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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Running title: Cholesterol and lipoprotein metabolism in salmon

Keywords: fish diets: aquaculture alternative diets: family genetic selection: liver transcriptome

Abstract

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

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

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

Early studies on dietary FO replacement in salmon suggested that high inclusion levels of certain VO might negatively affect fish health and resistance to stress by changing cardiac membrane fatty acid composition, and diets containing sunflower oil were reported to result in considerable cardiomyopathy, extensive thinning and necrosis of the ventricular muscle wall [6,7]. Other studies could not directly show an involvement of dietary fatty acid composition on the development of arteriosclerotic changes in Atlantic salmon but could not exclude it either [8]. This is therefore an area that is still open for discussion. On the other hand, a relationship between nutritional factors, especially dietary level of n-3 LC-PUFA, and risk of developing atherosclerosis has been well demonstrated in mammals and, furthermore, genetic polymorphisms/variants have been identified in several genes involved in cholesterol and lipoprotein metabolism that can explain different susceptibilities and responses to diet [9-12]. 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 [13] 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^3 net pens at the Ardnish Fish Trials Unit (Marine Harvest Scotland, Lochailort, Scotland; 500 fish pen^{-1}). 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

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

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

RNA extraction and purification

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

Microarray hybridisations and analysis

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

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

Antisense amplified RNA (aRNA) was produced from each total RNA isolation using the Amino Allyl MessageAmpTM II aRNA Amplification Kit (Ambion, Applied Biosystems), following the manufacturer's methodology, followed by Cy3 or Cy5 fluor incorporation through a dye-coupling reaction. Briefly, 500 ng of total RNA were amplified and column-purified according to manufacturer's instructions including a 17 h transcription step, and aRNA quantified and quality assessed as above. Cy dye suspensions (Cy3 & Cy5) in sufficient quantity for all labelling reactions were prepared by adding 36 µL high purity dimethyl sulphoxide (Stratagene, Hogehilweg, The Netherlands) to each tube of Cy dye (PA23001 or PA25001, GE HealthCare, Little Chalfont, UK). To attach the Cy dyes, 3 µg each aRNA sample was suspended in 6 µL nuclease-free H_2O and heated to 70 ºC for 2 min. When cooled to room temperature, 2 µL of coupling buffer (0.5 M NaHCO3; pH 9.2) and 2 µL of Cy3 dye suspension stock was added and then incubated for 1 h at 25ºC in the dark. For labelling the common pooled reference sample with Cy5, a scaled-up reaction was similarly performed. Unincorporated dye was removed by column purification (Illustra AutoSeq G-50 spin columns; GE Healthcare). Dye incorporation and aRNA yield were quantified by spectrophotometry (NanoDrop ND-1000) and quality controlled by separating 0.4 µL on a thin mini-agarose gel and visualising products on a fluorescence scanner (Typhoon Trio, GE Healthcare).

Microarray hybridisations were performed in a Lucidea semi-automated system (GE Healthcare), without a pre-hybridisation step. For hybridisation of each array, each labelled biological replicate and corresponding pooled reference (40 pmol each dye, c. 150 ng aRNA) were combined and volume made up to 25 µL with nuclease-free water. After heating the aRNA at 95 ºC for 3 min in a thermocycler, 225 µL of pre-heated (60 ºC) hybridisation solution, comprising 185 µL 0.7X UltraHyb buffer (Ambion), 20 µL poly(A) at l0 mg/mL (Sigma-Aldrich, Dorset, UK), 10 µL

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

Scanning was performed at 10 μm resolution using an Axon GenePix 4200AL Scanner (MDS Analytical Technologies, Wokingham, Berkshire, U.K.). Laser power was kept constant (80%) and the "auto PMT" function within the acquisition software (v.4) was enabled to adjust PMT for each channel such that less than 0.1% of features were saturated and that the mean intensity ratio of the Cy3 and Cy5 signals was close to one. BlueFuse software (BlueGnome, Cambridge, U.K.) was used to identify features and extract fluorescence intensity values from the resultant TIF images. Following a manual spot removal procedure and fusion of duplicate spot data (BlueFuse proprietary algorithm), the resulting fluorescence intensity data and quality annotations for the 17,102 gene features were exported into the GeneSpring GX version 10.0.2 analysis platform (Agilent Technologies, Wokingham, Berkshire, U.K.) after undergoing a block Lowess normalisation. All control features were excluded from subsequent analyses. Data transformation and quality filtering were as follows: (a) all intensity values <1 were set to 1 and (b) data were filtered using a Blue-Fuse spot confidence value >0.3 in at least 75 % of the values in any 2 out of 4 conditions and BlueFuse spot quality of ≥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 [15]. 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 [16]. 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) (Table 1). Amplification of three potential reference genes, *cofilin-2*, elongation factor-1α (*elf-1α*) and *β-actin*, was performed but only *cofilin-2* expression proved to be stable across treatments.

For RT-qPCR, one µg 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 (Quantica, Techne, Cambridge, U.K.) in a final volume of 20 µL containing either 5 µL (for most genes) or 2

208 µL (for the reference genes and other highly expressed genes) diluted (1/20) cDNA, 0.5 µM of each
209 primer and 10 µL AbsoluteTM QPCR SYBR® 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 Tm (Table 1) and 15 s at 72 °C. After the amplification phase, a melt curve of 0.5 °C
213 increments from 75 ºC 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-
217 quantification.info/), which employs a pair wise fixed reallocation randomisation test (10,000
218 randomisations) with efficiency correction [17], 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 [18,19] as described by Lie *et al.* [20] at 197 600 × *g* and 4°C (Beckman Optima^{TM}XL-100K
226 Ultracentrifuge and SW41Ti rotor). Density intervals were obtained by addition of solid KBr [21],
227 and run time for separation of lipoproteins was: VLDL, d<1.015 g/mL for 20 h; LDL, 1.015 g/mL
228 < d < 1.085 g/mL for 20 h and HDL, 1.085 g/mL < d < 1.21 g/mL for 44 h.
229
230 *Lipid class analyses*
231 Total lipids were extracted from flesh or liver according to Folch et al. [22] and tissue lipid class
232 compositions determined by single-dimension double-development high-performance thin-layer
233 chromatography (HPTLC) and densitometry as described previously [23].

Statistical analysis

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

Results

Microarray data

In order to identify genes involved in lipid metabolic processes whose expression is dependent on the combined effects of both diet and family, i.e., for which the effect of diet depends on family, the two-way ANOVA interaction list obtained from the analysis of the microarray data was examined. This list contained 529 features that were significantly differentially regulated of which 17 features (corresponding to 15 genes) were related to lipid metabolism (Table 2). The top 100 genes, sorted by p-value, were categorised according to function and the lipid metabolism category corresponded to 15 % of the total annotated genes (and excluding genes of miscellaneous function). The lipid metabolism genes found in the interaction list can be broadly described as being involved in the following processes: cholesterol/ isoprenoid biosynthesis (IPI-isopentenyl-diphosphate isomerise, SQLE-squalene monooxygenase/epoxidase and ACAT2-acetyl-CoA acetyltransferase 2), cholesterol transport/cellular efflux (ABCA1-ATP-binding cassete transporter A1), lipoprotein metabolism (Angptl4-angiopoietin-like 4, LPL-lipoprotein lipase, EL-endothelial lipase), β-oxidation (CRAT-carnitine O-acetyltransferase, ECH1-delta3,5-delta2,4-dienoyl-CoA isomerase and ECHS1-enoyl Coenzyme A hydratase 1), fatty acid synthesis (Δ5 and Δ6 fatty acyl desaturase, Fad) and transport (ACBP-acyl-Coenzyme A-binding protein), glycerophospholipid/

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

RT-qPCR analysis of gene expression

Relative gene expression of a series of genes involved in some of the preponderant lipid metabolism pathways mentioned above, mostly associated to cholesterol biosynthesis and its regulation and transport, fatty acid β-oxidation and lipoprotein metabolism, was determined by RT-qPCR (Table 3). This included some genes found in the significant interaction list from the microarray analysis (IPI, ABCA1, EL and LPL). Although the fold-changes obtained by both methods (microarray and RT-qPCR) differed, the general trend was similar for both IPI and ABCA1. In the case of IPI, although differences were not statistically significant there was a clear trend for it to be up-regulated when VO replaced FO in the diet in the Lean fish and down-regulated in the Fat group. This difference appears to result from a lower expression of IPI in Lean fish, compared to Fat, when they are fed the FO-diet. In contrast, the opposite was observed with ABCA1, with a trend for down-regulation in Lean fish fed VO compared to FO and an up-regulation in the Fat fish. In this case, gene expression was significantly lower in the Lean group compared to the Fat, when fed the VO diet. Agreement between the RT-qPCR and microarray results initially proved problematic for EL and LPL but, on closer examination, multiple transcripts for both genes were identified. In the case of EL, an EST was identified in the GenBank database (DY694576) that is 86% identical, in the aligned area, to the Atlantic salmon EL reference sequence (NM_001140535), and this is likely to have resulted in cross-hybridisation in the microarray. Indeed, an initially tested primer pair showed very similar fold changes as those obtained in the microarray experiment and was later found to have amplified both sequences. When RT-qPCR was repeated using primers specific for NM_001140535, quite different results were obtained, with significant up-regulation being observed in both Lean and Fat families fed VO compared to FO. On

286 the other hand, determination of LPL expression was initially performed using a primer pair
287 available in the literature (here termed LPLa) but this resulted in a pattern of expression not
288 corresponding to the microarray results. Further investigation revealed that the three LPL clones in
289 the microarray significant interaction list correspond to two different clusters (DFCI-The Gene
290 Index Project; http://compbio.dfci.harvard.edu/tgi/tgipage.html), TC67836 and TC84899, which are
291 85 % identical to each other in the aligned region (90 % query coverage), and which we here named
292 LPLb and LPLc, respectively. The published LPLa primers amplify a sequence corresponding to
293 TC91040, which does not align with TC sequences from LPLb or LPLc. The primers designed for
294 LPLb and LPLc gave comparable results to the microarray experiment, and broadly similar to each
295 other, with a trend for down-regulation in Lean fish when fed the VO diet instead of FO and an up-
296 regulation (significant for LPLc) in Fat fish fed the VO diet. In both cases, this was associated with
297 a significantly lower expression of these transcripts in Lean, compared to the Fat group, when fish
298 were fed the VO diet.

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

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

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

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

Lipid biochemical composition of plasma, lipoprotein classes, liver and flesh

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

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

Discussion

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

Cholesterol metabolism

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

cholesterol absorption [25]. Similarly, the lipid composition of liver, with higher levels of sterol in fish fed FO, probably also reflects dietary cholesterol intake.

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

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

Lipoprotein synthesis and hepatic TAG metabolism

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

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

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

Lipoprotein uptake and reverse cholesterol transport

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

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

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

The expression of ABCA1 also responded differently to diet depending on the genetic background of the fish with a trend for lower expression in the Lean fish fed VO, compared to FO. ABCA1 is a membrane transporter with roles in HDL synthesis and reverse cholesterol transport and thus this result may be related to either HDL metabolism or cholesterol biosynthesis [27]. However, its involvement in VLDL and LDL metabolism has recently been shown with deletion of the ABCA1 gene leading to increased VLDL production and elevated plasma TAG accompanied by enhanced LDL clearance through overexpression of hepatic LDLR [45]. Although we cannot exclude 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 [46,47]. 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 [48]. In addition, it increases flux of cholesterol to the liver through enhanced reverse transport from peripheral tissues [27]. 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 [49]. Finally, LPL and EL can both influence lipoprotein metabolism by catalysing the hydrolysis of TAG and PL, respectively, thus facilitating lipoprotein catabolism and clearance [39,43]. 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 [44]. 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 [6,7].

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

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

Conclusions

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

Acknowledgements

This study was funded by the EU FP6 IP "AQUAMAX" (Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for Consumers; 016249-2). SM was supported by a Marie Curie Intra European Fellowship (FP7-PEOPLE-2007-2-1-IEF, Proposal Nº219667) and JP by a Royal Thai Government Scholarship. The authors would like to acknowledge Landcatch Ltd for provision of the Lean and Fat smolts and to thank the staff of Marine Harvest Ltd, Ardnish FTU, for their assistance with fish husbandry and sample collection. Authors responsibilities were as follows: SM, JP and BET performed laboratory analyses; DRG was responsible for family selection; JBT

supported the microarray analysis; SM and BET analysed the data; SM wrote the manuscript; SM, JGB and DRT planned the research; DRG, JGB and DRT were project leaders. There are no conflicts of interest to report.

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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'-CCTTCAGCCATGAACTGGAT-3'	224 bp	60ºC	TC102374 [2]	Leaver et al. (2008)
	HMG-1R	5'-TCCTGTCCACAGGCAATGTA-3'				
MEV	MEV-1F	5'-CCCTTAATCAGGGTCCCAAT-3'	247 bp	60ºC	DW005667 [1]	Leaver et al. (2008)
	MEV-1R	5'-GGTGCTGGTTGATGTCAATG-3'				
IPI	23-3p jbt1F	5'-ACAGCCCTATGGTTATGTGTCATCTC-3'	230 bp	60ºC	CK875291 [1]	Leaver et al. (2008)
	23-3p jbt1R	5'-CAAGGTGAGGCGAATGTTTGAAC-3'				
DHCR7	7DCHR-1F	5'-CTTCTGGAATGAGGCATGGT-3'	230 bp	60ºC	TC99602 [2]	Leaver et al. (2008)
	7DCHR-1R	5'-ACAGGTCCTTCTGGTGGTTG-3'				
SREBP2	SREBP2-1F	5'-GACAGGCACAACACAAGGTG-3'	215 bp	60ºC	DY733476 [1]	Leaver et al. (2008)
	SREBP2-1R	5'-CAGCAGGGGTAAGGGTAGGT-3'				
ABCA1	ABCA1-UTR-F2	5'- GGACGAACCCTGTGTCTGTT -3'	203 bp	60ºC	EG836783 [1]	New design
	ABCA1-UTR-R2	5'- ATTTGCATTGCGTTTCAGTG -3'				
CPT1	CPT1-1F	5'-CCTGTACCGTGGAGACCTGT-3'	212 bp	60ºC	AM230810 [1]	Leaver et al. (2008)
	CPT1-1R	5'-CAGCACCTCTTTGAGGAAGG-3'				
ACO	ACO-2F	5'-AAAGCCTTCACCACATGGAC-3'	230 bp	60ºC	TC49531 [2]	Leaver et al. (2008)
	ACO-2R	5'-TAGGACACGATGCCACTCAG-3'				
apoAI	SsApoAI-F1	5'-CCATCAGCCAGGCCATAAA-3'	73 bp	60ºC	CB506105 [1]	Kleveland et al. (2006)
	SsApoAI-R1	5'-TGAGTGAGAAGGGAGGGAGAGA-3'				
apoCII	SsApoCII-F1	5'-GGAACCAGTCGCAGATGTTGA-3'	145 bp	60ºC	DN047858 [1]	Kleveland et al. (2006)
	SsApoCII-R1	5'-TGAGGACATTCGTGGCCTTC-3'				
apoB100	SsApoBfQ	5'-AGCCTTCGATGCTGTCGGCCA-3'	153 bp	60ºC	TC79364 [2*]	New design
	SsApoBrQ	5'-AGGAGCACAGGCAGGGTGGTT-3'				
SR-BI	SsSRBI-F1	5'-AACTCAGAGAAGAGGCCAAACTTG-3'	204 bp	60ºC	DQ266043 [1]	Kleveland et al. (2006)
	SsSRBI-R1	5'-TGCGGCGGTGATGATG-3'				
LDLR	SsLDLR-F1	5'-GCATGAACTTTGACAATCCAGTGTAC-3'	78 bp	60ºC	AJ003118 [1]	Kleveland et al. (2006)
	SsLDLR-R1	5'-TGGAGGAGTGCCTGCTGATAT-3'				
EL	SsEL-F4	5'-CCGGTGCTGCTGGAGGAAGC-3'	378 bp	60ºC	NM_001140535 [1]	New design
	SsEL-R5	5'-CGACATGCAGGTCATCGGT-3'				
LPLa	SsLPL-F1	5'-TGCTGGTAGCGGAGAAAGACAT-3'	114 bp	60ºC	BI468076 [1**]	Kleveland et al. (2006)
	SsLPL-R1	5'-CTGACCACCAGGAAGACACCAT-3'				
LPLb	SsLPL-F4	5'-GGCAGCCCTACATGATAACC-3'	172 bp	60ºC	TC67836 [2***]	New design
	SsLPL-R4	5'-TCTGTCCAAAGCCACTCACA-3'				
LPLc	SsLPL-F6	5'-AGGGCGTTAATCCATGTCAG-3'	223 bp	60ºC	TC84899 [2]	New design
	SsLPL-R6	5'-GACCTTTCAAAAGGGCATGA-3'				
Reference genes:						
Elf-1α	ELF-1A jbt2	5'-CTGCCCCTCCAGGACGTTTACAA-3'	175 bp	60ºC	AF321836 [1]	Leaver et al. (2008)
	ELF-1A jbt2	5'-CACCGGGCATAGCCGATTCC-3'				
β-actin	BACT-F	5'-ACATCAAGGAGAAGCTGTGC-3'	141 bp	56ºC	AF012125 [1]	Leaver et al. (2008)
	BACT-R	5'-GACAACGGAACCTCTCGTTA-3'				
Cofilin-2	B2F	5'-AGCCTATGACCAACCCACTG-3'	224 bp	60ºC	TC63899 [2]	Leaver et al. (2008)
	B2R	5'-TGTTCACAGCTCGTTTACCG-3'				

[1] GenBank (http://www.ncbi.nlm.nih.gov/)

[2] 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 × 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, β-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.

	VO/FO				Lean/Fat			
	Lean		Fat		FO		VO	
Genes	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
β-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; μmol/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 × family".

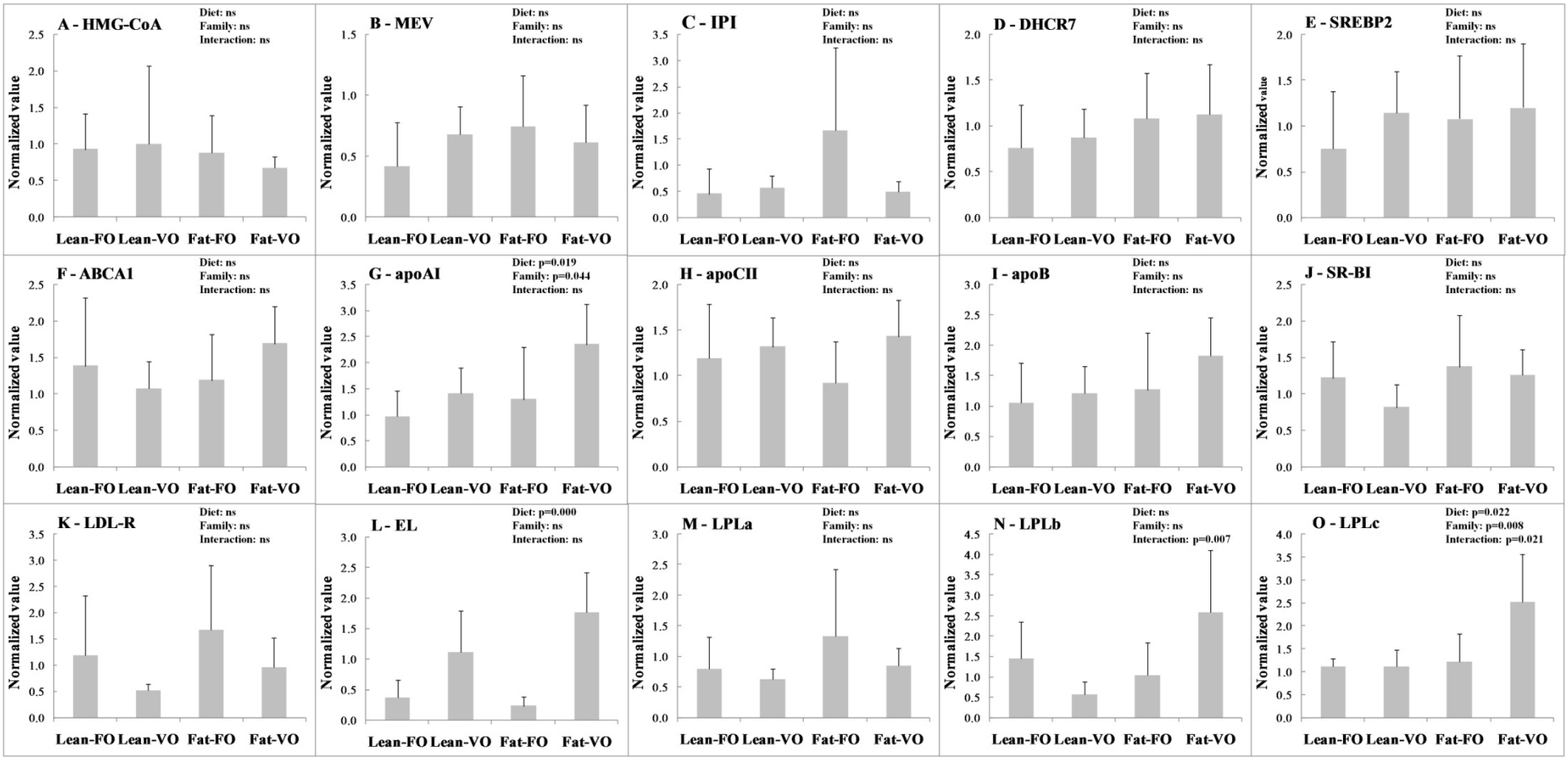
	Lean				Fat				ANOVA p-value		
	FO		VO		FO		VO		Diet	Family	D×F
	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
Cholesterol											
Plasma	8.87	0.66	6.92	0.15	8.10	0.80	6.87	0.23	0.014	0.340	0.396
VLDL	0.11	0.01	0.06	0.00	0.06	0.02	0.06	0.01	0.062	0.075	0.046
LDL	0.96	0.10	0.65	0.10	0.64	0.17	0.55	0.12	0.089	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
TAG											
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			
Liver											
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
Flesh											
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 c*ofilin-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).


A - HMG-CoA
Diet: ns
Family: ns
Interaction: ns
B - MEV
Diet: ns
Family: ns
Interaction: ns
C - IPI
Diet: ns
Family: ns
Interaction: ns
D - DHCR7
Diet: ns
Family: ns
Interaction: ns
E - SREBP2
Diet: ns
Family: ns
Interaction: ns
F - ABCA1
Diet: ns
Family: ns
Interaction: ns
G - apoAI
Diet: p=0.019
Family: p=0.044
Interaction: ns
H - apoCII
Diet: ns
Family: ns
Interaction: ns
I - apoB
Diet: ns
Family: ns
Interaction: ns
J - SR-BI
Diet: ns
Family: ns
Interaction: ns
K - LDL-R
Diet: ns
Family: ns
Interaction: ns
L - EL
Diet: p=0.000
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Interaction: ns
M - LPLa
Diet: ns
Family: ns
Interaction: ns
N - LPLb
Diet: ns
Family: ns
Interaction: p=0.007
O - LPLc
Diet: p=0.022
Family: p=0.008
Interaction: p=0.021
Normalized value
Lean-FO Lean-VO Fat-FO Fat-VO

Fig. 1