Carmona-Antoñanzas G, Tocher DR, Martinez-Rubio L & Leaver M (2014) Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals, *Gene*, 534 (1), pp. 1-9.

This is the peer reviewed version of this article

Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals

Greta Carmona-Antoñanzas [a §], Douglas R. Tocher, Laura Martinez-Rubio and Michael J. Leaver

[a] School of Natural Sciences, University of Stirling, Institute of Aquaculture, Stirling FK9 4LA, UK

[§] Corresponding author

GCA: g.e.carmonaantonanzas@stir.ac.uk; Tel.: +44 (0) 178644 6593

DRT: d.r.tocher@stir.ac.uk

LMR: laura.martinez@stir.ac.uk

MJL: m.j.leaver@stir.ac.uk

Abstract

Lipid content and composition in aquafeeds have changed rapidly as a result of the recent drive to replace ecologically limited marine ingredients, fishmeal and fish oil (FO). Terrestrial plant products are the most economic and sustainable alternative; however, plant meals and oils are devoid of physiologically important cholesterol and long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic (EPA), docosahexaenoic (DHA) and arachidonic (ARA) acids. Although replacement of dietary FO with vegetable oil (VO) has little effect on growth in Atlantic salmon (*Salmo salar*), several studies have shown major effects on the activity and expression of genes involved in lipid metabolism. In vertebrates, sterols and LC-PUFA play crucial roles in lipid metabolism by direct interaction with lipid-sensing transcription factors (TF) and consequent regulation of target genes. The primary aim of the present study was to elucidate the role of key TFs in the transcriptional regulation of lipid metabolism in fish by transfection and overexpression of TFs. The results show that the expression of genes of LC-PUFA biosynthesis (*elovl* and *fads2*) and cholesterol metabolism (*abca1*) are regulated by Lxr and Srebp TFs in salmon, indicating highly conserved regulatory mechanism across vertebrates. In addition, *srebp1* and *srebp2* mRNA respond to replacement of dietary FO with VO. Thus, Atlantic salmon adjust lipid metabolism in response to dietary lipid composition through the transcriptional regulation of gene expression. It may be possible to further increase efficient and effective use of sustainable alternatives to marine products in aquaculture by considering these important molecular interactions when formulating diets.

Key words

Atlantic salmon; fatty acid; gene expression; lipid; pyloric caeca; transcription factor

1. Introduction

Lipid content and composition in feeds for farmed fish has experienced a recent and rapid change, because, in order to sustain growth of the aquaculture industry, ecologically limited marine fish meal and fish oil (FO) ingredients have been replaced by terrestrial plant-derived meals and oils. Although replacement of up to 100% dietary FO with vegetable oil (VO) has little effect on growth in Atlantic salmon (*Salmo salar*) [1], some studies have shown major effects on the expression and regulation of genes involved in fatty acid and cholesterol metabolism [2,3]. This impact was consistent with the major compositional changes caused by feeding VO to fish including decreased levels of dietary cholesterol and long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoate (EPA; 20:5n-3), docosahexaenoate (DHA; 22:6n-3) and arachidonate (ARA, 20:4n-6), which are absent in terrestrial plants [2]. Cholesterol and LC-PUFA are critical functional components of cellular membranes and are important precursors of bioactive lipids required for homeostasis, cell signaling, immune and inflammatory responses [4], and the long-term health and welfare effects of reductions in these essential dietary nutrients in fish, including salmon, are unknown.

Much effort has been directed toward the understanding of effects of dietary imbalances in LC-PUFA and cholesterol in humans and mammalian models because of the links between dyslipidemia and a range of highly prevalent cardiovascular, metabolic and inflammatory diseases [5]. These studies have shown that cholesterol and fatty acids and their metabolic derivatives can exert major effects on physiology by interactions with a range of transcription factors [6]. Particular attention has focused on liver X receptor (LXR), peroxisome proliferator-activated receptors (PPAR) and sterol regulatory element binding proteins (SREBP) and their activities in liver and monocyte cells. LXR has a pivotal role in the control of intermediary metabolism mediating cross-regulation between fatty acid and sterol metabolism [7]. LXR activity is activated by binding oxysterol ligands, catabolic products of cholesterol [8]. In response to cholesterol overloading, and consequent oxysterol production, LXR modulates intracellular cholesterol levels by transactivating the expression of cholesterol ester transfer

71 protein, apolipoproteins, cholesterol 7alpha-hydroxylase (CYP7α1) and the ATP-binding cassette
72 transporter 1 (ABCA1), which regulate cholesterol efflux from cells [7].

73 In response to cholesterol depletion, SREBPs, a family of membrane-bound transcription factors, are
74 activated. SREBP1 plays a crucial role in the regulation of many lipogenic genes and SREBP2
75 primarily regulates the transcription of cholesterogenic enzymes [9]. Interactions between these
76 pathways are to some extent mediated through LXR activating SREBP1 transcription, inducing the
77 expression of enzymes involved in the synthesis of fatty acids, triacylglycerols and phospholipids. In
78 addition, some important lipid metabolizing genes, such as fatty acid synthase (FAS), are both LXR
79 and SREBP1 targets [10]. PPARs, encoded by three genes in mammals, are activated by binding fatty
80 acids or their oxidized derivatives and act to regulate expression of genes of lipid degradation and
81 biosynthesis. PPARα and PPARβ, regulate the expression of genes encoding mitochondrial and
82 peroxisomal fatty acid-catabolizing enzymes, whilst PPARγ has a central role in fat storage by
83 promoting and maintaining the adipocyte phenotype [6]. Thus, LXR, SREBP and PPAR transcription
84 factors act as lipid sensors that translate changes in cellular sterol and fatty acid content and
85 composition into metabolic activity.

86 Compared to mammals, few studies have addressed the existence or roles of these transcriptional
87 regulators of lipid metabolism in fish. Our contention is that a greater understanding of lipid-mediated
88 gene regulatory networks in Atlantic salmon will facilitate the efficient, effective and safe use of
89 sustainable alternatives to marine products in aquaculture feeds. Recently the genes for Atlantic
90 salmon Lxr, Srebp1 and Srebp2 and Ppars have been characterized [11-13]. In addition, studies on an
91 Atlantic salmon cell line (SHK-1) have shown that several lipid metabolic genes are transcriptionally
92 regulated in response to changes in lipid composition of the medium [13]. The primary aim of the
93 present study was to elucidate Lxr, Srebp and Ppar gene regulatory mechanisms and key lipid
94 metabolic target genes in Atlantic salmon and to determine the extent to which dietary modulation of
95 lipid and fatty acid metabolism in salmon reflects or varies from the patterns of gene regulation
96 described for mammals.

2. Materials & Methods

2.1. Cell lines and cell culture

The established Atlantic salmon cell line derived from head kidney (SHK-1) was grown at 22 °C in an atmosphere of 4 % carbon dioxide in Dulbecco's modified eagle medium (DMEM) containing 3 g L^{-1} D-glucose and 55 mg L^{-1} sodium pyruvate, and supplemented with 10% foetal bovine serum (FBS), 50 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, 40 μM 2-mercaptoethanol and 4 mM L-glutamine. For gene promoter transactivation assays, fathead minnow (*Pimephales promelas*; FHM) epithelial cells were maintained at 22 °C in Leibovitz's L-15 with GlutaMAX^{TM}-1 medium containing 900 mg L^{-1} D+ galactose and 550 mg L^{-1} sodium pyruvate and 10% FBS. All media and supplements were obtained from Life Technologies (Glasgow, UK).

For subculturing, the cell monolayer was washed twice with phosphate buffer saline (PBS) without $CaCl_2$ or $MgCl_2$ (Invitrogen, UK), cells detached by incubation with 0.05 % trypsin/0.02 % EDTA and re-suspended in medium. Viable cells were counted after harvesting using a Neubauer haemocytometer, 0.4 % Trypan blue (Sigma, Dorset, UK) and an inverted microscope (IMT-2, Olympus).

For transcription factor (TF) ligand treatments, SHK-1 cells were seeded in 6-well clear plates (Nunc, Denmark) at a density of 4 x 10^5 cells per well in a volume of 3 ml Leibovitz's L-15 medium. Cells were approximately 70 % confluent after 48 h growth, when medium was aspirated, cells washed twice with PBS and fresh medium containing the various treatments as ethanol solutions was added. Final concentrations were cholesterol (20 μM), WY14643 (25 μM), 2-bromopalmitate (25 μM), or LXR agonists (GW3965 and T0901317, 10 μM) or with ethanol carrier alone (100% ethanol). After 24 h, the medium was aspirated, the cell monolayer washed twice (PBS) and cells scraped from each well in 0.5 ml of PBS. Cells were centrifuged for 5 min at 3000 x g, PBS discarded and replaced by

0.5 ml of TriReagent (Ambion, UK), followed by vigorous mixing to lyse and digest cells. Cells from two wells were pooled to produce three replicates per treatment.

2.2. Fish, diets and sampling protocols

Four diets (4 mm pellets) with the same basal protein composition, but coated with four different oils were formulated at Skretting Technology Centre (Stavanger, Norway) to satisfy the nutritional requirements of salmonid fish [14]. The oils used were FO (anchovy oil), or 100 % replacement with rapeseed oil (RO), linseed oil (LO) or soybean oil (SO). Atlantic salmon post-smolts (130 g) were randomly distributed into 16 tanks at the Skretting Aquaculture Research Centre (Stavanger, Norway). After a conditioning period of 3 weeks during which the fish received a commercial diet containing FO, the fish were fed the experimental diets to satiation for a period of 16 weeks. Full descriptions of the diet compositions and experimental conditions were reported previously [2]. At the end of the trial fish were anaesthetized with metacain (50 mg/L) and pyloric caeca (intestine), a major organ involved in uptake and transport of lipids, were dissected from five randomly selected fish from each dietary treatment. Samples of 0.5 g of caeca were immediately and rapidly disrupted in 5 ml of TriReagent (Ambion, UK) using an Ultra-Turrax homogenizer (Fisher Scientific, UK), and stored at - 80 ºC prior to RNA extraction. The dietary trial and all procedures on Atlantic salmon conformed to European ethical regulations regarding the care and use of farmed animals in research.

2.3. Atlantic salmon LXR activation assay

The ligand binding domain (LBD) (amino acid residues 191-462) [GenBank:FJ470290] of Lxr was amplified by PCR from salmon pyloric caeca cDNA using primers (Supplementary Table 1) with 5' restriction sites to allow in-frame subcloning between the *Bam*HI and *Xba*I sites of the pBIND vector (Promega, Southampton, UK), which contains the yeast GAL4 DNA-binding domain [8]. The resulting pBIND-Lxr chimeras were co-transfected with a reporter gene plasmid in which the *Firefly* luciferase gene is under the control of a promoter containing UAS (upstream activation sequence), which is recognized by Gal4. To control differences in transfection efficiency, a constitutively

146 expressed control reporter construct encoding for *Renilla* luciferase was included. Ligand activation
147 of Lxr was determined by a luciferase-based functional assay using the FHM cell line as described
148 previously [15]. Twenty-four hours prior to transfection, 2 x 10^4 cells per well were seeded in a 96-
149 well black-sided, clear-bottom microtitre plate (Corning, NY, USA). Transfection mixtures contained,
150 per well; 100 ng pBIND-Lxr construct, 60 ng of luc2P/GAL4UAS reporter plasmid (pGL4.31,
151 Promega), 20 ng of hRluc/CMV internal control reporter plasmid (pGL4.75, Promega) and 1.5 µl of
152 Polyfect transfection reagent (Qiagen) in 100 µl of L-15 medium. Within each experiment, treatments
153 were performed in triplicate. Experimental controls included treatments in which the pBIND-Lxr
154 construct was replaced by empty pBIND vectors during transfection, as well as cells transfected with
155 the appropriate pBIND-Lxr construct and reporters and treated with ethanol carrier only. After 24 h,
156 transfection mixes were removed and replaced with media containing treatment vehicle (ethanol),
157 cholesterol, or one of the following LXR agonists: natural oxysterols 20(S)-hydroxycholesterol (20S-
158 OH) and 22(R)-hydroxycholesterol (22R-OH), synthetic agonists T0901317 and GW3965, and the
159 fungal molecule paxilline (Sigma Aldrich, UK). Compounds of interest were diluted into L-15 from
160 ethanolic stock and 100 µl of the dilution was added per well and incubated for 24 h. Cells were lysed
161 by 10 min incubation in 75 µl per well of 1x Passive Lysis Buffer (Promega), and *Firefly* and *Renilla*
162 luciferase activities quantified using an assay protocol described previously [15]. Transactivation
163 activities were obtained using VICTOR X Multilabel plate reader (PerkinElmer, USA) and data were
164 normalized to the *Renilla* luciferase activities. Data are presented as means of raw transactivation
165 activities of three independent assays. The significance of effects of treatments were tested by one-
166 way analysis of variance (ANOVA), followed by multiple comparisons using Tukey's *post hoc* test at
167 a significance level of $P \leq 0.05$ (PASWS 18.0, SPSS Inc., USA).

168 *2.5. Quantitative RT-PCR (qPCR)*

169 Salmon caecal samples from fish fed different oil sources (n = 5), or SHK-1 cells exposed to Lxr- or
170 Ppar-ligands (n = 3) were used for relative and absolute qPCR analyses, respectively. For gene
171 expression analysis, samples were immediately and rapidly disrupted in TriReagent, and stored at - 80

ºC prior RNA extraction. Total RNA was extracted following the manufacturer's instructions (Ambion, UK), and the quantity and quality of isolated RNA determined by electrophoresis and spectrophotometry (Nanodrop 1000, Thermo Scientific, USA). One microgram of total RNA was reverse transcribed into cDNA using the Verso cDNA synthesis kit (Thermo Scientific, UK) and primed with random hexamers and oligo(dT) in a 3:1 molar ratio. The resulting cDNA was diluted 20-fold with nuclease-free water. For quantitative qPCR, oligonucleotide primers for target genes and housekeeping genes (*elf-1α* and *β-actin*) (Supplementary Table 1) were used at 0.3 μM with one-fortieth of the cDNA synthesis reaction (5 μl of a 1:20 dilution) and 10 μl of SYBR-green qPCR mix (ABgene, UK) in a total volume of 20 μl. Reactions were run in a Mastercycler RealPlex^2 (Eppendorf, UK). Amplifications were carried out including systematic negative controls containing no cDNA (NTC, no template control) and omitting reverse transcriptase enzyme (-RT) to check for DNA contamination. Thermal cycle and melting curves were performed as described previously with specific annealing temperatures for each primer pair shown in Supplementary Table 1 [16]. The qPCR product sizes were checked by agarose gel electrophoresis and the identity of random samples confirmed by sequencing. Absolute quantification was achieved by including a parallel set of reactions containing spectrophotometrically-determined standards consisting of serial dilutions of known copy numbers of linearised plasmid that contain the predicted amplification product for each measured gene. Results were expressed as mean normalized ratios (± SE) between the copy number of target genes and the mean copy number of the reference genes (*elf-1α* and *β-actin*). Differences in the expression of target genes among different treatments were determined by one-way analysis of variance (ANOVA), followed by multiple comparisons using Tukey's *post hoc* test (PASWS 18.0, SPSS Inc., USA). In contrast, the effects of diet on TF expression were analyzed for statistical significance using the relative expression software tool (REST-MCS, 2009 V2 [http://www.gene-quantification.de/rest-2009.html]), and normalized by two housekeeping genes (*elf-1α* and *β-actin*). Gene expression was presented as the relative expression ratio of each gene in fish fed one of the VOs or FO. A significance of $P \leq 0.05$ was applied to all statistical tests performed.

2.6. Expression constructs and cell transfection assays

Atlantic salmon transcription factors Lxr [GenBank: FJ470290], Pparα [GenBank: AM230809], Pparβ1a [GenBank: AJ416953], Srebp1 [GenBank: HM561860] and Srebp2 [GenBank: HM561861NM_004599] had been previously described [12,13]. The entire open reading frame of Lxr (Lxr ORF, 1-462 amino acids), Pparα (Pparα ORF, 1-464 aa), Pparβ1a (Pparβ ORF, 1-443 aa), and the soluble N-terminal domains of Srebp1 (nSrebp1, 1-476 aa) and Srebp2 (nSrebp2, 1-460 aa) were amplified from caecal cDNA samples using the primers detailed in Supplementary Table 1, which included suitable restriction sites, *Eco*RI and *Xho*I, for subsequent insertion into the CMV-based constitutive expression vector pcDNA3 (Invitrogen, UK). Positive clones were selected by enzymatic digestion and sequenced (CEQ-8800 Beckman Coulter Inc., USA). The putative promoter regions, first non-coding exon, first intron and the ATG start codon of Atlantic salmon *elovl5a* (-3618, [GenBank:GU238431]), *elovl5b* (-3141, [GenBank:GU324549]), and *fads2d6*a (-1791, [GenBank:AY736067]) were amplified from genomic DNA using high-fidelity PfuTurbo DNA Polymerase (Statagene, UK) and primers containing restriction sites (Supplementary Table 1). These fragments were cloned into *Sac*I and *Nco*I or *Xho*I sites encompassing the luciferase start codon of a promoterless reporter plasmid [pGL4.10, *luc2*] (Promega, USA), which encodes *Firefly* luciferase.

For luciferase assays, FHM cells were co-transfected, under the conditions described previously, with the salmon *elovl5* or *fads2d6*a reporter constructs, Lxr, Pparα, Pparβ1a, nSrebp1 or nSrebp2 expression constructs to overexpress the protein product. Briefly, stock FHM cells were seeded in 96-well opaque plates at a density of 2×10^4 per well 24 h prior to transfection. Transfection mixtures consisted of 50 ng of pGL4.10 reporter construct (empty pGL4.10 vector in controls), 30 ng of pcDNA3 expression construct (empty pcDNA3 vector in controls), 20 ng of *Renilla* pGL4.75 and 1 µl of Polyfect transfection reagent to 100 µl of L-15 medium. Within each transfection experiment, each treatment was performed in triplicate. After 24 h, the medium was aspirated, monolayer washed twice with PBS, cells lysed, and *Firefly* and *Renilla* luciferase activities quantified as described previously [15]. Transactivation activities were obtained, data normalized and statistical analyses performed as described above.

225

226 **3. Results**

227 *3.1. Ligand specificity of Atlantic salmon LXR*

228 Prior to testing for Lxr-dependent gene expression the ligand binding specificity of *S. salar* Lxr was
229 determined. The salmon Lxr/Gal4 chimera was activated by synthetic Lxr ligands, including
230 T0901317, GW3965 and paxilline, and also by the physiological oxysterols, 22(R)-OH and 20(S)-OH
231 (Fig.1). The concentration-response curve for activation of Lxr indicated that T0901317 and GW3965
232 were the most potent agonists (Fig. 1). T0901317 and GW3965 activated salmon Lxr at micromolar
233 concentrations with maximal effect observed at 10 μM. The synthetic agonist paxilline exhibited the
234 lowest effect, whereas cholesterol had no effect. No response was detected at 100 μM for most
235 agonists, perhaps due to toxic effects.

236 *3.2. Gene expression in SHK-1 cells*

237 Salmon SHK-1 cells were incubated with Lxr (T091317 and GW3965) and Ppar (Wy14643 and 2-
238 bromopalmitate) agonists to identify respective target genes. Incubation with synthetic Lxr ligands
239 caused potent induction of *srebp* genes, with up to 9-fold increase for *srebp1* and 2-fold for *srebp2*
240 (Fig. 2). Lxr agonists GW3965 and T091317 increased the expression of *fas* mRNA 3- and 6-fold,
241 respectively (Fig. 3). Fatty acyl desaturases, *fads2d6a* (~3.3-fold), *fads2d6b* (~2.4-fold) and *fads2d5*
242 (4-fold) mRNAs were increased by GW3965. Only *fads2d5* expression was affected by T091317
243 being increased < 2-fold (Fig. 3). The expression of mRNA for *abca1* transporter, which regulates
244 cholesterol efflux, was strongly upregulated by both Lxr agonists, with ~14-fold increases (Fig. 3). In
245 the presence of Ppar agonists, only *srebp2* expression responded, relatively weakly, to 2-
246 bromopalmitate (Fig. 2), which is a dual Pparα and Pparβ agonist. However, Wy1465, a specific
247 Pparα agonist had no significant effect on *srebp2* expression. The expression of acyl-CoA oxidase
248 (*acox*), HMG-CoA reductase (*hmgCoAR*) and fatty acid elongases, *elovl4*, *elovl5a* and *elovl5b* (Fig. 3)
249 *and ppars* (Fig. 2) did not change significantly after incubation with Lxr or Ppar ligands.

3.3. Trans-regulation of genes related to LC-PUFA metabolism by Srebp, Lxr and Ppar

To investigate the regulatory role of Srebp, Lxr and Ppar on the expression of key genes of LC-PUFA biosynthesis, we established Atlantic salmon promoter luciferase assays in FHM cells. The Srebp transcriptional regulation of duplicated *elovl5a* and *elovl5b* elongases, and *fads2d6a* desaturase promoters was assessed by co-transfecting with the active nuclear DNA-binding region of either Srebp1 or Srebp2. Consistent with previous *in vitro* observations that f*ads2d6a* contains an SRE response element in the promoter region [17] the data indicate that both Srebp1 and Srebp2 promote the expression of *fads2d6a*. The promoters of both *elovl5a* and *elovl5b* duplicated genes were also highly activated (Fig. 4) and Srebp2 showed higher activity than Srebp1 for *elovl5b* and *fads2d6a*, whereas *elovl5a* was activated equally by both Srebp mature proteins. Co-transfection of FHM cells with ligand-activated salmon Ppars did not stimulate expression from *elovl5a*, *elovl5b*, or *fads2d6a*; promoters, however the *fads2d6a* promoter was significantly activated by Lxr (Fig. 5).

3.4. Nutritional regulation of the expression of transcription factors

Regulation of TF genes in response to dietary lipid composition was examined in pyloric caeca from salmon post-smolts fed diets containing either FO rich in EPA, DHA and cholesterol, or VOs rich in C18 FA, 18:1n-9 (RO), 18:2n-6 (SO), or 18:3n-3 (LO) but lacking LC-PUFA and cholesterol [2]. Compared to the FO-fed group, there was a significant increase of *srebp1* and *srebp2* transcripts in the pyloric caeca of fish fed the RO and LO diets (Fig. 6). The same trend was observed in fish fed the SO diet suggesting biological significance although not statistically. No differences were found between the dietary groups with regard to the expression of *lxr* or *ppar* α, β or γ.

4. Discussion

Elucidating the regulation of lipid and fatty acid metabolism in fish at a fundamental level is critical to understanding the relationships between lipid biosynthesis and metabolism and dietary lipid supply in fish, and will be crucially important to sustain the growth of aquaculture against a background of

static or diminishing supplies of fish oil derived from wild fisheries. To address these relationships we have undertaken this study to investigate the role of key lipid-regulated TFs on several salmon genes in an Atlantic salmon cell line, SHK-1, with a focus on genes of the LC-PUFA biosynthetic pathway. The SHK-1 cell line was initially developed from salmon head kidney tissue and is possibly leucocyte-derived, showing both macrophage- or dendritic-like phenotypes [18]. However, as a suitable salmon liver- or intestine-like cell line is lacking, the SHK-1 cell line was recently successfully utilized as a model for studying salmon lipid metabolism [13] on the basis that the critical transcriptional regulators of macrophage lipid homeostasis were the same as those in liver and they regulated similar target genes [19,20]. Thus, the response of this cell line to supplementation or depletion of cholesterol or fatty acids suggested the involvement of Srebp and Lxr in regulating several critical lipid biosynthetic genes [13]. To extend these studies and investigate the roles of individual TFs and their target genes more specifically we first characterized the ligand-activation dependency of Lxr and developed promoter/reporter gene constructs to investigate the role of Srebp, Lxr and Ppar in Atlantic salmon.

4.1. Lxr ligand activation

A single Lxr subtype has been identified in various fish species (compared to two in mammals), and all show a highly conserved structure across teleost fish, amphibians, birds and mammals [8]. Although a salmon *lxr* cDNA had been characterized the response of the corresponding receptor to activating ligands had not been tested [12]. Accordingly, concentration-response curves for Lxr ligands indicated that natural oxysterols including 20S-OH and 22R-OH (but not cholesterol) and the fungal metabolite paxilline were activators of Atlantic salmon Lxr. As with other vertebrate LXRs [8], the synthetic agonists T0901317 and GW3965 were strong activators of Atlantic salmon Lxr. Thus the synthetic agonists T0901317 and GW3965 were considered suitable tools for cellular assays of Lxr function.

4.2. Transcriptional regulation of genes of the lipid metabolism

The genetic control of lipid metabolism involves a complex interplay of transcription factors and regulatory loops acting on many genes of lipid metabolism and transport. For example, in mammals, LXR can mediate the regulation of lipogenesis through the direct activation of genes involved in lipid biosynthesis, or in a SREBP1c-dependent manner [10,21,22]. In mammals, two SREBP1 isoforms, SREBP1a and SREBP1c, are encoded by a single gene through the use of alternative start transcription sites, while a separate gene encodes Srebp2 [23]. Given this complexity, elucidated from studies in human and mammalian models, the present study aimed to determine if similar regulatory systems exist in fish due to the increasing importance of understanding basic lipid metabolic processes in modern aquaculture. In agreement with recent studies performed in rainbow trout (*Oncorhynchus mykiss*) [24,25], treatment of Atlantic salmon SHK-1 cells with Lxr agonists resulted in the upregulation of mRNAs for several important lipid metabolic genes, including *fas* involved in *de novo* biosynthesis of fatty acids and *abca1*, which controls the reverse cholesterol efflux (See diagram in Fig. 7). Ligand-activated Lxr also induced the expression of both *srebp1* and *srebp2* in SHK-1 cells. Similarly, *SREBP1c* and also *FAS* and *ABCA1* are established direct LXR targets in various human and rodent systems [10,21,26]. However, the LXR-mediated induction of *Srebp2* is not observed in mammals [22], and may be specific to Atlantic salmon. According to our results, several groups have reported LXR autoregulatory behavior in mammals through binding to LXRE (LXR response element) present in LXRα promoter [26].

Although vertebrates have the capability for endogenous synthesis of monounsaturated fatty acids from the saturated fatty acid products of FAS through the action of stearoyl CoA desaturase (SCD or Δ9 desaturase), they are incapable of creating LC-PUFA *de novo*. Vertebrates thus require either dietary LC-PUFA directly or, depending upon species, they can produce the physiologically important ARA, EPA and DHA by desaturation and elongation of dietary shorter chain PUFA, linoleic (18:2n-6) and α-linolenic (18:3n-3) acids, through the action of Fads1 and 2 and Elovl5 [27]. Intense interest has been paid to vertebrate LC-PUFA enzymes given their hormonal, ontogenetic and/or nutritional regulation in vertebrates [28,29]. Salmon Elovl5 enzymes are encoded by two very similar duplicated genes [3]. Neither mRNA was affected by Lxr agonist in SHK-1 cells, despite the *elovl5* gene

promoters being activated by co-transfection with nSrebps, which indicates that, although Srebps were induced in the cell line, under the experimental condition used they were not processed to transcriptionally active forms. In mammals, *Elovl5* is also not an LXR target, but is secondarily activated through LXR effects on Srebp1c [30]. Salmon *fads2* was significantly increased by Lxr agonist *in vitro*, and strongly activated by nSrebps indicating that this gene is a direct target of both transcription factors. In mammals, LXR agonist increased *Fads1* and *Fads2* expression although the results were attributed to a secondary LXR-dependent induction of SREBP1c [31]. The promoter element of the *Fads2* gene conferring Srebp1 response is highly conserved in several vertebrates including Atlantic salmon, with a potential SRE site and a NF-Y cofactor site [17].

Notably, Atlantic salmon *elovl5* duplicates exhibit differential tissue expression patterns and respond differently to dietary nutrients [3,32]. Accordingly, our results show that *elovl5a* is equally regulated by both Srebp1 and Srebp2, whereas *elovl5b* regulation by Srebp2 is 2-fold greater than that exhibited in response to Srebp1, possibly as result of sequence divergence in the promoter regions of these genes.

PPARs are also central to the transcriptional control of lipid metabolism and several interactions with LXRs and SREBPs have been proposed [33,34]. Mice knockout assays and subsequent *in silico* analysis confirmed that PPARα induces *Srebp1* and *Srebp2* expression through the interaction with PPRE (PPAR response element) in the mammalian *Srebp2* promoter [34]. In SHK-1 cells, few effects were observed upon treatment with WY14643, a Pparα specific ligand, or 2-bromopalmitate, a non-metabolizable fatty acid activator of teleost Pparα and Pparβ [15]. However, bromopalmitate induced the expression of *srebp2*, whereas WY14643 had no effect. This suggests a Pparβ-mediated effect, although it is also possible that the lack of response to the Pparα-specific WY14643 is due to a limited *ppar* subtype expression profile in SHK-1 cells.

4.3. Dietary regulation of lipid transcription factors

The *in vivo* significance of the results from the SHK-1 cell line and promoter assays was previously shown by the observation that hepatic *srebp2* mRNA levels and LC-PUFA biosynthetic gene expression and biosynthesis increased in salmon fed diets containing high levels of VO [2]. In this study, similar results for the expression of LC-PUFA biosynthetic genes were observed in the intestine (pyloric caeca) of salmon fed VO [3]. The pyloric caeca of salmon constitutes the major tissue in terms of nutrient uptake, and lipid digestion and transport [35], and is also the tissue that exhibited the highest level of expression of *srebp*s and *lxr* mRNAs in Atlantic salmon [13]. Therefore, if the LC-PUFA biosynthetic genes, *elovl5* and *fads2*, are driven by Srebps *in vivo*, then the same increase in Srebps should be observed in intestine from VO-fed fish. As the present study has shown, this was indeed the case. Our results indicated that, among the transcription factors studied, only *srebp1* and *srebp2* expression was increased significantly in the pyloric caeca of Atlantic salmon fed rapeseed oil (RO) and linseed oil (LO) compared to the expression in fish fed FO. Plant-derived products constitute the perfect activation ground for SREBPs. In the absence of sterols, the TF amino-terminus is proteolytically released from the endoplasmic reticulum membrane and transported into the nucleus where it binds SRE (serum response element) of specific sets of target genes [36]. In mammals, SREBP1 recognizes SRE sites in the promoters of *Srepb1* and *Srebp2* [37], thus it is likely that salmon Srebp1 activated in low sterol conditions, induced by the inclusion of VO, promoted its own expression and that of *srebp2* in a positive-feedback mechanism. Despite TF responses to dietary lipid variations in fish have been reported at specific developmental stages [12], no effects of VO inclusion were observed in the expression of *lxr* or *ppar* in this study.

5. Conclusions

The results of the present study showed that the fatty acyl elongases and desaturases responsible for endogenous production of LC-PUFA from PUFA in Atlantic salmon were primarily regulated by Srebps, and that Lxr may also be involved in regulating desaturases, but not elongases, whereas there was no evidence for a direct role of Ppars, at least in the salmon cell line tested. ABCA1, a gene central to the process of reverse cholesterol transport, and which is a direct LXR target in mammals,

was also a target of Lxr in salmon. Furthermore *fas* and both *srebp* genes, responsible for the major steps of lipogenesis, are direct Lxr targets in salmon as they are in mammals. Thus, overall, the transcriptional regulatory systems that drive cholesterol transport from the cellular space to lipoprotein, initiate lipogenesis, and regulate LC-PUFA biosynthesis in mammals are largely conserved in Atlantic salmon. This knowledge will be key to deriving a conceptual framework for future experiments designed to answer more applied questions related to lipid metabolism and nutrition with regard to the development and optimization of more sustainable aquaculture feeds. Importantly the present results also showed that these lipid regulatory factors and the genes that they target are ancient, likely to be have arisen early in vertebrate evolution. Thus, further basic studies of evolutionarily conserved pathways of lipid metabolic control across vertebrates might also elucidate, through definition of fundamental regulatory mechanisms, medically relevant aspects of human lipid nutrition and metabolism.

Acknowledgements

We thank Dr. Sofia Morais who kindly provided Atlantic salmon pyloric caeca RNA samples. This research was supported by a Horizon Scholarship to Greta Carmona-Antoñanzas awarded by the University of Stirling (Scotland).

Conflict of interest

The authors declare that there are no conflicts of interest.

References

[1] B.E. Torstensen, J.G. Bell, G. Rosenlund, R.J. Henderson, I.E. Graff, D.R. Tocher, Ø. Lie, J.R. Sargent, Tailoring of Atlantic salmon (*Salmo salar* L.) flesh lipid composition and sensory

401 quality by replacing fish oil with a vegetable oil blend, J. Agric. Food Chem. 53 (2005) 10166-
402 10178.
403 [2] M.J. Leaver, L.A.N. Villeneuve, A. Obach, L. Jensen, J.E. Bron, D.R. Tocher, J.B. Taggart,
404 Functional genomics reveals increased cholesterol and highly unsaturated fatty acid
405 biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo*
406 *salar*), BMC Genomics 9 (2008) 299.
407 [3] S. Morais, O. Monroig, X.Z. Zheng, M.J. Leaver, D.R. Tocher, Highly unsaturated fatty acid
408 synthesis in Atlantic salmon: Characterization of ELOVL5-and ELOVL2-like elongases, Mar.
409 Biotechnol. 11 (2009) 627-639.
410 [4] A.P. Simopoulos, The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular
411 disease and other chronic diseases, Exp. Biol. Med. (Maywood) 233 (2008) 674-688.
412 [5] P.W. Wilson, R.B. D'Agostino, H. Parise, L. Sullivan, J.B. Meigs, Metabolic syndrome as a
413 precursor of cardiovascular disease and type 2 diabetes mellitus, Circulation 112 (2005) 3066-
414 3072.
415 [6] B. Desvergne, L. Michalik, W. Wahli, Transcriptional regulation of metabolism, Physiol. Rev.
416 86 (2006) 465-514.
417 [7] T. Jakobsson, E. Treuter, J.Å. Gustafsson, K.R. Steffensen, Liver X receptor biology and
418 pharmacology: new pathways, challenges and opportunities, Trends Pharmacol. Sci. 33 (2012)
419 394-404.
420 [8] E.J. Reschly, N. Ai, W.J. Welsh, S. Ekins, L.R. Hagey, M.D. Krasowski, Ligand specificity and
421 evolution of liver X receptors, J. Steroid Biochem. Mol. Biol. 110 (2008) 83-94.
422 [9] T.I. Jeon, T.F. Osborne, SREBPs: metabolic integrators in physiology and metabolism, Trends
423 Endocrinol. Metab. 23 (2012) 65-72.
424 [10] S.B. Joseph, B.A. Laffitte, P.H. Patel, M.A. Watson, K.E. Matsukuma, R. Walczak, J.L. Collins,
425 T.F. Osborne, P. Tontonoz, Direct and indirect mechanisms for regulation of fatty acid synthase
426 gene expression by liver X receptors, J. Biol. Chem. 277 (2002) 11019-11025.

427 [11] M.J. Leaver, M.T. Ezaz, S. Fontagne, D.R. Tocher, E. Boukouvala, G. Krey, Multiple
428 peroxisome proliferator-activated receptor β subtypes from Atlantic salmon (*Salmo salar*), J.
429 Mol. Endocrinol. 33 (2007) 391-400.
430 [12] L. Cruz-Garcia, M. Minghetti, I. Navarro, D.R. Tocher, Molecular cloning, tissue expression
431 and regulation of liver X receptor (LXR) transcription factors of Atlantic salmon (*Salmo salar*)
432 and rainbow trout (*Oncorhynchus mykiss*), Comp. Biochem. Physiol. 153 (2009) 81-88.
433 [13] M. Minghetti, M.J. Leaver, D.R. Tocher, Transcriptional control mechanisms of genes of lipid
434 and fatty metabolism in the Atlantic salmon (*Salmo salar* L.) established cell line, SHK-1,
435 Biochim. Biophys. Acta 1811 (2011) 194-202.
436 [14] National Research Council (NRC), Nutrient requirements of fish and shrimp, National
437 Academies Press, Washington D.C., 2011.
438 [15] L. Colliar, A. Sturm, M.J. Leaver, Tributyltin is a potent inhibitor of piscine peroxisome
439 proliferator-activated receptor α and β, Comp. Biochem. Physiol. 153 (2011) 168-173.
440 [16] G. Carmona-Antoñanzas, O. Monroig, J.R. Dick, A. Davie, D.R. Tocher, Biosynthesis of very
441 long-chain fatty acids (C > 24) in Atlantic salmon: Cloning, functional characterisation, and
442 tissue distribution of an Elovl4 elongase, Comp. Biochem. Physiol. B Comp. Biochem. 159
443 (2011) 122-129.
444 [17] X. Zheng, M.J. Leaver, D.R. Tocher, Long-chain polyunsaturated fatty acid synthesis in fish:
445 Comparative analysis of Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.)
446 Δ6 fatty acyl desaturase gene promoters, Comp. Biochem. Physiol. 154 (2009) 255-263.
447 [18] B.H. Dannevig, B.E. Brudeseth, T. Gjøen, M. Rode, H.I. Wergeland, Ø. Evensen, C.M. Press,
448 Characterisation of a long-term cell line (SHK-1) developed from the head kidney of Atlantic
449 salmon (*Salmo salar* L.), Fish Shellfish Immunol. 7 (1997) 213-226.
450 [19] A. Castrillo, P. Tontonoz, Nuclear receptors in macrophage biology: at the crossroads of lipid
451 metabolism and inflammation, Annu. Rev. Cell Dev. Biol. 20 (2004) 455-480.

[20] A. Caimari, P. Oliver, J. Keijer, A. Palou, Peripheral blood mononuclear cells as a model to study the response of energy homeostasis-related genes to acute changes in feeding conditions, OMICS 14 (2010) 129-141.

[21] J.J. Repa, S.D. Turley, J.A. Lobaccaro, J. Medina, L. Li, K. Lustig, B. Shan, R.A. Heyman, J.M. Dietschy, D.J. Mangelsdorf, Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers, Science 289 (2000) 1524-1529.

[22] J.J. Repa, G. Liang, J. Ou, Y. Bashmakov, J.M. Lobaccaro, I. Shimomura, B. Shan, M.S. Brown, J.L. Goldstein, D.J. Mangelsdorf, Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta, Genes Dev. 14 (2000) 2819-2830.

[23] X. Hua, J. Wu, J.L. Goldstein, M.S. Brown, H.H. Hobbs, Structure of the human gene encoding sterol regulatory element binding protein-1 (SREBF1) and localization of SREBF1 and SREBF2 to chromosomes 17p11.2 and 22q13, Genomics 25 (1995) 667-673.

[24] L. Cruz-Garcia, J. Sánchez-Gurmaches, J. Gutiérrez, I. Navarro, Regulation of LXR by fatty acids, insulin, growth hormone and tumor necrosis factor-a in rainbow trout myocytes, Comp. Biochem. Physiol. 160 (2011) 125-136.

[25] L. Cruz-Garcia, J. Sánchez-Gurmaches, J. Gutiérrez, I. Navarro, Role of LXR in trout adipocytes: Target genes, hormonal regulation, adipocyte differentiation and relation to lipolysis, Comp. Biochem. Physiol. 163 (2012), 120-126.

[26] B.A. Laffitte, S.B. Joseph, R. Walczak, L. Pei, D.C. Wilpitz, J.L. Collins, P. Tontonoz, Autoregulation of the human liver X receptor alpha promoter, Mol. Cell. Biol. 21 (2001) 7558-7568.

[27] D.R. Tocher, Metabolism and functions of lipids and fatty acids in teleost fish, Rev. Fish. Sci. 11 (2003) 107-184.

[28] Y. Wang, D. Botolin, B. Christian, J. Busik, J. Xu, D.B. Jump, Tissue-specific, nutritional, and developmental regulation of rat fatty acid elongases, J. Lipid Res. 46 (2005) 706-715.

[29] Y. Wang, D. Botolin, J. Xu, B. Christian, E. Mitchell, B. Jayaprakasam, M.G. Nair, J.M. Peters, J.V. Busik, L.K. Olson, D.B. Jump, Regulation of hepatic fatty acid elongase and desaturase expression in diabetes and obesity, J. Lipid Res. 47 (2006) 2028-2041.

[30] Y. Qin, K.T. Dalen, J.A. Gustafsson, H.I. Nebb, Regulation of hepatic fatty acid elongase 5 by LXRalpha-SREBP-1c, Biochim. Biophys. Acta 1791 (2009) 140-147.

[31] T.Y. Nara, W.S. He, C. Tang, S.D. Clarke, M.T. Nakamura, The E-box like sterol regulatory element mediates the suppression of human Delta-6 desaturase gene by highly unsaturated fatty acids, Biochem. Biophys. Res. Commun. 296 (2002) 111-117.

[32] G. Carmona-Antoñanzas, D.R. Tocher, J.B. Taggart, M.J. Leaver, An evolutionary perspective on Elovl5 fatty acid elongase: comparison of Northern pike and duplicated paralogs from Atlantic salmon, BMC Evol. Biol. 13 (2013) 85.

[33] L. Michalik, J. Auwerx, J.P. Berger, V.K. Chatterjee, C.K. Glass, F.J. González, P.A. Grimaldi, T. Kadowaki, M.A. Lazar, S. O'Rahilly, C.N.A. Palmer, J. Plutzky, J.K. Reddy, B.M. Spiegelman, B. Staels, W. Wahli, International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors, Pharmacol. Rev. 58 (2006) 726-741.

[34] K. Martens, E. Ver Loren van Themaat, M.F. van Batenburg, M. Heinäniemi, S. Huyghe, P. Van Hummelen, C. Carlberg, P.P. Van Veldhoven, A. Van Kampen, M. Baes, Coordinate induction of PPAR alpha and SREBP2 in multifunctional protein 2 deficient mice, Biochim. Biophys. Acta 1781 (2008) 694-702.

[35] Å. Krogdahl, S. Nordrum, M. Sørensen, L. Brudeseth, C. Røsjø, Effects of diet composition on apparent nutrient absorption along the intestinal tract and of subsequent fasting on mucosal disaccharidase activities and plasma nutrient concentration in Atlantic salmon *Salmo salar* L., Aquacult. Nutr. 5 (1999) 121-133.

[36] P.J. Espenshade, SREBPs: sterol-regulated transcription factors, J. Cell Sci. 119 (2006) 973-976.

[37] M. Amemiya-Kudo, H. Shimano, A.H. Hasty, N. Yahagi, T. Yoshikawa, T. Matsuzaka, H. Okazaki, Y. Tamura, Y. Iizuka, K. Ohashi, J.I. Osuga, K. Harada, T. Gotoda, R. Sato, S.

Kimura, S. Ishibashi, N. Yamada, Transcriptional activities of nuclear SREBP-1a, -1c, and -2 to different target promoters of lipogenic and cholesterogenic genes, J. Lipid Res. 43 (2002) 1220-1235.

Figure captions

Figure 1. Activation of Atlantic salmon LXR.

Concentration-response curve for activation of Atlantic salmon LXR ligand-binding domain by T091317, GW3965, paxilline, cholesterol and oxysterols (20S-OH and 22R-OH). FHM cells were transfected with Gal4-LXR constructs, firefly luciferase reported plasmid pGL4.31 and an internal *Renilla* luciferase reporter to correct for transfection efficiencies. The ordinate represents activation of LXR as arbitrary units of firefly luciferase normalized to *Renilla* luciferase. Data points represent the mean of three independent experiments (n = 9).

Figure 2. Effects LXR and PPAR agonists on transcription factor gene expression in Atlantic salmon SHK-1 cells.

Expression of *lxr*, *srebp1*, *srebp2*, *pparα*, *pparβ* and *pparγ* in SHK-1 cells exposed to 20 μM cholesterol (Chol), 10 μM of LXR agonists T0901317 (T0) or GW3965 (GW), 25 μM of PPAR agonists 2-bromopalmitate (2-BP) or WY14643 (WY), or carrier alone (EtOH). Gene expression is expressed as mean normalized ratios (n = 3, ± SE) between the copy numbers of target genes and the mean copy number of the reference genes (*elf-1α* and *β-actin*). Bars bearing different letters are significantly different (ANOVA; Tukey's test; $P < 0.05$).

Figure 3. Effects LXR and PPAR agonists on lipid metabolic gene expression in Atlantic salmon SHK-1 cells.

Expression of Atlantic salmon lipid metabolic genes in SHK-1 cells exposed to 20 μM cholesterol (Chol), 10 μM of LXR agonists T0901317 (T0) or GW3965 (GW), 25 μM of PPAR agonists 2-

bromopalmitate (2-BP) or WY14643 (WY), or carrier alone (EtOH). Gene expression was expressed as mean normalized ratios (n = 3, ± SE) between the copy numbers of target genes and the mean copy number of the reference genes (*elf-1α* and *β-actin*). Bars bearing different letters are significantly different (ANOVA; Tukey's test; $P < 0.05$).

Figure 4. Effects of SREBPs on LC-PUFA gene promoters.

Co-transfection in FHM cells with Srebp1, or Srebp2 expression constructs (empty pcDNA3 expression vector as control), and reporter pGL4.10 [*luc2*] directed by the promoters of salmon *elovl5a* (- 3618 nt), *elovl5b* (- 3141 nt), or *fads2d6a* (- 1791 nt). Error bars indicate SE between data points (n = 3) of independent luciferase assays. * Significant differences between treatments are indicated (ANOVA; Tukey's test; $P < 0.05$).

Figure 5. Effects of LXR and PPARs on LC-PUFA gene promoters.

Co-transfection in FHM cells with Pparα or Pparβ expression constructs (empty pcDNA3 expression vector as control), and reporter pGL4.10 [*luc2*] directed by the promoters of salmon *elovl5a* (- 3618 nt), *elovl5b* (- 3141 nt), or *fads2d6a* (- 1791 nt). After transfection, FHM cells were incubated with Ppar agonists WY14643 (25 μM) or 2-bromopalmitate (2-BP, 25 μM), Lxr synthetic agonist GW3965 (10 μM), or ethanol carrier (EtOH). Error bars indicate SE between data points (n = 3) of independent luciferase assays. * Significant differences between treatments are indicated (ANOVA; Tukey's test; $P < 0.05$).

Figure 6. Nutritional regulation of transcription factor mRNA in Atlantic salmon intestine.

lxr, *srebp1*, *srebp2*, *pparα*, *pparβ* and *pparγ* mRNA expression in Atlantic salmon fed diets containing fish oil (FO), rapeseed oil (RO), soybean oil (SO), or linseed oil (LO) in the pyloric caeca determined by RT-qPCR. The results shown are means (n = 5) ± SE of normalized expression (β-actin and elongation factor-1alpha reference genes) in relative units (RU). Bars bearing different letters represent significant differences between dietary treatments for the respective transcripts (REST-MCS 2009 V2; $P < 0.05$).

Figure 7. Diagram indicating LXR and SREBP target genes.

A) SHK-1 cells incubated with synthetic Lxr agonists (T0901317 and GW3965) showed that Lxr regulates multiple genes of the lipid biosynthetic pathway, mediating cross-regulation between cholesterol and fatty acid biosynthesis. Transcriptional expression of *srebp* was induced by Lxr agonist *in vitro* and dietary lipids (vegetable oils) in salmon intestine. B) Co-transfection assays in FHM cells indicated that Srebp1 and Srebp2 promote the transcription of *elovl5* and *fads2*, genes of the LC-PUFA biosynthesis; ER, endoplasmic reticulum; n, amino-terminus.

Figure1

Figure 1.

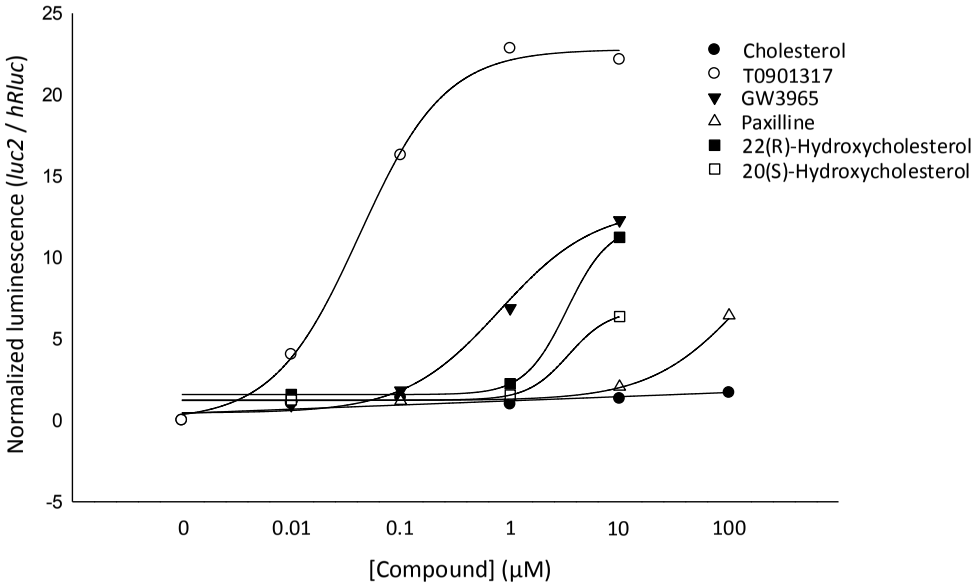


Figure2

Figure 2.

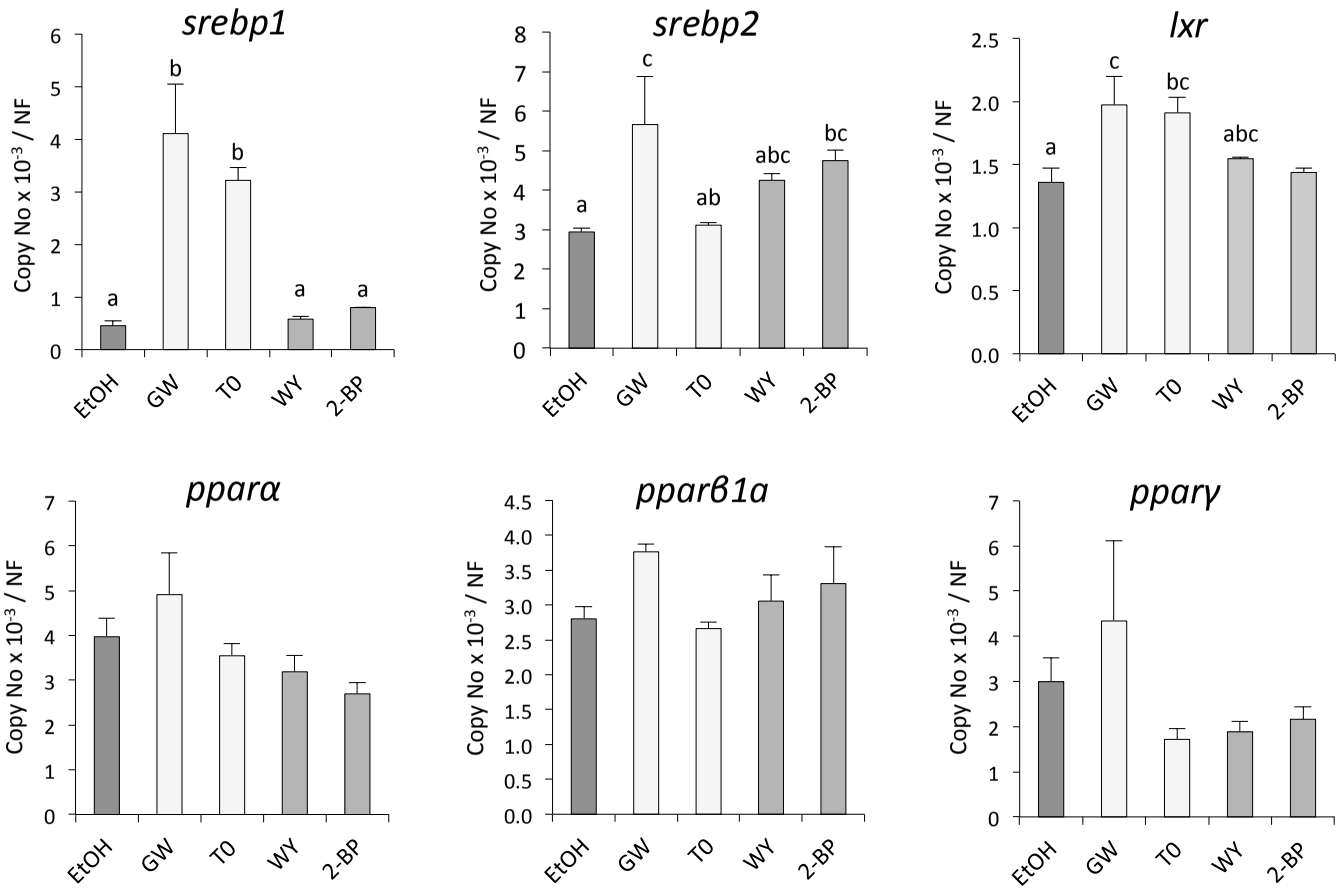


Figure3

Figure 3.

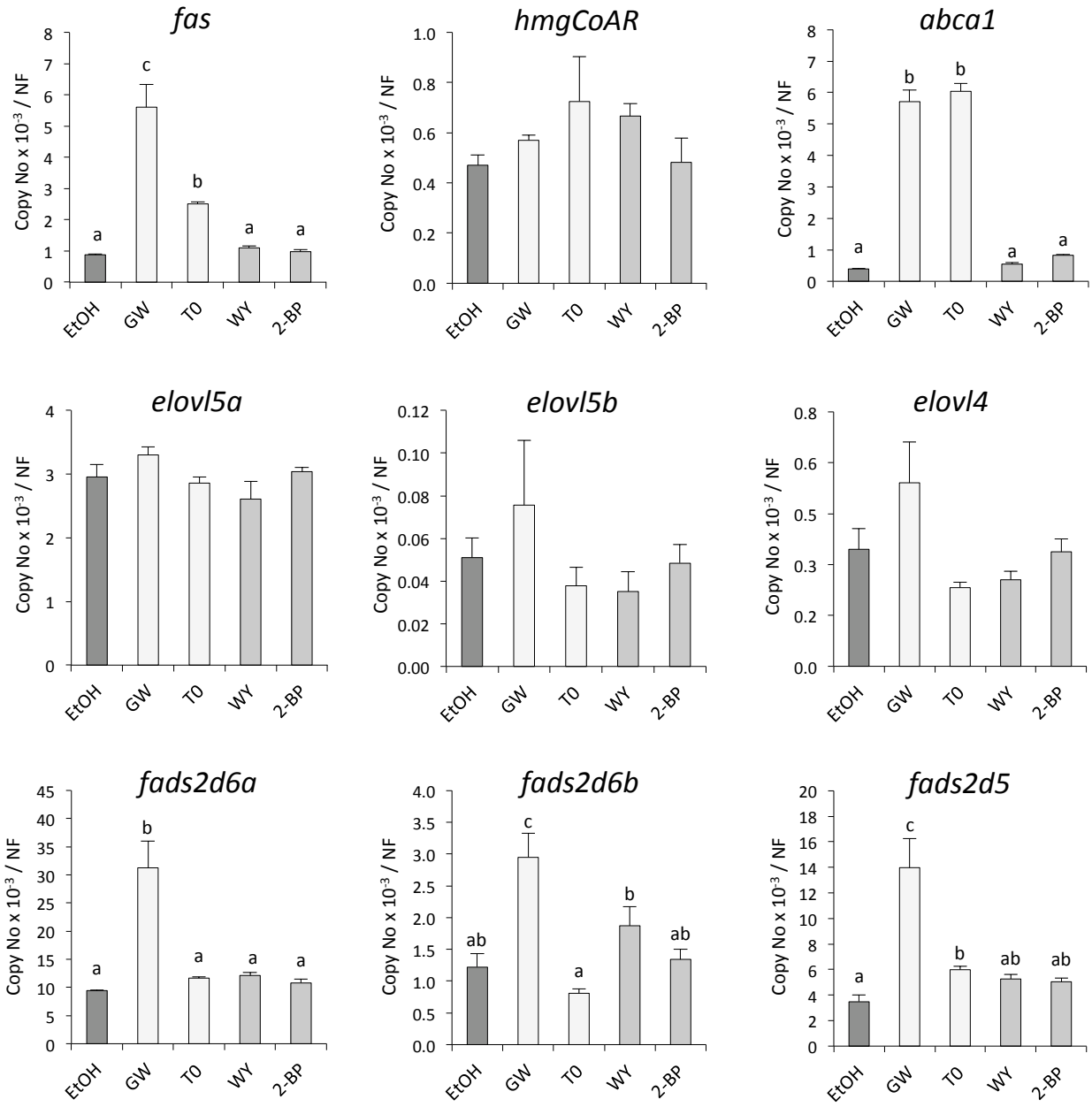


Figure 4.

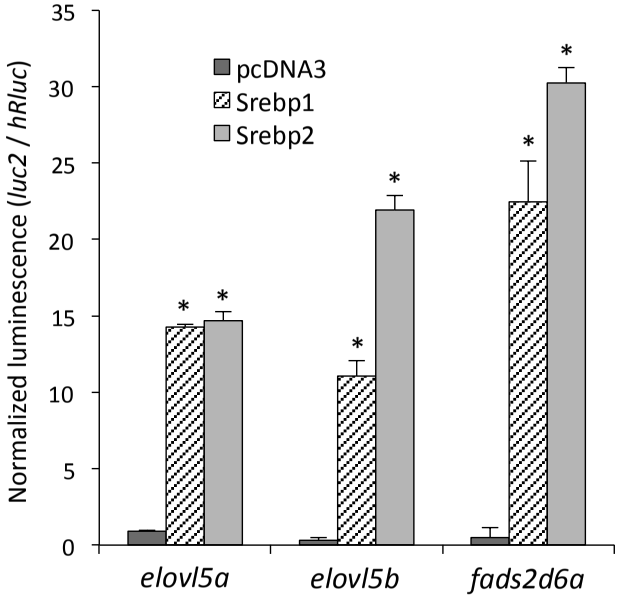

35
30
25
20
15
10
5
0
Normalized luminescence (*luc2* / *hRluc*)
pcDNA3
Srebp1
Srebp2
*
*
*
*
*
*
elovl5a
elovl5b
fads2d6a

Figure5

Figure 5.

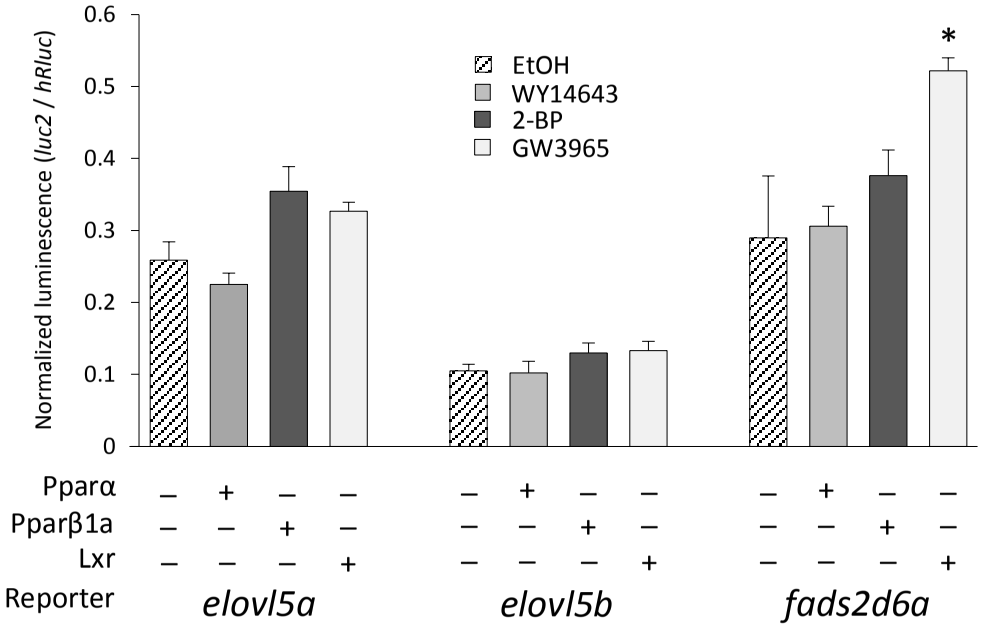


Figure6

Figure 6.

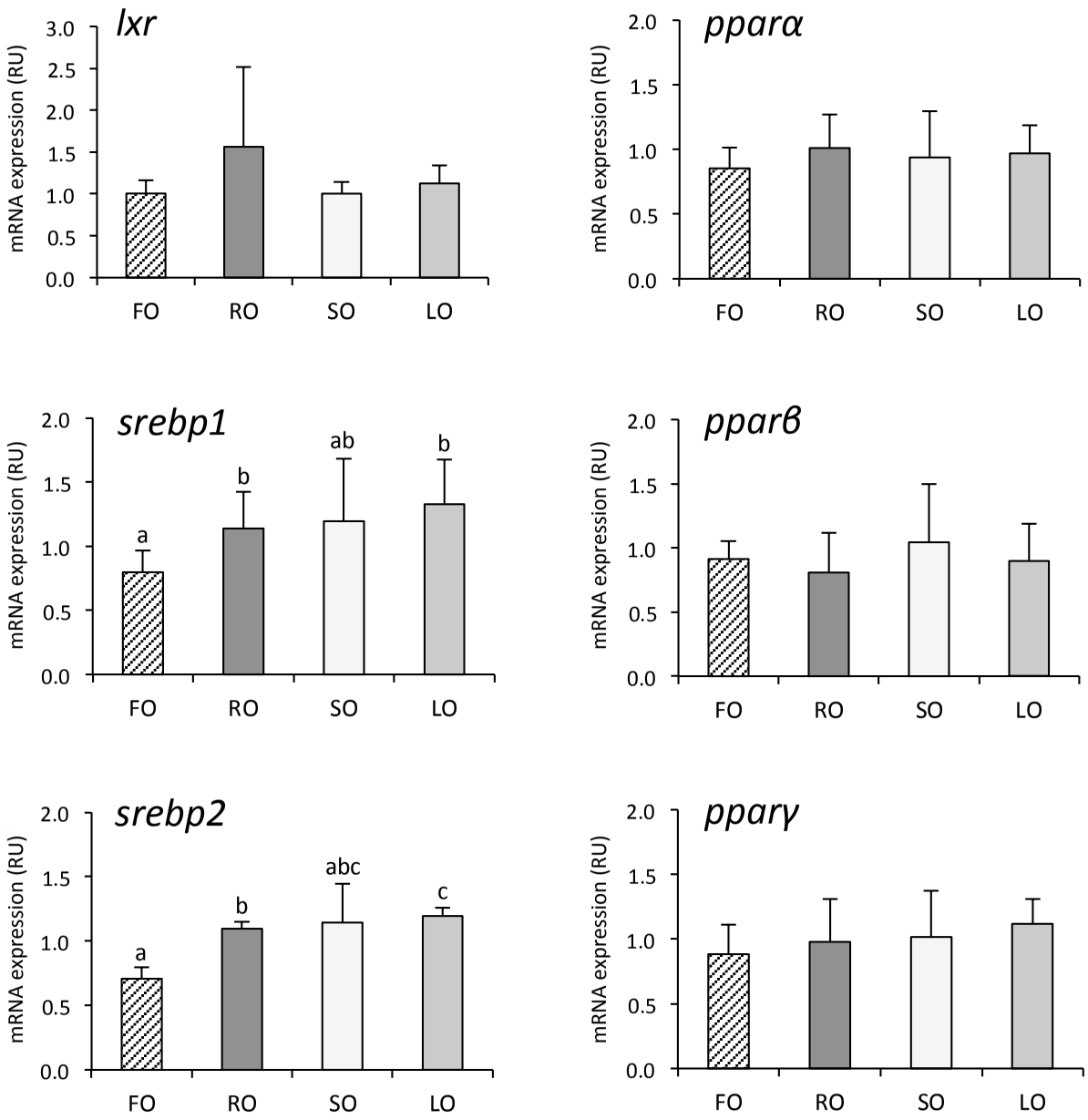
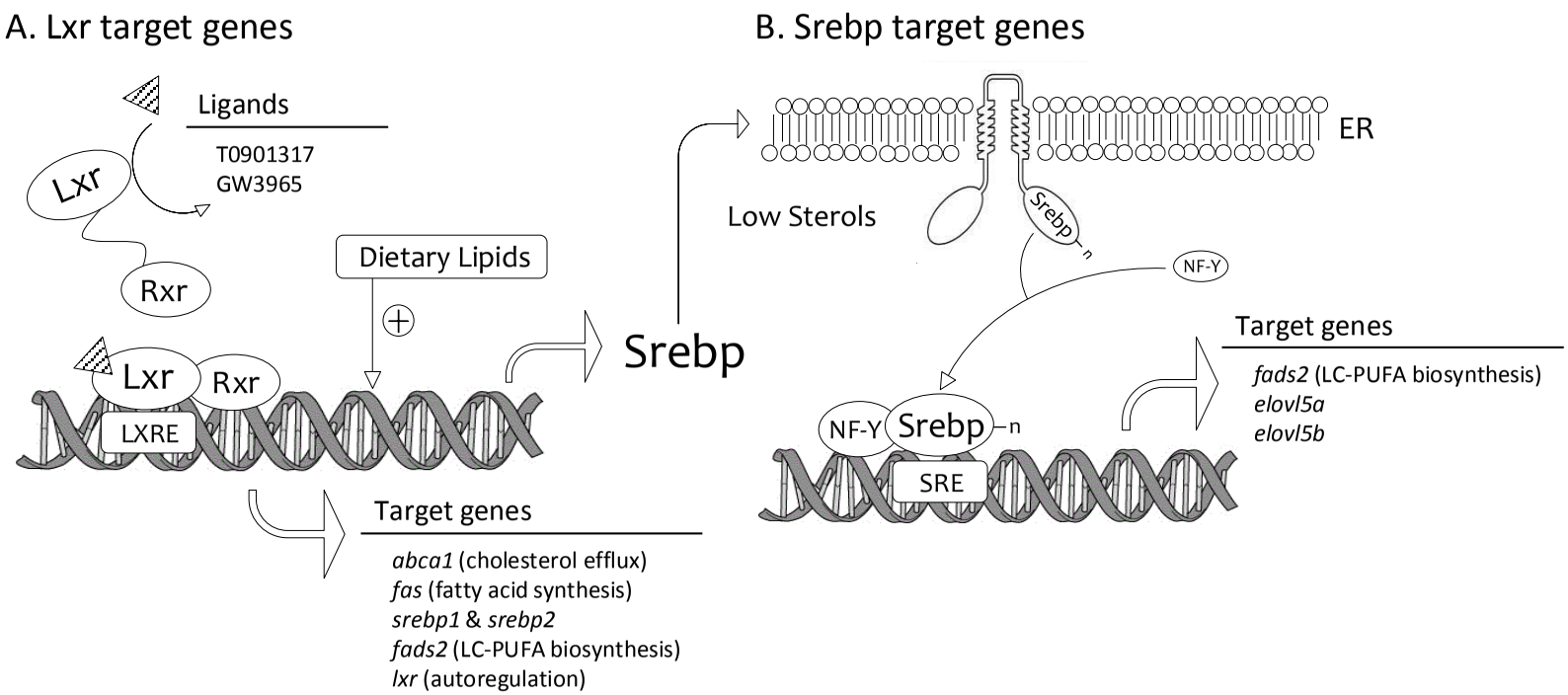


Figure7

Figure 7.



Supplementary table1

Click here to download Supplementary material for on-line publication only: Supplementary Table 1.pdf