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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

Sofia Morais[1]*, Jarunan Pratoomyot[1], Bente E. Torstensen[2], John B. Taggart[1], Derrick R. Guy[3], J. Gordon Bell[1] and Douglas R. Tocher[1]

[1]*Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK*
[2]*National Institute of Nutrition and Seafood Research, PO Box 2029 Nordnes, Bergen N-5817, Norway*
[3]*Landcatch Natural Selection, Cooperage Way Business Centre, Alloa FK10 3LP, Scotland, UK*

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Abstract

The present study investigates the 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 a fish oil (FO) or a vegetable oil (VO) blend indicated that pathways of cholesterol and lipoprotein metabolism might be differentially affected by the diet depending on the genetic background of the fish, and this was further investigated by real-time quantitative PCR, 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 with those fed VO in both family groups. Significantly lower levels of plasma TAG and LDL-TAG were measured in the fat group that was 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. A significantly lower expression of genes considered anti-atherogenic in mammals (ATP-binding cassette transporter A1, apoAI, scavenger receptor class B type 1, lipoprotein lipase (LPL)b (TC67836) and LPLc (TC84899)) was found in lean fish, compared with fat fish, when fed VO. Furthermore, the lean family group appeared to show a greater response to diet composition in the cholesterol biosynthesis pathway, mediated by sterol-responsive element-binding protein 2. Finally, the presence of three different transcripts for LPL, with differential patterns of nutritional regulation, was demonstrated.

Key words: Fish diets: Aquaculture alternative diets: Family genetic selection: Liver transcriptome

As the worldwide demand for seafood continues to grow and traditional fisheries are at best stable or in decline, aquaculture production needs 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 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 fish meal 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 vegetable oil (VO) can replace up to 100 % of FO in salmonid diets without compromising fish growth or condition, but above 50 % of FO replacement there is a significant reduction that is observed in the tissue levels of *n*-3 long-chain PUFA (LC-PUFA), namely EPA and DHA, diminishing the beneficial, health-promoting, nutritional profile for human consumption[(2–4)]. There is now evidence that *n*-3 LC-PUFA level in flesh 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 fish meal 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

Abbreviations: ABCA1, ATP-binding cassette transporter A1; aRNA, amplified RNA; cDNA, complementary DNA; EL, endothelial lipase; FO, fish oil; IPI, isopentenyl diphosphate isomerise; LC-PUFA, *n*-3 long-chain PUFA; LPLa, lipoprotein lipase (TC91040); LPLb, lipoprotein lipase b (TC67836); LPLc, lipoprotein lipase c (TC84899); RT-qPCR, real-time quantitative PCR; SR-BI, scavenger receptor class B type 1; SREBP2, sterol-responsive element-binding protein 2; VO, vegetable oil.

* **Corresponding author:** S. Morais, fax +44 1786 472133, email sofia.morais@stir.ac.uk

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 have 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 have been 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 in 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 the 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 present study was to further explore the potential influence of dietary oil source on cholesterol and lipoprotein metabolism, which may ultimately affect the 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 from microarray analysis of the liver 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 Limited breeding programme (Argyll, Scotland). Choice was based on estimated breeding values of the parents for high or low flesh adiposity, assessed by Torry Fatmeter (Distell Industries, Bathgate, 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 estimated breeding value distribution for flesh lipid content ('lean') and two families from the extreme upper end of the distribution ('fat'). The average estimated breeding values 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.

A total of 2000 fish of each group were stocked into eight $12 \times 5\,m^3$ net pens at the Ardnish Fish Trials Unit (500 fish/pen; Marine Harvest Scotland, Lochailort, Scotland). Each group was fed one of the two experimental diets (Skretting ARC, Stavanger, Norway) formulated to fully satisfy the nutritional requirements of salmonid fish for 55 weeks until reaching approximately 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 or as a VO blend comprising rapeseed, palm and Camelina oils in a ratio of 5:3:2. Diets contained similar levels of PUFA (approxiamately 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, twenty-five fish were sampled per pen, killed by a blow to the head following anaesthesia using MS222 (tricaine methanesulfonate). Samples of the liver were collected for molecular analyses and stored at −80°C. Additionally, ten samples of liver and flesh (Norwegian Quality Cut) were collected per pen and stored at −20°C pending biochemical analysis. For flesh and liver lipid analyses, four pools comprising five fish per pool were prepared from the duplicate pens per family and diet[13]. Blood was collected from the caudal vein using EDTA vacutainers from five 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 the present 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 2 ml of TRI Reagent (Ambion/Applied Biosystems, Warrington, UK). Total RNA was isolated following the manufacturer's instructions; 100 μg of total RNA were further purified by mini spin-column (RNeasy Mini Kit; Qiagen, Crawley, West Sussex, UK), and RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE, USA).

Microarray hybridisations and analysis

The TRAITS/SGP (Transcriptome Analysis of Important Traits of Salmon/Salmon Genome Project; version 2.1) salmon 17k complementary DNA (cDNA) microarray (ARK-Genomics, Roslin Institute, Roslin, UK) 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, containing equal amounts of all samples, which permits valid statistical comparisons to be made across all treatments. The entire experiment comprised twenty-four hybridisations: two lipid phenotype groups (lean/fat) × two diets (FO/VO) × six biological replicates.

Antisense amplified RNA (aRNA) was produced from each total RNA that was isolated using the Amino Allyl

MessageAmpTM II aRNA Amplification Kit (Ambion/Applied Biosystems), following the manufacturer's methodology, followed by cyanine (Cy) 3 or Cy5 fluor incorporation through a dye-coupling reaction. Briefly, 500 ng of total RNA were amplified and column-purified according to the manufacturer's instructions including a 17 h transcription step, and aRNA quantified and quality were assessed as described earlier. Cy dye suspensions (Cy3 and 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, Bucks, UK). To attach the Cy dyes, 3 μg each of the aRNA sample were suspended in 6 μl nuclease-free H_2O and heated to 70°C for 2 min. When cooled to room temperature, 2 μl of coupling buffer (0·5 M-$NaHCO_3$; pH 9·2) and 2 μl of Cy3 dye suspension stock were added and then incubated for 1 h at 25°C 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; Thermo Scientific) 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 the hybridisation of each array, each labelled biological replicate and corresponding pooled reference (40 pmol of each dye, about 150 ng aRNA) were combined, and the volume was made up to 25 μl with nuclease-free water. After heating the aRNA at 95°C for 3 min in a thermocycler, 225 μl of pre-heated (60°C) hybridisation solution, comprising 185 μl 0·7 × UltraHyb buffer (Ambion/Applied Biosystems), 20 μl poly(A) at 10 mg/ml (Sigma-Aldrich, Gillingham, Dorset, UK), 10 μl herring sperm at about 10 mg/ml (Sigma-Aldrich) and 10 μl ultra-pure bovine serum albumin at 10 mg/ml (Sigma-Aldrich), were added, and the mixture was kept at 60°C in the dark until being applied to the microarray. After loading the slides and hybridisation solution into the Lucidea chambers (heated at 60°C), chamber temperature was raised to 70°C for 10 min and then lowered to 42°C, at which temperature hybridisation was continued for 17 h with pulse mixing every 15 min. We performed two post-hybridisation automatic washes (800 μl/slide at 8 μl/s) with 1·0 × saline-sodium citrate (SSC), 0·1 % SDS (wash 1) and 0·3 × SSC, 0·2 % SDS (wash 2), after which the temperature was lowered to 40°C. The slides were then manually washed using the EasyDipTM Slide staining system (Canemco, Inc., Gore, QC, Canada): two times with wash 2 solution for 3 min each (125 rpm; 45°C), followed by three times with 0·2 × SSC for 2 min each (125 rpm; 45°C) and a final 20 s dip (room temperature) in 0·1 × SSC. The 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 a 10 μm resolution using an Axon GenePix 4200AL Scanner (MDS Analytical Technologies, Wokingham, Berkshire, UK). Laser power was kept constant (80 %), and the 'auto PMT' (auto photo-multiplier tube) function within the acquisition software (version 4; MDS Analytical Technologies, Wokingham, Berkshire, UK) was enabled to adjust PMT for each channel such that less than 0·1 % of the features were saturated and that the mean intensity ratio of the Cy3 and Cy5 signals was close to 1. BlueFuse software (BlueGnome, Great Shelford, Cambridge, UK) was used to identify features and extract fluorescence intensity values from the resultant TIF (tagged image file format) 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 17 102 gene features were exported into the GeneSpring GX version 10.0.2 analysis platform (Agilent Technologies, Wokingham, Berkshire, UK) after undergoing a block Lowess normalisation. All control features were excluded from subsequent analyses. Data transformation and quality filtering were as follows: (1) all intensity values <1 were set to 1 and (2) data were filtered using a BlueFuse spot confidence value $>0{\cdot}3$ in at least 75 % of the values in any two out of four conditions and BlueFuse spot quality of $\geq 0{\cdot}5$ in at least 75 % of the values in any two out of four conditions. This gave a list of 14 772 genes eligible for statistical analysis. Experimental annotations complied fully with minimum information about microarray experiment guidelines[15]. The experimental hybridisations are archived on the European Bioinformatics Institute (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 real-time quantitative PCR (RT-qPCR), indicate that such corrections are overconservative for these types 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 focused 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 are presented.

Real-time quantitative PCR

The 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 quantitative RT-PCR. Primers were either found in the literature or designed from expressed sequence tag 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* and *β-actin*, was performed, but only *cofilin-2* expression proved to be stable across treatments.

For RT-qPCR, 1 μg of column-purified total RNA per sample was reverse transcribed into cDNA using the VersoTM cDNA kit (ABgene, Epsom, Surrey, UK), following the manufacturer's instructions, using a mixture of random hexamers and anchored oligo-dT (3:1, v/v). Negative controls (no enzyme) were prepared to check for genomic DNA contamination.

Table 1. Primers used for real-time quantitative PCR

Transcript	Primer name	Primer sequence	Amplicon (bp)	T_m (°C)	Accession no.	Source
HMG-CoA	HMG-1F	5′-CCTTCAGCCATGAACTGGAT-3′	224	60	TC102374*	Leaver *et al.*[16]
	HMG-1R	5′-TCCTGTCCACAGGCAATGTA-3′				
MEV	MEV-1F	5′-CCCTTAATCAGGGTCCCAAT-3′	247	60	DW005667†	Leaver *et al.*[16]
	MEV-1R	5′-GGTGCTGGTTGATGTCAATG-3′				
IPI	23-3p jbt1F	5′-ACAGCCCTATGGTTATGTGTCATCTC-3′	230	60	CK875291†	Leaver *et al.*[16]
	23-3p jbt1R	5′-CAAGGTGAGGCGAATGTTTGAAC-3′				
DHCR7	7DCHR-1F	5′-CTTCTGGAATGAGGCATGGT-3′	230	60	TC99602*	Leaver *et al.*[16]
	7DCHR-1R	5′-ACAGGTCCTTCTGGTGGTTG-3′				
SREBP2	SREBP2-1F	5′-GACAGGCACAACACAAGGTG-3′	215	60	DY733476†	Leaver *et al.*[16]
	SREBP2-1R	5′-CAGCAGGGGTAAGGGTAGGT-3′				
ABCA1	ABCA1-UTR-F2	5′-GGACGAACCCTGTGTCTGTT-3′	203	60	EG836783†	New design
	ABCA1-UTR-R2	5′-ATTTGCATTGCGTTTCAGTG-3′				
CPT1	CPT1-1F	5′-CCTGTACCGTGGAGACCTGT-3′	212	60	AM230810†	Leaver *et al.*[16]
	CPT1-1R	5′-CAGCACCTCTTTGAGGAAGG-3′				
ACO	ACO-2F	5′-AAAGCCTTCACCACATGGAC-3′	230	60	TC49531*	Leaver *et al.*[16]
	ACO-2R	5′-TAGGACACGATGCCACTCAG-3′				
ApoAI	SsApoAI-F1	5′-CCATCAGCCAGGCCATAAA-3′	73	60	CB506105†	Kleveland *et al.*[50]
	SsApoAI-R1	5′-TGAGTGAGAAGGGAGGGAGAGA-3′				
ApoCII	SsApoCII-F1	5′-GGAACCAGTCGCAGATGTTGA-3′	145	60	DN047858†	Kleveland *et al.*[50]
	SsApoCII-R1	5′-TGAGGACATTCGTGGCCTTC-3′				
ApoB100	SsApoBfQ	5′-AGCCTTCGATGCTGTCGGCCA-3′	153	60	TC79364*‡	New design
	SsApoBrQ	5′-AGGAGCACAGGCAGGGTGGTT-3′				
SR-BI	SsSRBI-F1	5′-AACTCAGAGAAGAGGCCAAACTTG-3′	204	60	DQ266043†	Kleveland *et al.*[50]
	SsSRBI-R1	5′-TGCGGCGGTGATGATG-3′				
LDLR	SsLDLR-F1	5′-GCATGAACTTTGACAATCCAGTGTAC-3′	78	60	AJ003118†	Kleveland *et al.*[50]
	SsLDLR-R1	5′-TGGAGGAGTGCCTGCTGATAT-3′				
EL	SsEL-F4	5′-CCGGTGCTGCTGGAGGAAGC-3′	378	60	NM_001140535†	New design
	SsEL-R5	5′CGACATGCAGGTCATCGGT-3′				
LPLa	SsLPL-F1	5′-TGCTGGTAGCGGAGAAAGACAT-3′	114	60	BI468076†§	Kleveland *et al.*[50]
	SsLPL-R1	5′-CTGACCACCAGGAAGACACCAT-3′				
LPLb	SsLPL-F4	5′-GGCAGCCCTACATGATAACC-3′	172	60	TC67836*‖	New design
	SsLPL-R4	5′-TCTGTCCAAAGCCACTCACA-3′				
LPLc	SsLPL-F6	5′AGGGCGTTAATCCATGTCAG-3′	223	60	TC84899*	New design
	SsLPL-R6	5′-GACCTTTCAAAAGGGCATGA-3′				
Reference genes						
Elf-1α	ELF-1A jbt2	5′-CTGCCCCTCCAGGACGTTTACAA-3′	175	60	AF321836†	Leaver *et al.*[16]
	ELF-1A jbt2	5′-CACCGGGCATAGCCGATTCC-3′				
β-Actin	BACT-F	5′-ACATCAAGGAGAAGCTGTGC-3′	141	56	AF012125†	Leaver *et al.*[16]
	BACT-R	5′-GACAACGGAACCTCTCGTTA-3′				
Cofilin-2	B2F	5′-AGCCTATGACCAACCCACTG-3′	224	60	TC63899*	Leaver *et al.*[16]
	B2R	5′-TGTTCACAGCTCGTTTACCG-3′				

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, subfamily A, member 1; CPT1, carnitine palmitoyltransferase I; ACO, acyl-CoA oxidase; SR-BI, scavenger receptor class B type 1; LDLR, LDL-receptor; EL, endothelial lipase; LPLa, lipoprotein lipase (TC91040); LPLb, lipoprotein lipase (TC67836); LPLc, lipoprotein lipase (TC84899); Elf-1α, elongation factor-1α.

* Atlantic salmon gene index (http://compbio.dfci.harvard.edu/tgi/).

† GenBank (http://www.ncbi.nlm.nih.gov/).

‡ Primer was designed in the region of the sequence corresponding only to the C terminal half of ApoB100 (i.e. not containing the N-terminal region that is common to ApoB48).

§ Corresponding to TC91040 (Atlantic salmon gene index), which does not align with TC sequences from LPLb or LPLc.

‖ LPLb and LPLc have 85 % identity in the aligned region (90 % query coverage).

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, UK) in a final volume of 20 μl containing either 5 μl (for most genes) or 2 μl (for the reference genes and other highly expressed genes) diluted (1:20) cDNA, 0·5 μM of each primer and 10 μl AbsoluteTM QPCR SYBR® Green mix (ABgene). Amplifications were carried out with a systematic negative control. The qPCR profiles contained an initial activation step at 95°C for 15 min, followed by thirty to forty cycles: 15 s at 95°C, 15 s at the specific primer pair annealing T_m (Table 1) and 15 s at 72°C. After the amplification phase, a melt curve analysis of 0·5°C increments from 75 to 90°C was performed, confirming amplification of single products. RT-qPCR product sizes were examined by agarose gel electrophoresis, and identities were confirmed by sequencing. Non-occurrence of primer–dimer formation in the negative control was also verified. The results were analysed using the relative expression software tool (REST 2008; http://www.gene-quantification.info/), which employs a pairwise fixed reallocation randomisation test (10 000 randomisations) with efficiency correction[17], to determine the statistical significance of expression ratios (or gene expression fold changes) between the two treatments.

Plasma and lipoprotein lipid analysis

Plasma and lipoprotein lipids were analysed by means of a clinical bioanalyser (Maxmat PL analyser, Montpellier, France). VLDL, LDL and HDL in plasma were obtained by sequential centrifugal flotation[18,19], as described by Lie *et al.*[20], at 197 600 ***g*** and 4°C (Beckman Optima™ XL-100K Ultracentrifuge and SW41Ti rotor; Beckman, Brea, CA, USA). Density intervals were obtained by the addition of solid KBr[21], and run time for the separation of lipoproteins was as follows: VLDL, $d < 1{\cdot}015$ g/ml for 20 h; LDL, $1{\cdot}015$ g/ml $< d < 1{\cdot}085$ g/ml for 20 h; HDL, $1{\cdot}085$ g/ml $< d < 1{\cdot}21$ g/ml for 44 h.

Lipid class analyses

Total lipids were extracted from the flesh or liver according to Folch *et al.*[22], and tissue lipid class compositions were determined by single-dimension, double-development, high-performance TLC and densitometry, as described previously[23].

Statistical analysis

Differences in lipid class composition in the liver and flesh and the levels of cholesterol and TAG in the plasma and lipoproteins were assessed by two-way ANOVA, at a significance level of $P<0{\cdot}05$. The RT-qPCR data were analysed both using the $\Delta\Delta C_t$ 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 the 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 seventeen features (corresponding to fifteen 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 (isopentenyl diphosphate isomerise (IPI), squalene mono-oxygenase/epoxidase and acetyl-CoA acetyltransferase 2), cholesterol transport/cellular efflux (ATP-binding cassette transporter A1 (ABCA1)), lipoprotein metabolism (angiopoietin-like 4 (Angptl4), lipoprotein lipase (LPL) and endothelial lipase (EL)), β-oxidation (carnitine *O*-acetyltransferase, Δ3,5-Δ2,4-dienoyl-CoA isomerase 1 and enoyl CoA hydratase 1), fatty acid synthesis (Δ5 and Δ6 fatty acyl desaturase) and transport (acyl-CoA-binding protein), glycerophospholipid/phosphatidylcholine biosynthesis (phosphatidylethanolamine *N*-methyltransferase) and regulation of energy metabolism through the switch on/off of multiple catabolic/anabolic pathways (5′-AMP-activated protein kinase subunit γ-3).

Real-time quantitative PCR analysis of gene expression

Relative gene expression of a series of genes involved in some of the preponderant lipid metabolism pathways mentioned earlier, mostly associated with 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

Table 2. Genes involved in lipid metabolism whose expression in the liver transcriptome showed a significant diet × family interaction*, revealing transcripts whose level of expression is dependent on the combined effects of both factors

		VO:FO†		Lean:fat†			
Accession no.	Gene	Lean	Fat	FO	VO	*P*	Position‡
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 subfamily A member 1	−2·0	1·1	−1·0	−2·2	0·0051	45
CK883097	5′-AMP-activated protein kinase subunit γ-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-CoA-binding protein	1·7	1·0	−1·5	1·1	0·0103	89
CK880279	δ3,5-δ2,4-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	Δ-5 Fatty acyl desaturase	2·1	1·2	−1·7	1·0	0·0179	159
CK879648	Squalene mono-oxygenase (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	Δ-6 Fatty acyl desaturase	2·1	1·4	−1·3	1·1	0·0317	320
BM413811	Enoyl CoA hydratase 1	−1·1	1·3	1·3	−1·1	0·0403	420

VO, vegetable oil; FO, fish oil.
* Identified by two way-ANOVA.
† 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 position of the feature in the interaction list (total features, *n* 529) in the order of by ascending *P* value.

Table 3. Relative analysis of gene expression* of genes involved in cholesterol biosynthesis and its regulation, cholesterol transport/cellular efflux, β-oxidation and lipoprotein metabolism† in the liver of two groups of Atlantic salmon (lean and fat families), after a year of feeding diets containing either 100 % fish oil (FO) or 100 % vegetable oil (VO)

(Ratios and *P* values)‡

	VO:FO				Lean:fat			
	Lean		Fat		FO		VO	
Genes	Ratio	*P*	Ratio	*P*	Ratio	*P*	Ratio	*P*
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, subfamily A, member 1; ACO, acyl-CoA oxidase; CPT1, carnitine palmitoyltransferase I; SR-BI, scavenger receptor class B type 1; LDLR, LDL-receptor; EL, endothelial lipase; LPLa, lipoprotein lipase (TC91040); LPLb, lipoprotein lipase (TC67836); LPLc, lipoprotein lipase (TC84899).

* Analysed by REST 2008.

† Assayed by real-time quantitative PCR.

‡ 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.

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 the 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 group and down-regulated in the fat fish group. This difference appears to result from a lower expression of IPI in lean fish, compared with fat fish, 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 with those fed FO and an up-regulation in fat fish. In this case, gene expression was significantly lower in the lean group compared with the fat group, 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 expressed sequence tag 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 to those obtained in the microarray experiment and was later found to have amplified both the 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 with those fed FO. On the other hand, determination of LPL expression was initially performed using a primer pair available in the literature (termed LPLa (TC91040) in the present study), 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 (Dana-Farber Cancer Institute-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 named LPLb (TC67836) and LPLc (TC84899), respectively, in the present study. 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 of 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 the lean group, compared with 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 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, mevalonate kinase (Table 3) and squalene mono-oxygenase/epoxidase (Table 2), with a pronounced up-regulation in lean fish when VO replaced FO in the diet, coupled with lower expression in lean salmon, compared with fat salmon, when fed FO.

The microarray experiment had indicated potential differential regulation of fatty acid β-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 the expression of two genes involved in the β-oxidation pathway including acyl-CoA oxidase and carnitine palmitoyltranferase 1, responsible for facilitating the transfer of long-chain fatty acids into the mitochondria and thus a common indicator of β-oxidation[16]. However, no significant changes were observed for these genes.

To further analyse the physiological mechanisms related to lipoprotein metabolism, quantification of apo genes (apoAI, apoCII and apoB) and lipoprotein receptors (scavenger receptor class B type 1 (SR-BI) and LDL-receptor) was performed. In general, dietary FO replacement by VO tends to increase the expression of the three apo genes and to reduce that of the two lipoprotein receptors assayed in both the 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 with the fat group, 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. 1(g), (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 with lean fish. In both cases, and particularly for LPLc, the fat group fed VO presented the highest up-regulation and thus had the greatest influence. Finally, LPLb showed a significant interaction as a result of the lowest and highest levels of expression being measured in lean and fat fish fed VO, respectively (Fig. 1(n)).

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

To assess the 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 h after the last meal were analysed (Tables 4 and 5). Plasma cholesterol was significantly affected by the diet, with higher levels found in fish fed FO, independent of the family (Table 4). Cholesterol in VLDL showed a 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 the liver, significantly lower proportions of TAG, and correspondingly higher percentages of phospholipids and sterols, were found in fish fed FO, independent of the family (Table 5). A significantly higher relative phospholipid level was also found in the flesh of fish fed FO, but, contrary to the 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 a 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 inter-related. 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 apo, 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 the 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 with 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 3-hydroxy-3-methylglutaryl-CoA reductase, which was not significantly regulated, cholesterol biosynthesis genes and SREBP2 were all over twofold 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 probably

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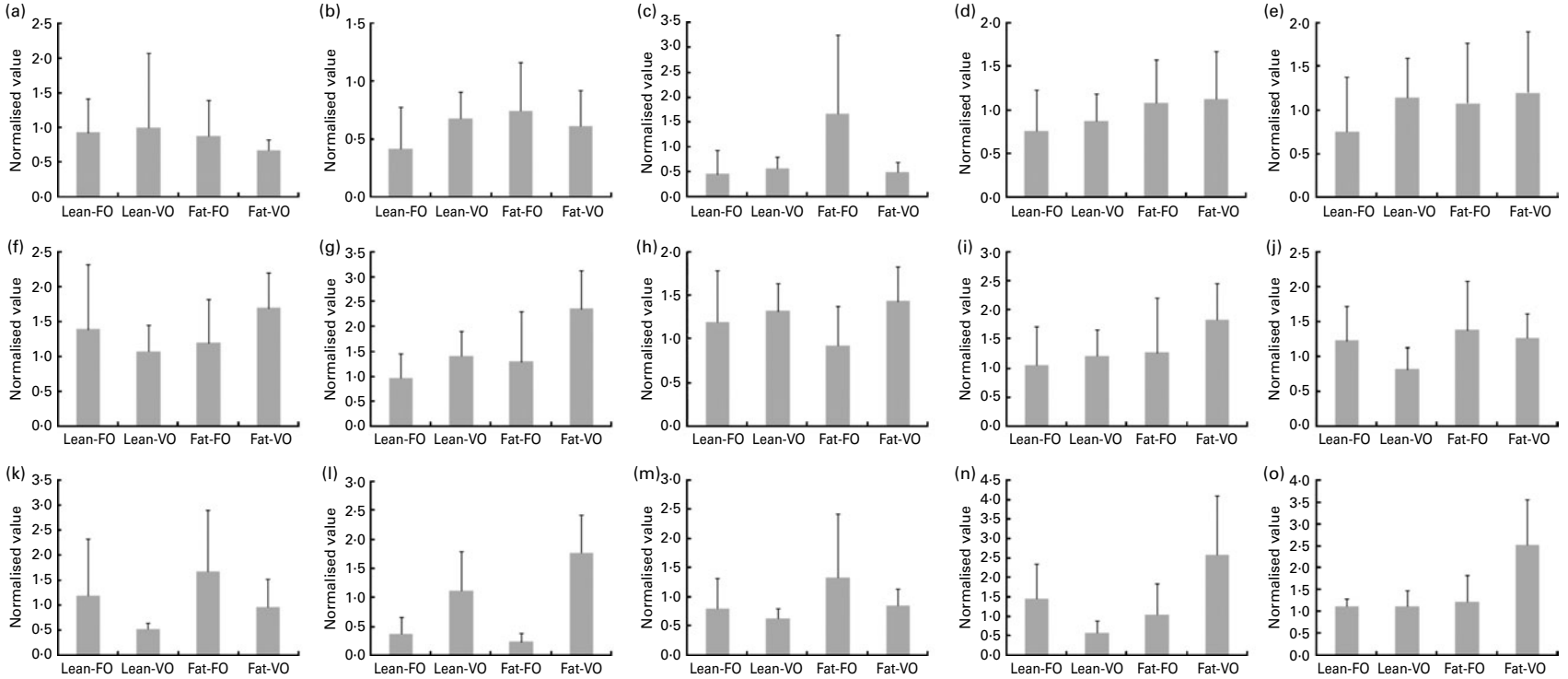

Fig. 1. Normalised gene expression levels (obtained by dividing the number of copies of the target gene by the number of copies of *cofilin-2*) involved in cholesterol biosynthesis and its regulation, cholesterol transport/cellular efflux and lipoprotein metabolism, determined by real-time quantitative PCR in the liver of two groups of Atlantic salmon (lean and fat families), after a year of feeding diets containing either 100 % fish oil (FO) or 100 % vegetable oil (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, subfamily A, member 1 (ABCA1); (g) apoAI; (h) apoCII; (i) apoB100; (j) scavenger receptor class B type 1 (SR-BI); (k) LDL-receptor (LDLR); (l) endothelial lipase (EL); (m) lipoprotein lipase TC91040 (LPLa); (n) lipoprotein lipase TC67836 (LPLb); (o) lipoprotein lipase TC84899; (LPLc). Mean values were not significantly different for HMG-CoA, MEV, IPI, DHCR7, SREBP2, ABCA1, apoCII, apoB, SR-BI, LDLR and LPLa (for 'diet', 'family' and interaction 'diet × family'). Mean values were significantly different for apoAI (for diet and family): $P=0{\cdot}019$ and $P=0{\cdot}044$. Mean values were significantly different for EL (for diet): $P<0{\cdot}000$. Mean values were significantly different for LPLb (for diet × family interaction): $P=0{\cdot}007$. Mean values were significantly different for LPLc (for diet, family and diet × family interaction): $P=0{\cdot}022$, $P=0{\cdot}008$ and $P=0{\cdot}021$. Mean values were significantly different for the factors 'diet', 'family' and interaction 'diet × family' ($P<0{\cdot}05$; two-way ANOVA).

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Table 4. Levels of circulating plasma (mM) or lipoprotein (VLDL, LDL and HDL (μmol/ml plasma)) cholesterol and TAG in Atlantic salmon lean and fat families, determined after a year of feeding diets containing either 100 % fish oil (FO) or 100 % vegetable oil (VO)
(Mean values and standard deviations)

	Lean				Fat						
	FO		VO		FO		VO		ANOVA *P**		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Diet	Family	Diet × family
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

* Mean values were significantly different for the factors 'diet', 'family' and interaction 'diet × family' (two-way ANOVA).

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 VO as described 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 twelve enzymes of the cholesterol biosynthetic pathway[26], shows a greater increase in lean fish, compared with 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 the family groups seem to arise mostly when feeding the more 'natural' FO diet containing higher cholesterol, with 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 with 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.

Table 5. Liver and flesh total lipids (g/100 g of wet weight) and lipid class composition (percentage of total lipid) in Atlantic salmon lean and fat families, determined by TLC, after a year of feeding diets containing either fish oil (FO) or vegetable oil (VO)
(Mean values and standard deviations)

	Lean				Fat						
	FO		VO		VO		FO		ANOVA *P*		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Diet	Family	Diet × family
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
TAG	29·8	0·8	37·6	2·2	26·7	3·1	39·8	3·8	0·000	0·776	0·072
NEFA	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
TAG	74·1	1·4	74·6	1·1	72·5	2·8	73·7	0·5	0·326	0·153	0·683
NEFA	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

* Steryl esters were not detected in flesh.

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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 the 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 with the lean family irrespective of diet. Reduced levels of VLDL and LDL-TAG in mammals can be caused by several complex and inter-related 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 the 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 by 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 hypotriacylglycerolaemic 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 the inhibition of SREBP-1c and PPAR activation, respectively[33–35]. As previously observed in salmon[29,36], phospholipid:TAG ratios were affected by diet, with FO inducing lower TAG and correspondingly higher phospholipids, which is attributed to similar hypotriacylglycerolaemic mechanisms of *n*-3 LC-PUFA in FO to those described in mammals. Consistent with this, lower expression of fatty acid synthase, which plays a key role in lipogenesis, was measured in fish fed FO, independent of the family (S. Morais, unpublished results). On the other hand, microarray data suggested an interaction between diet and family, affecting hepatic β-oxidation (carnitine *O*-acetyltransferase, Δ3,5-Δ2,4-dienoyl-CoA isomerase and enoyl CoA hydratase 1) and the expression of 5′-AMP-activated protein kinase, a metabolic 'sensor' responsible for regulating energy homeostasis[37]. However, fold changes were marginal, and when the expression of β-oxidation genes acyl-CoA oxidase and carnitine palmitoyltranferase 1 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 the liver, which does not enable the 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 the 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 the 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 has also been 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. It was worth noting that the change in the expression of an angiopoietin-like 4 cDNA 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 the 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 phospholipid-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 lean fish fed VO, compared with those fed 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 the overexpression of hepatic LDL-receptor[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 LDL-receptor 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 with 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 phospholipids 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 the bile for excretion and recycling of apo, particularly in hepatic and steroidogenic cells[49]. Finally, LPL and EL can both influence lipoprotein metabolism by catalysing the hydrolysis of TAG and phospholipids, 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 LDL-receptor, 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 a faster lipoprotein turnover when fed VO, but the physiological and health effects of this, including the development of arteriosclerotic changes when VO replaces FO, require elucidation[6,7].

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

The present study 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 the 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 the genetic background. In red sea bream, two LPL genes have been reported[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 the 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 NEFA and 2-monoacylglycerols for tissue utilisation, either storage or oxidation, depending on tissue and the 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 in 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 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 factors and 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 fat fish, compared with 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 probably 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 with those fed VO in both groups. Lean fish also showed

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a stronger response in the cholesterol biosynthesis pathway, mediated by SREBP2, to dietary lipid composition.

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