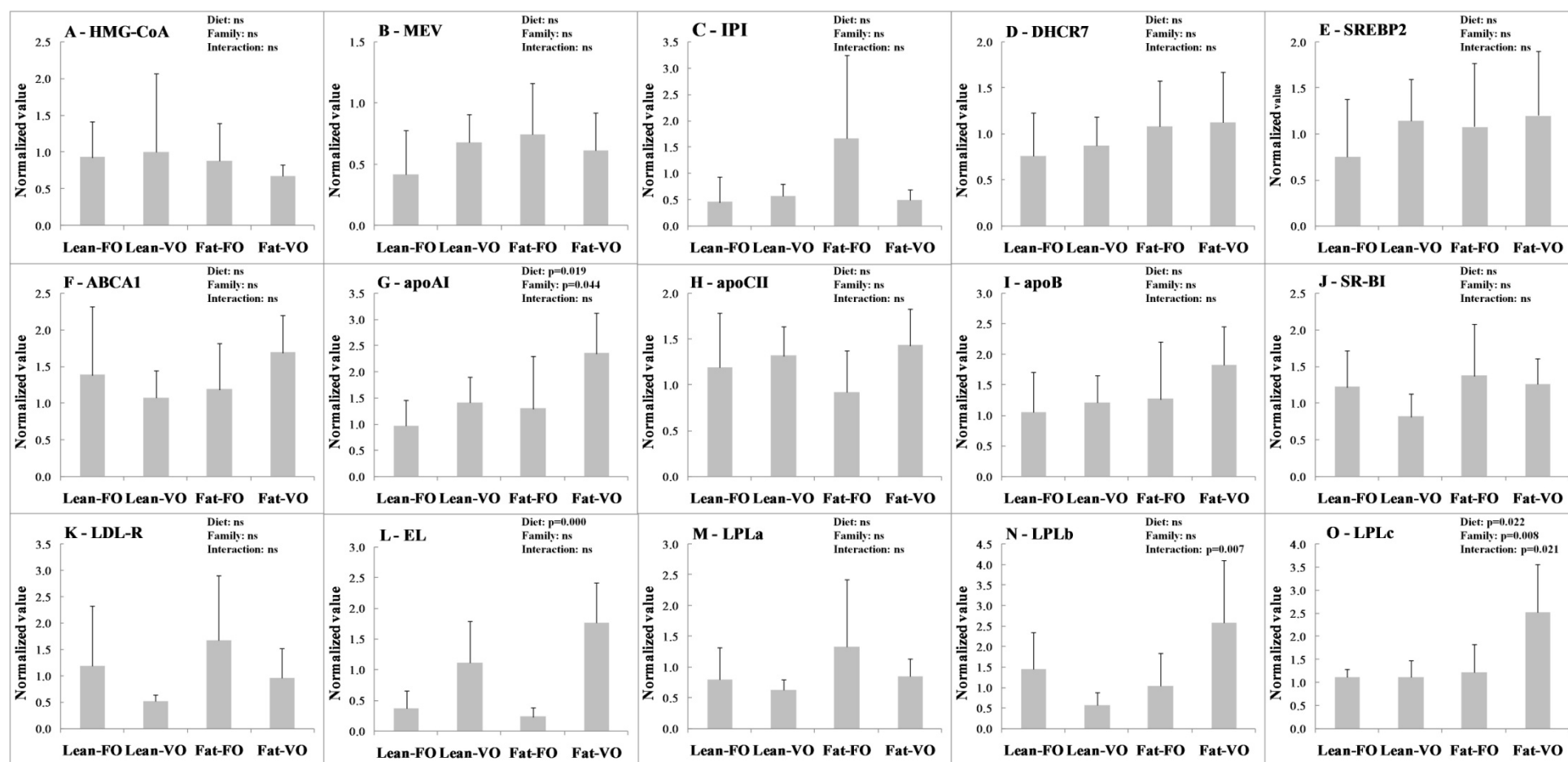


Fig. 1