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1 **Conservation of lipid metabolic gene transcriptional regulatory networks**

2 **in fish and mammals**

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4

5 **Greta Carmona-Antoñanzas^{a §}, Douglas R. Tocher, Laura Martinez-Rubio and Michael J.**

6 **Leaver**

7

8 ^a School of Natural Sciences, University of Stirling, Institute of Aquaculture, Stirling FK9 4LA, UK

9

10 [§] Corresponding author

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

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

13 LMR: laura.martinez@stir.ac.uk

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

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21 **Abstract**

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

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42 **Key words**

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

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46 **1. Introduction**

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

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

71 protein, apolipoproteins, cholesterol 7 α -hydroxylase (CYP7 α 1) 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 cholesterologenic 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. PPAR α and PPAR β , regulate the expression of genes encoding mitochondrial and
82 peroxisomal fatty acid-catabolizing enzymes, whilst PPAR γ 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.

97

98 **2. Materials & Methods**

99 *2.1. Cell lines and cell culture*

100 The established Atlantic salmon cell line derived from head kidney (SHK-1) was grown at 22 °C in an
101 atmosphere of 4 % carbon dioxide in Dulbecco's modified eagle medium (DMEM) containing 3 g L⁻¹
102 D-glucose and 55 mg L⁻¹ sodium pyruvate, and supplemented with 10% foetal bovine serum (FBS),
103 50 U mL⁻¹ penicillin, 50 µg mL⁻¹ streptomycin, 40 µM 2-mercaptoethanol and 4 mM L-glutamine. For
104 gene promoter transactivation assays, fathead minnow (*Pimephales promelas*; FHM) epithelial cells
105 were maintained at 22 °C in Leibovitz's L-15 with GlutaMAXTM-1 medium containing 900 mg L⁻¹ D+
106 galactose and 550 mg L⁻¹ sodium pyruvate and 10% FBS. All media and supplements were obtained
107 from Life Technologies (Glasgow, UK).

108 For subculturing, the cell monolayer was washed twice with phosphate buffer saline (PBS) without
109 CaCl₂ or MgCl₂ (Invitrogen, UK), cells detached by incubation with 0.05 % trypsin/0.02 % EDTA
110 and re-suspended in medium. Viable cells were counted after harvesting using a Neubauer
111 haemocytometer, 0.4 % Trypan blue (Sigma, Dorset, UK) and an inverted microscope (IMT-2,
112 Olympus).

113 For transcription factor (TF) ligand treatments, SHK-1 cells were seeded in 6-well clear plates (Nunc,
114 Denmark) at a density of 4 x 10⁵ cells per well in a volume of 3 ml Leibovitz's L-15 medium. Cells
115 were approximately 70 % confluent after 48 h growth, when medium was aspirated, cells washed
116 twice with PBS and fresh medium containing the various treatments as ethanol solutions was added.
117 Final concentrations were cholesterol (20 µM), WY14643 (25 µM), 2-bromopalmitate (25 µM), or
118 LXR agonists (GW3965 and T0901317, 10 µM) or with ethanol carrier alone (100% ethanol). After
119 24 h, the medium was aspirated, the cell monolayer washed twice (PBS) and cells scraped from each
120 well in 0.5 ml of PBS. Cells were centrifuged for 5 min at 3000 x g, PBS discarded and replaced by

121 0.5 ml of TriReagent (Ambion, UK), followed by vigorous mixing to lyse and digest cells. Cells from
122 two wells were pooled to produce three replicates per treatment.

123 *2.2. Fish, diets and sampling protocols*

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

138 *2.3. Atlantic salmon LXR activation assay*

139 The ligand binding domain (LBD) (amino acid residues 191-462) [GenBank:FJ470290] of Lxr was
140 amplified by PCR from salmon pyloric caeca cDNA using primers (Supplementary Table 1) with 5'
141 restriction sites to allow in-frame subcloning between the *Bam*HI and *Xba*I sites of the pBIND vector
142 (Promega, Southampton, UK), which contains the yeast GAL4 DNA-binding domain [8]. The
143 resulting pBIND-Lxr chimeras were co-transfected with a reporter gene plasmid in which the *Firefly*
144 luciferase gene is under the control of a promoter containing UAS (upstream activation sequence),
145 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, 2×10^4 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 μ l of
152 Polyfect transfection reagent (Qiagen) in 100 μ l 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 μ l of the dilution was added per well and incubated for 24 h. Cells were lysed
161 by 10 min incubation in 75 μ l 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

172 °C prior RNA extraction. Total RNA was extracted following the manufacturer's instructions
173 (Ambion, UK), and the quantity and quality of isolated RNA determined by electrophoresis and
174 spectrophotometry (Nanodrop 1000, Thermo Scientific, USA). One microgram of total RNA was
175 reverse transcribed into cDNA using the Verso cDNA synthesis kit (Thermo Scientific, UK) and
176 primed with random hexamers and oligo(dT) in a 3:1 molar ratio. The resulting cDNA was diluted 20-
177 fold with nuclease-free water. For quantitative qPCR, oligonucleotide primers for target genes and
178 housekeeping genes (*elf-1α* and *β-actin*) (Supplementary Table 1) were used at 0.3 μM with one-
179 fortieth of the cDNA synthesis reaction (5 μl of a 1:20 dilution) and 10 μl of SYBR-green qPCR mix
180 (ABgene, UK) in a total volume of 20 μl. Reactions were run in a Mastercycler RealPlex² (Eppendorf,
181 UK). Amplifications were carried out including systematic negative controls containing no cDNA
182 (NTC, no template control) and omitting reverse transcriptase enzyme (-RT) to check for DNA
183 contamination. Thermal cycle and melting curves were performed as described previously with
184 specific annealing temperatures for each primer pair shown in Supplementary Table 1 [16]. The qPCR
185 product sizes were checked by agarose gel electrophoresis and the identity of random samples
186 confirmed by sequencing. Absolute quantification was achieved by including a parallel set of
187 reactions containing spectrophotometrically-determined standards consisting of serial dilutions of
188 known copy numbers of linearised plasmid that contain the predicted amplification product for each
189 measured gene. Results were expressed as mean normalized ratios (\pm SE) between the copy number
190 of target genes and the mean copy number of the reference genes (*elf-1α* and *β-actin*). Differences in
191 the expression of target genes among different treatments were determined by one-way analysis of
192 variance (ANOVA), followed by multiple comparisons using Tukey's *post hoc* test (PASWS 18.0,
193 SPSS Inc., USA). In contrast, the effects of diet on TF expression were analyzed for statistical
194 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*).
195 Gene expression was presented as the relative expression ratio of each gene in fish fed one of the VOs
196 or FO. A significance of $P \leq 0.05$ was applied to all statistical tests performed.

198 *2.6. Expression constructs and cell transfection assays*

199 Atlantic salmon transcription factors Lxr [GenBank: FJ470290], Ppara [GenBank: AM230809],
200 Ppar β 1a [GenBank: AJ416953], Srebp1 [GenBank: HM561860] and Srebp2 [GenBank:
201 HM561861NM_004599] had been previously described [12,13]. The entire open reading frame of Lxr
202 (Lxr ORF, 1-462 amino acids), Ppara (Ppara ORF, 1-464 aa), Ppar β 1a (Ppar β ORF, 1-443 aa), and
203 the soluble N-terminal domains of Srebp1 (nSrebp1, 1-476 aa) and Srebp2 (nSrebp2, 1-460 aa) were
204 amplified from caecal cDNA samples using the primers detailed in Supplementary Table 1, which
205 included suitable restriction sites, *EcoRI* and *XhoI*, for subsequent insertion into the CMV-based
206 constitutive expression vector pcDNA3 (Invitrogen, UK). Positive clones were selected by enzymatic
207 digestion and sequenced (CEQ-8800 Beckman Coulter Inc., USA). The putative promoter regions,
208 first non-coding exon, first intron and the ATG start codon of Atlantic salmon *elovl5a* (-3618,
209 [GenBank:GU238431]), *elovl5b* (-3141, [GenBank:GU324549]), and *fads2d6a* (-1791,
210 [GenBank:AY736067]) were amplified from genomic DNA using high-fidelity PfuTurbo DNA
211 Polymerase (Statagene, UK) and primers containing restriction sites (Supplementary Table 1). These
212 fragments were cloned into *SacI* and *NcoI* or *XhoI* sites encompassing the luciferase start codon of a
213 promoterless reporter plasmid [pGL4.10, *luc2*] (Promega, USA), which encodes *Firefly* luciferase.

214 For luciferase assays, FHM cells were co-transfected, under the conditions described previously, with
215 the salmon *elovl5* or *fads2d6a* reporter constructs, Lxr, Ppara, Ppar β 1a, nSrebp1 or nSrebp2
216 expression constructs to overexpress the protein product. Briefly, stock FHM cells were seeded in 96-
217 well opaque plates at a density of 2×10^4 per well 24 h prior to transfection. Transfection mixtures
218 consisted of 50 ng of pGL4.10 reporter construct (empty pGL4.10 vector in controls), 30 ng of
219 pcDNA3 expression construct (empty pcDNA3 vector in controls), 20 ng of *Renilla* pGL4.75 and 1 μ l
220 of Polyfect transfection reagent to 100 μ l of L-15 medium. Within each transfection experiment, each
221 treatment was performed in triplicate. After 24 h, the medium was aspirated, monolayer washed twice
222 with PBS, cells lysed, and *Firefly* and *Renilla* luciferase activities quantified as described previously
223 [15]. Transactivation activities were obtained, data normalized and statistical analyses performed as
224 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 μ M. The synthetic agonist paxilline exhibited the
234 lowest effect, whereas cholesterol had no effect. No response was detected at 100 μ M 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 Ppar α and Ppar β agonist. However, Wy1465, a specific
247 Ppara 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.

250 3.3. *Trans-regulation of genes related to LC-PUFA metabolism by Srebp, Lxr and Ppar*

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

262 3.4. *Nutritional regulation of the expression of transcription factors*

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

270

271 **4. Discussion**

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

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

289 *4.1. Lxr ligand activation*

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

299 *4.2. Transcriptional regulation of genes of the lipid metabolism*

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

318 Although vertebrates have the capability for endogenous synthesis of monounsaturated fatty acids
319 from the saturated fatty acid products of FAS through the action of stearoyl CoA desaturase (SCD or
320 $\Delta 9$ desaturase), they are incapable of creating LC-PUFA *de novo*. Vertebrates thus require either
321 dietary LC-PUFA directly or, depending upon species, they can produce the physiologically important
322 ARA, EPA and DHA by desaturation and elongation of dietary shorter chain PUFA, linoleic (18:2n-
323 6) and α -linolenic (18:3n-3) acids, through the action of Fads1 and 2 and Elov15 [27]. Intense interest
324 has been paid to vertebrate LC-PUFA enzymes given their hormonal, ontogenetic and/or nutritional
325 regulation in vertebrates [28,29]. Salmon Elov15 enzymes are encoded by two very similar duplicated
326 genes [3]. Neither mRNA was affected by Lxr agonist in SHK-1 cells, despite the *elov15* gene

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

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

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

350 *4.3. Dietary regulation of lipid transcription factors*

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

371 **5. Conclusions**

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

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

389

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394

395 **Conflict of interest**

396 The authors declare that there are no conflicts of interest.

397

398 **References**

399 [1] B.E. Torstensen, J.G. Bell, G. Rosenlund, R.J. Henderson, I.E. Graff, D.R. Tocher, Ø. Lie, J.R.
400 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 $\Delta 6$ 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.

- 452 [20] A. Caimari, P. Oliver, J. Keijer, A. Palou, Peripheral blood mononuclear cells as a model to
453 study the response of energy homeostasis-related genes to acute changes in feeding conditions,
454 OMICS 14 (2010) 129-141.
- 455 [21] J.J. Repa, S.D. Turley, J.A. Lobaccaro, J. Medina, L. Li, K. Lustig, B. Shan, R.A. Heyman, J.M.
456 Dietschy, D.J. Mangelsdorf, Regulation of absorption and ABC1-mediated efflux of cholesterol
457 by RXR heterodimers, Science 289 (2000) 1524-1529.
- 458 [22] J.J. Repa, G. Liang, J. Ou, Y. Bashmakov, J.M. Lobaccaro, I. Shimomura, B. Shan, M.S.
459 Brown, J.L. Goldstein, D.J. Mangelsdorf, Regulation of mouse sterol regulatory element-
460 binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRA and LXRbeta, Genes
461 Dev. 14 (2000) 2819-2830.
- 462 [23] X. Hua, J. Wu, J.L. Goldstein, M.S. Brown, H.H. Hobbs, Structure of the human gene encoding
463 sterol regulatory element binding protein-1 (SREBF1) and localization of SREBF1 and
464 SREBF2 to chromosomes 17p11.2 and 22q13, Genomics 25 (1995) 667-673.
- 465 [24] L. Cruz-Garcia, J. Sánchez-Gurmaches, J. Gutiérrez, I. Navarro, Regulation of LXR by fatty
466 acids, insulin, growth hormone and tumor necrosis factor- α in rainbow trout myocytes, Comp.
467 Biochem. Physiol. 160 (2011) 125-136.
- 468 [25] L. Cruz-Garcia, J. Sánchez-Gurmaches, J. Gutiérrez, I. Navarro, Role of LXR in trout
469 adipocytes: Target genes, hormonal regulation, adipocyte differentiation and relation to
470 lipolysis, Comp. Biochem. Physiol. 163 (2012), 120-126.
- 471 [26] B.A. Laffitte, S.B. Joseph, R. Walczak, L. Pei, D.C. Wilpitz, J.L. Collins, P. Tontonoz,
472 Autoregulation of the human liver X receptor alpha promoter, Mol. Cell. Biol. 21 (2001) 7558-
473 7568.
- 474 [27] D.R. Tocher, Metabolism and functions of lipids and fatty acids in teleost fish, Rev. Fish. Sci.
475 11 (2003) 107-184.
- 476 [28] Y. Wang, D. Botolin, B. Christian, J. Busik, J. Xu, D.B. Jump, Tissue-specific, nutritional, and
477 developmental regulation of rat fatty acid elongases, J. Lipid Res. 46 (2005) 706-715.

- 478 [29] Y. Wang, D. Botolin, J. Xu, B. Christian, E. Mitchell, B. Jayaprakasam, M.G. Nair, J.M. Peters,
479 J.V. Busik, L.K. Olson, D.B. Jump, Regulation of hepatic fatty acid elongase and desaturase
480 expression in diabetes and obesity, *J. Lipid Res.* 47 (2006) 2028-2041.
- 481 [30] Y. Qin, K.T. Dalen, J.A. Gustafsson, H.I. Nebb, Regulation of hepatic fatty acid elongase 5 by
482 LXRA α -SREBP-1c, *Biochim. Biophys. Acta* 1791 (2009) 140-147.
- 483 [31] T.Y. Nara, W.S. He, C. Tang, S.D. Clarke, M.T. Nakamura, The E-box like sterol regulatory
484 element mediates the suppression of human Delta-6 desaturase gene by highly unsaturated fatty
485 acids, *Biochem. Biophys. Res. Commun.* 296 (2002) 111-117.
- 486 [32] G. Carmona-Antoñanzas, D.R. Tocher, J.B. Taggart, M.J. Leaver, An evolutionary perspective
487 on Elovl5 fatty acid elongase: comparison of Northern pike and duplicated paralogs from
488 Atlantic salmon, *BMC Evol. Biol.* 13 (2013) 85.
- 489 [33] L. Michalik, J. Auwerx, J.P. Berger, V.K. Chatterjee, C.K. Glass, F.J. González, P.A. Grimaldi,
490 T. Kadowaki, M.A. Lazar, S. O'Rahilly, C.N.A. Palmer, J. Plutzky, J.K. Reddy, B.M.
491 Spiegelman, B. Staels, W. Wahli, International Union of Pharmacology. LXI. Peroxisome
492 proliferator-activated receptors, *Pharmacol. Rev.* 58 (2006) 726-741.
- 493 [34] K. Martens, E. Ver Loren van Themaat, M.F. van Batenburg, M. Heinäniemi, S. Huyghe, P.
494 Van Hummelen, C. Carlberg, P.P. Van Veldhoven, A. Van Kampen, M. Baes, Coordinate
495 induction of PPAR α and SREBP2 in multifunctional protein 2 deficient mice, *Biochim.*
496 *Biophys. Acta* 1781 (2008) 694-702.
- 497 [35] Å. Krogdahl, S. Nordrum, M. Sørensen, L. Brudeseth, C. Røsjø, Effects of diet composition on
498 apparent nutrient absorption along the intestinal tract and of subsequent fasting on mucosal
499 disaccharidase activities and plasma nutrient concentration in Atlantic salmon *Salmo salar* L.,
500 *Aquacult. Nutr.* 5 (1999) 121-133.
- 501 [36] P.J. Espenshade, SREBPs: sterol-regulated transcription factors, *J. Cell Sci.* 119 (2006) 973-
502 976.
- 503 [37] M. Amemiya-Kudo, H. Shimano, A.H. Hasty, N. Yahagi, T. Yoshikawa, T. Matsuzaka, H.
504 Okazaki, Y. Tamura, Y. Iizuka, K. Ohashi, J.I. Osuga, K. Harada, T. Gotoda, R. Sato, S.

505 Kimura, S. Ishibashi, N. Yamada, Transcriptional activities of nuclear SREBP-1a, -1c, and -2 to
506 different target promoters of lipogenic and cholesterogenic genes, J. Lipid Res. 43 (2002) 1220-
507 1235.

508

509 **Figure captions**

510 **Figure 1. Activation of Atlantic salmon LXR.**

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

517 **Figure 2. Effects LXR and PPAR agonists on transcription factor gene expression in Atlantic** 518 **salmon SHK-1 cells.**

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

525 **Figure 3. Effects LXR and PPAR agonists on lipid metabolic gene expression in Atlantic salmon** 526 **SHK-1 cells.**

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

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

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

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

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

540 Co-transfection in FHM cells with *Ppara α* or *Ppara β* expression constructs (empty pcDNA3 expression
541 vector as control), and reporter pGL4.10 [*luc2*] directed by the promoters of salmon *elovl5a* (- 3618
542 nt), *elovl5b* (- 3141 nt), or *fads2d6a* (- 1791 nt). After transfection, FHM cells were incubated with
543 *Ppar* agonists WY14643 (25 μ M) or 2-bromopalmitate (2-BP, 25 μ M), *Lxr* synthetic agonist GW3965
544 (10 μ M), or ethanol carrier (EtOH). Error bars indicate SE between data points ($n = 3$) of independent
545 luciferase assays. * Significant differences between treatments are indicated (ANOVA; Tukey's test;
546 $P < 0.05$).

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

548 *lxr*, *srebp1*, *srebp2*, *ppara*, *ppar β* and *ppar γ* mRNA expression in Atlantic salmon fed diets containing
549 fish oil (FO), rapeseed oil (RO), soybean oil (SO), or linseed oil (LO) in the pyloric caeca determined
550 by RT-qPCR. The results shown are means ($n = 5$) \pm SE of normalized expression (*β -actin* and
551 elongation factor-1alpha reference genes) in relative units (RU). Bars bearing different letters
552 represent significant differences between dietary treatments for the respective transcripts (REST-MCS
553 2009 V2; $P < 0.05$).

554 **Figure 7. Diagram indicating LXR and SREBP target genes.**

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

Figure 1.

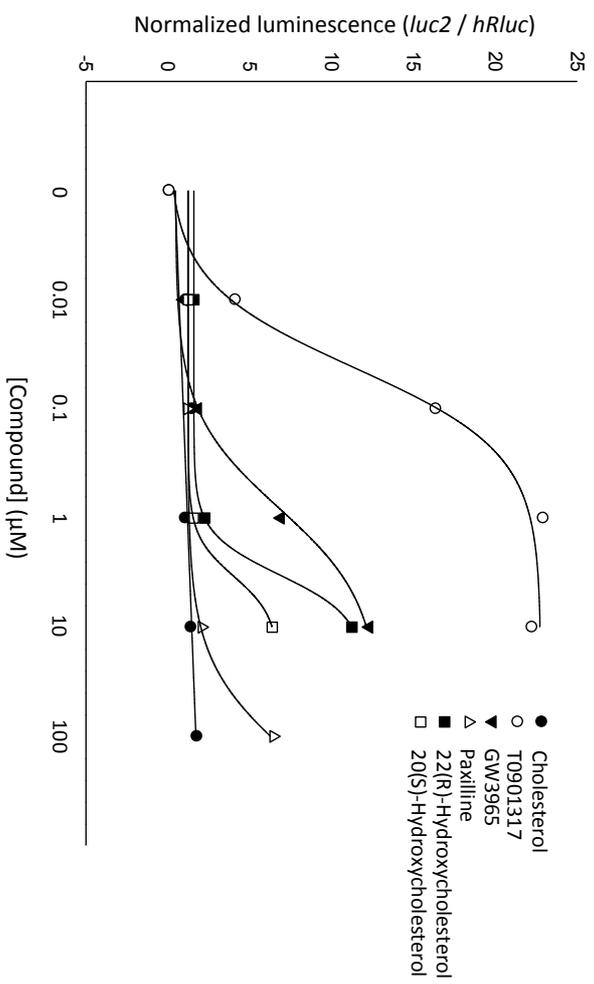


Figure 2.

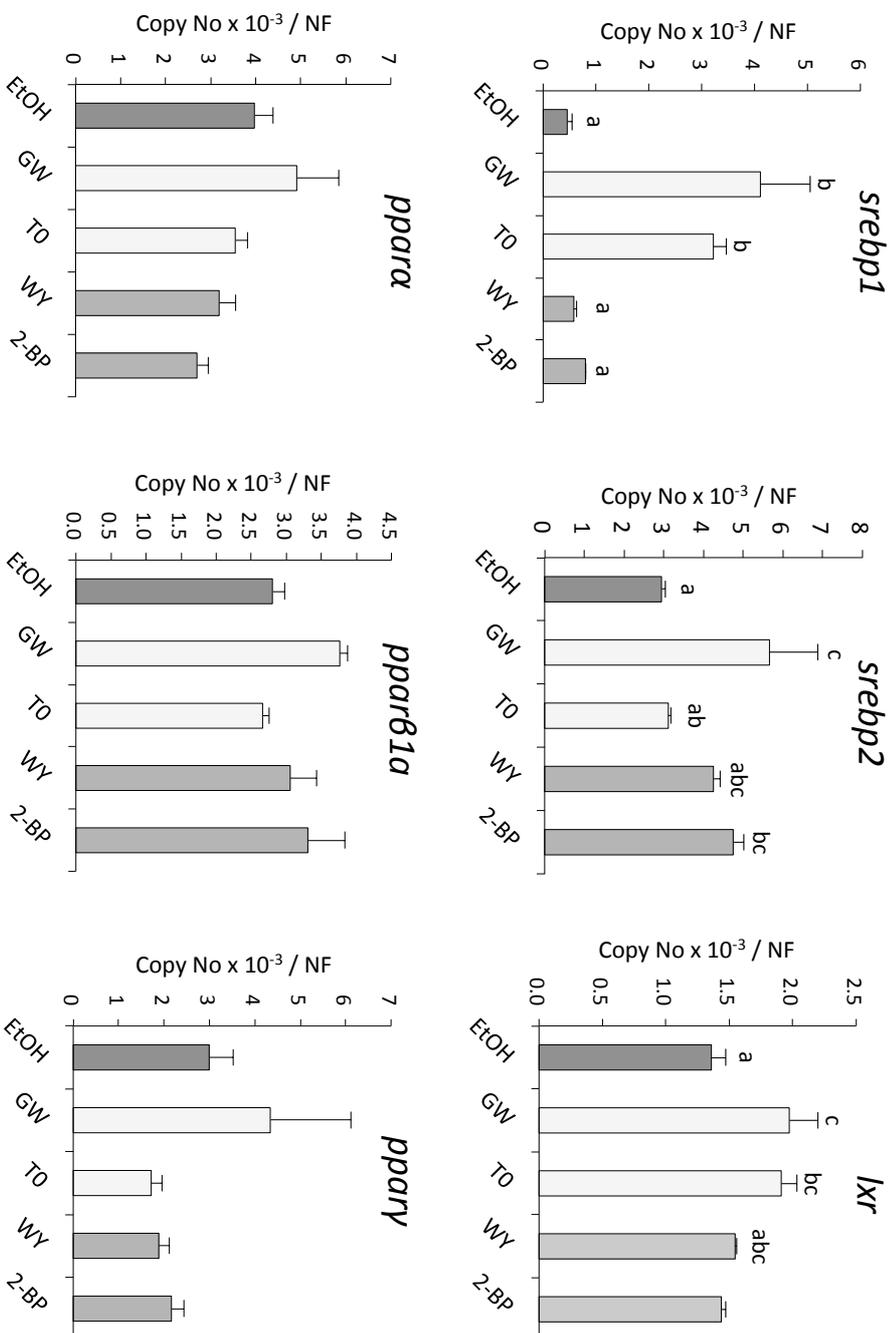


Figure3

Figure 3.

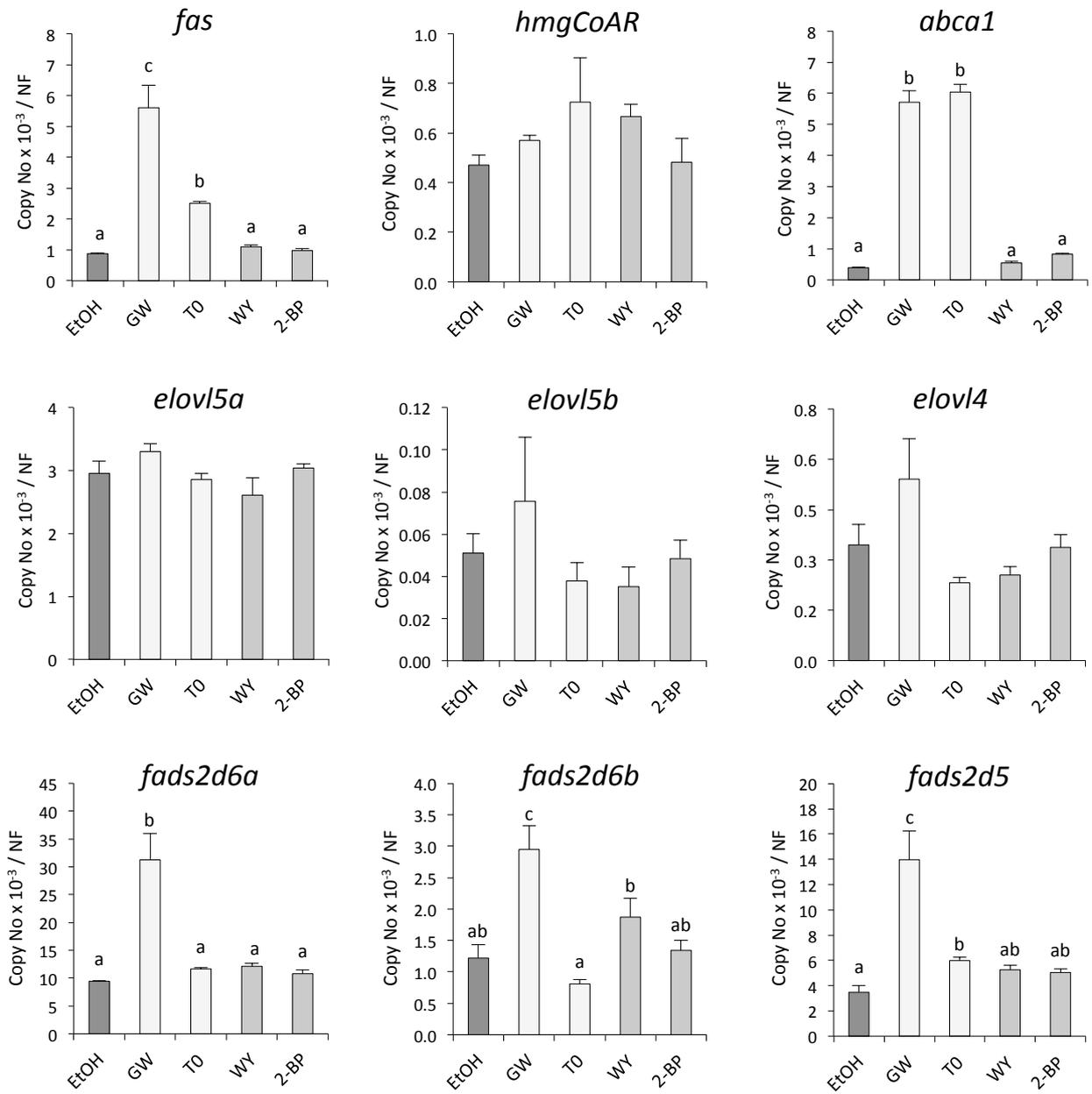


Figure 4.

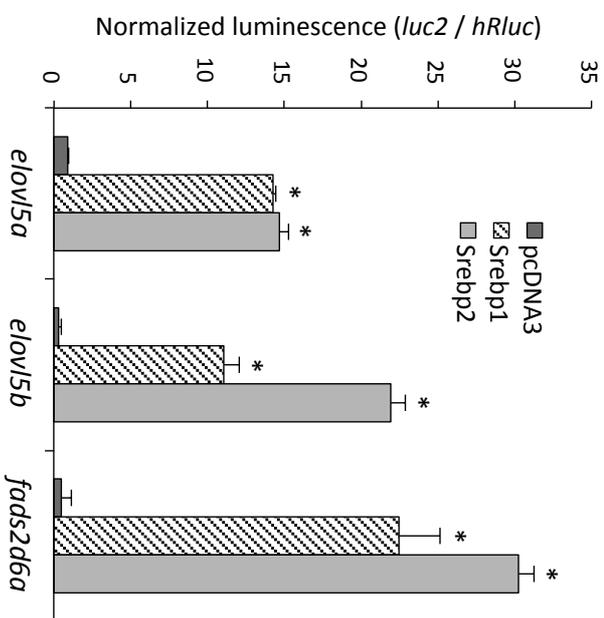


Figure 5.

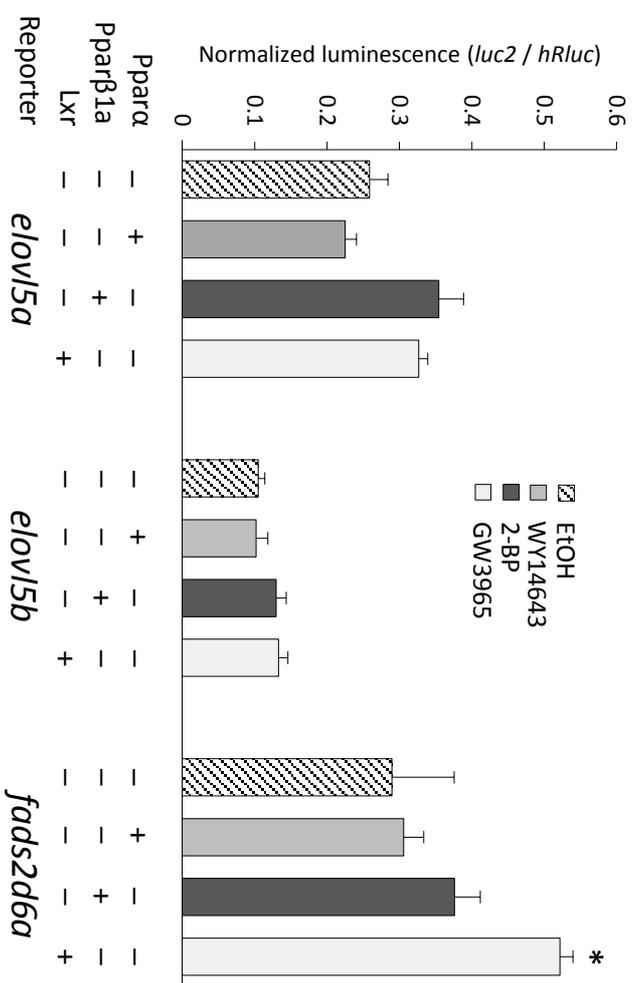


Figure 6.

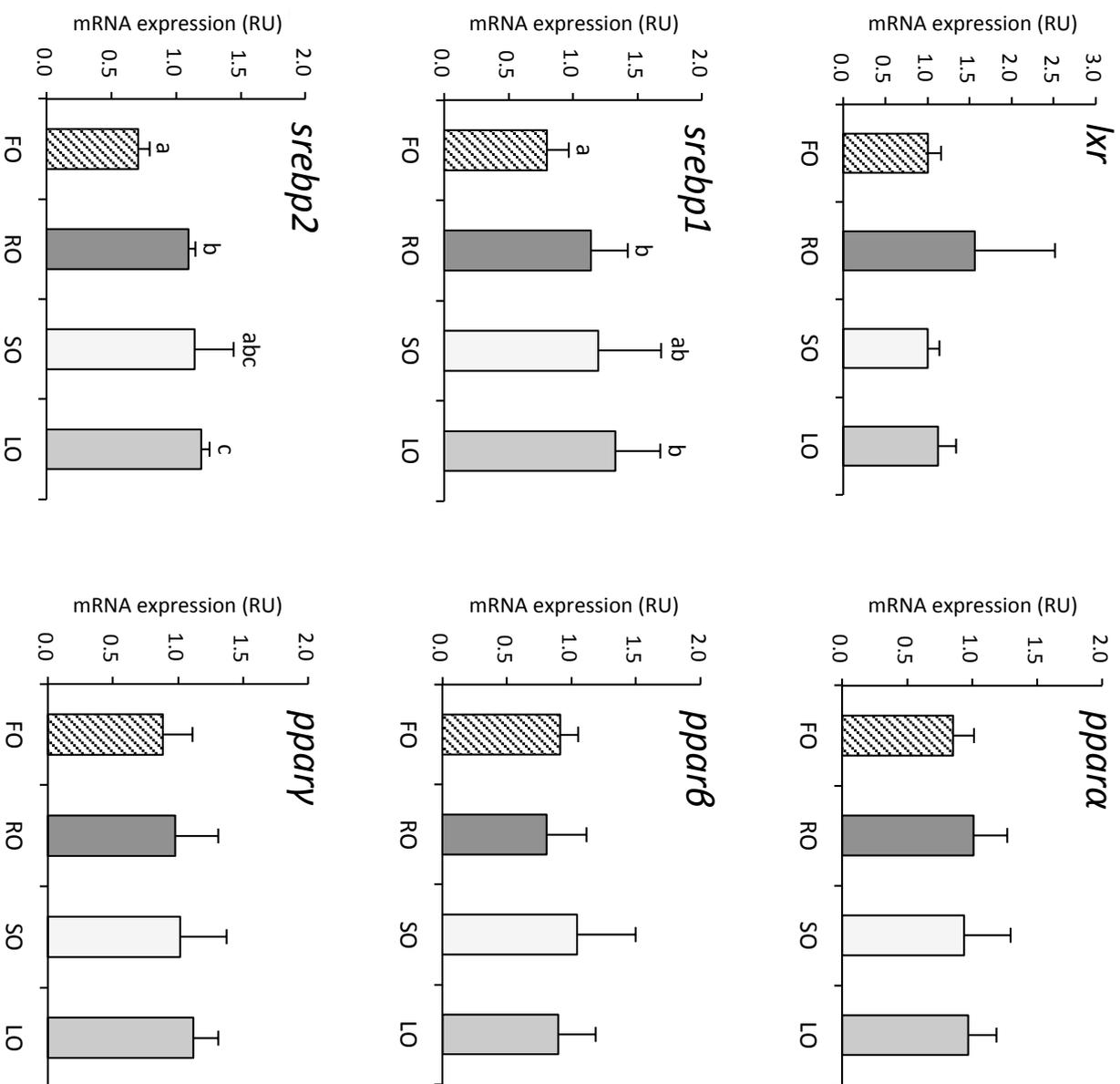
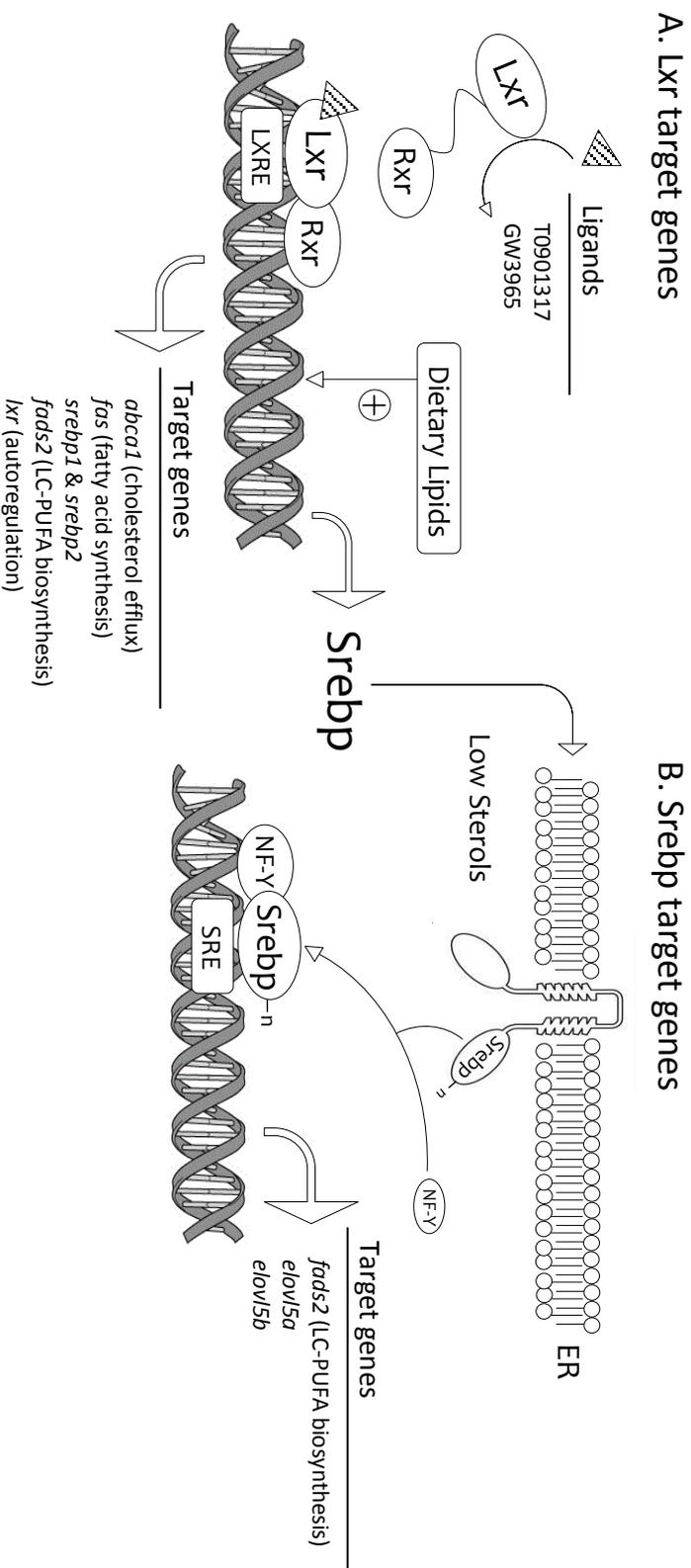


Figure 7.



Supplementary table1

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