Title Page

Authors names

Qinghao Zhang [a, 1], Cuihong You [a, 1], Fang Liu [a], Wendi Zhu [a], Shuqi Wang [a], Dizhi Xie [a], Óscar Monroig [b], Douglas R. Tocher [b], Yuanyou Li [a]*

[1] Joint first authorship

Title

Cloning and characterization of Lxr and Srebp1, and their potential roles in regulation of LC-PUFA biosynthesis in rabbitfish *Siganus canaliculatus*

Affiliations and addresses of the authors

[a] *Marine Biology Institute & Guangdong Provincial Key Laboratory of Marine Biotechnology, Shantou University, Shantou, Guangdong, China*

[b] *Institute of Aquaculture, University of Stirling, Stirling, Scotland, UK*

***Corresponding author**

E–mail: yyli@stu.edu.cn

Tel: + 86-754-86503157

Fax: +86-754-86500613

Keywords

Elovl; Fad; LC-PUFA biosynthesis; Lxr; Rabbitfish *Siganus canaliculatus*; Srebp1

Title

Cloning and characterization of Lxr and Srebp1, and their potential roles in regulation of LC-PUFA biosynthesis in rabbitfish *Siganus canaliculatus*

Abstract

Rabbitfish *Siganus canaliculatus* was the first marine teleost demonstrated to have the ability to biosynthesize C_{20-22} long-chain polyunsaturated fatty acids (LC-PUFA) from C_{18} PUFA precursors, which is generally absent or low in marine teleosts. Thus, understanding the molecular basis of LC-PUFA biosynthesis in rabbitfish will contribute to efforts aimed at optimizing LC-PUFA biosynthesis in teleosts, especially marine species. In the present study, the importance of the transcription factors liver X receptor (Lxr) and sterol regulatory element-binding protein 1 (Srebp1) in regulation of LC-PUFA biosynthesis in rabbitfish was investigated. First, full-length cDNAs of *Lxr* and *Srebp1* were cloned and characterized. The *Lxr* mRNA displayed a ubiquitous tissue expression pattern while *Srebp1* was highly expressed in eyes, brain and intestine. In rabbitfish primary hepatocytes treated with Lxr agonist T0901317, the expression of *Lxr* and *Srebp1* was activated, accompanied by elevated mRNA levels of Δ4 and Δ6/Δ5 fatty acyl desaturases (Fad), key enzymes of LC-PUFA biosynthesis, as well as peroxisome proliferator-activated receptor *γ* (Pparγ). In addition, *Srebp1* displayed higher expression levels in liver of rabbitfish fed a vegetable oil diet or reared at 10 ppt salinity, which were conditions reported to increase the liver expression of Δ4 and Δ6/Δ5 *Fad* and LC-PUFA biosynthetic ability, than fish fed a

fish oil diet or reared at 32 ppt, respectively. These results suggested that Lxr and Srebp1 are involved in regulation of LC-PUFA biosynthesis probably by promoting the expression of two *Fads* in rabbitfish liver, which, to our knowledge, is the first report in marine teleosts.

Abbreviations

ARA	Arachidonic acid (20:4n-6)
cDNA	Complementary deoxyribonucleic acid
DHA	Docosahexaenoic acid (22:6n-3)
DMSO	Dimethyl Sulphoxide
Elovl	Elongases of very long-chain fatty acids
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid (20:5n-3)
EtOH	Ethanol
Fad	Fatty acyl desaturases
FBS	Fetal bovine serum
FO	Fish oil
LC-PUFA	Long-chain polyunsaturated fatty acids
Lxr	Liver X receptor
NAMBS	Nan Ao Marine Biology Station
PCR	Polymerase chain reaction
Ppar	Peroxisome proliferator-activated receptors
PUFA	Polyunsaturated fatty acids
Srebp	Sterol regulatory element binding proteins
VO	Vegetable oil
22HC	22(R)-hydroxycholesterol

Introduction

Long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids, are essential fatty acids (EFA) for marine teleosts which, compared to freshwater and salmonid species, are historically regarded as species with limited capability for LC-PUFA biosynthesis from C_{18} PUFA precursors [1,2]. Hence in aquaculture, LC-PUFA-rich fish oil (FO) is utilized in feeds to satisfy the EFA requirement of marine teleosts [1]. However, FO is derived from wild capture fisheries that have reached their maximum sustainable level, and this has paradoxically constrained the development of marine aquaculture. Consequently, feasible and sustainable alternatives to FO are urgently required and vegetable oils (VO) have been the prime candidates [3]. In contrast to FO, VOs are rich in C_{18} PUFA but devoid of n-3 LC-PUFA and so replacement of dietary FO by VO results in reduced flesh n-3 LC-PUFA contents of farmed fish, which compromises their nutritional quality for humans [3]. Considerable attention therefore has focused on improving our understanding of the molecular basis for LC-PUFA biosynthesis, which will faciliate the efficient and effective utilization of sustainable plant lipid sources while maintaining the nutritional quality of farmed fish, especially marine teleosts.

In vertebrates, LC-PUFA biosynthesis initiates from C_{18} PUFA and requires a series of desaturation and elongation steps catalyzed by fatty acid desaturases (Fad) and elongases of very long-chain fatty acids (Elovl) [4,5]. In teleosts, all *Fad* genes cloned to date are homologous to mammlian *Fads2* and their enzyme products display

93 Δ4, Δ5 or Δ6 specificity, respectively, or act in a bifunctional manner [5]. Δ4 Fad
94 from vertebrates was first identified in rabbitfish *Siganus canaliculatus* by our group
95 [6], followed by Senegalese sole (*Solea senegalensis*) [7], Mexican whitefish
96 (*Chirostoma estor*) [8] and striped snakehead (*Channa striata*) [9], and separate Δ5
97 Fad have been identified in Atlantic salmon (*Salmo salar*) [10] and rainbow trout
98 (*Oncorhynchus mykiss*) [11]. Bifunctional Δ5/Δ6 Fad were identified in zebrafish
99 (*Danio rerio*) [12], rabbitfish [6] and Mexican whitefish [8], while Δ6 Fad have been
100 found in many fish species [5,13]. In addition, elongase genes in teleosts include
101 *Elovl2*, *Elovl4* and *Elovl5*, among which *Elovl5* genes have been identified in most
102 teleosts studied to date [5]. Based on the presence and substrate specificity of the key
103 enzymes involved in LC-PUFA biosynthesis, studies increasingly focused on the
104 promoter of *Fads* where response elements for transcription factors were identified,
105 such as sterol regulatory element binding proteins (Srebp), nuclear factor Y (NF-Y)
106 and specificity protein 1 (Sp1) [14-16]. However, the mechanisms of transcriptional
107 regulation for LC-PUFA biosynthesis are largely unexplored, especially in marine
108 teleosts.

109 The Srebp family includes three members, Srebp1a and 1c, and Srebp2 in
110 mammals and, among them, Srebp1 plays a major role in regulation of fatty acid
111 synthesis [17]. Furthermore, Srebp1c can be activated by Liver X receptor (Lxr), a
112 class I nuclear receptor that can be activated by oxysterols derived from cholesterol
113 oxidation [18-20]. In mouse liver, it was established that Lxr and Srebp1 formed a
114 functional pathway (called "Lxr-Srebp1 pathway") that stimulates the production of

115 PUFA by transcriptional activation of Δ5 and Δ6 desaturase (encoded by *Fads1* and
116 *Fads2* genes, respectively) and Elovl5 [21]. In teleosts, the promoting effect of the
117 Lxr-Srebp1 pathway on LC-PUFA biosynthesis has been implied in anadromous
118 Atlantic salmon by increasing the expression of Δ5 and Δ6 *Fad* genes [16,22-24].
119 However, little is known about the presence or efficacy of Lxr-Srebp1 pathway in
120 marine teleosts.

121 Besides Lxr and Srebp1, the peroxisome proliferator-activated receptors (Ppar)
122 are also important regulators of lipid metabolism [25]. The Ppar family consists of
123 three subtypes encoded by discrete genes [25]. In mammals, Pparα and Pparβ are
124 activated by fatty acids or their derivatives, and play pleiotropic roles in lipid
125 metabolism, i.e. stimulating the expression of genes related to peroxisomal and
126 mitochondrial fatty acid oxidation, and LC-PUFA biosynthesis [26]. Pparγ has been
127 associated with adipocyte differentiation and lipogenesis [27]. In mammals, Pparα and
128 Lxr interact in controlling *Srebp1* activation [28,29]. However, our understanding of
129 the interaction between Ppar and Lxr in LC-PUFA biosynthesis in teleosts remains
130 limited.

131 Rabbitfish *Siganus canaliculatus*, a true herbivore consuming algae and seagrass
132 in nature, is widespread along the Indo-West Pacific coast and there is a commercial
133 fishery. In aquaculture, rabbitfish can be domesticated to accept formulated feeds and,
134 in recent years, rabbitfish farming has expanded rapidly in southeastern Asia,
135 including China, due to its popularity in markets. It is noteworthy that *S. canaliculatus*
136 was the first marine teleost demonstrated to have the ability of bioconverting C_{18}

PUFA to LC-PUFA, and now all the key enzymes required for LC-PUFA biosynthesis have been characterized in this species, including Δ4 Fad, Δ6/Δ5 Fad, Elovl4 and Elovl5 [6,30,31]. Thus, rabbitfish provides a favourable model to investigate the regulatory mechanisms of LC-PUFA biosynthesis in teleosts. Besides, previous studies showed that dietary lipid source and ambient salinity influenced the LC-PUFA biosynthetic ability of rabbitfish as well as the liver expression of the key enzymes mentioned above [31,32], which suggested a repertoire of regulatory machinery responsible for liver LC-PUFA biosynthesis that is largely unexplored.

Accordingly, the present study aimed to investigate the potential role of Lxr and Srebp1 in the regulation of LC-PUFA biosynthesis in rabbitfish *S. canaliculatus*. First, the cDNAs encoding Lxr and Srebp1 were cloned and characterized, followed by determination of their tissue distribution. Subsequently, the expression levels of *Lxr* and *Srebp1* were measured in rabbitfish primary hepatocytes incubated with Lxr agonists, including T0901317, GW3965 and 22(R)-hydroxycholesterol (22HC), along with other genes related to LC-PUFA biosynthesis including Δ4 *Fad*, Δ6/Δ5 *Fad*, *Elovl5*, *Pparα*, *Pparβ* and *Pparγ*. Furthermore, the mRNA level of *Lxr* and *Srebp1* were evaluated in livers of rabbitfish reared at different ambient salinities with diets containing VO or FO. The results provide further insight into the regulatory mechanisms of LC-PUFA biosynthesis in rabbitfish and teleosts, and also provide a foundation to optimize the LC-PUFA biosynthetic pathway in teleosts, which could facilitate the efficient and effective utilization of sustainable vegetable lipid resources in marine aquaculture.

Materials and Methods

Diets and feeding trials

Two iso-proteic and iso-lipidic diets containing 35 % crude protein and 8 % crude lipid were formulated using either fish oil (rich in LC-PUFA) or a blend of canola oil and perilla oil (LC-PUFA-free) as lipid sources and named as FO and VO diets, respectively. Dietary compositions, experimental animal preparation and the feeding trial have all been described in detail previously, along with the detailed parameters on the growth performance and lipid composition of fish [32]. Briefly, juvenile rabbitfish (body mass approximately 13 g, sex indistinguishable visually) were caught off the coast near Nan Ao Marine Biology Station (NAMBS) of Shantou University, Southern China. Initially, 500 fish were maintained in an indoor seawater (32 ppt) pool for one month to adapt to the laboratory conditions. Subsequently, 250 fish were gradually acclimated to brackish water (10 ppt) for one month, followed by a further two weeks' period at 10 ppt while the other half remained in seawater throughout. The fish were then starved for 24 h, anesthetized with 0.01 % 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA), individually weighed and 20 fish each allocated randomly to 12 cylindrical tanks (90 cm diameter, 100 cm depth) with triplicate tanks for each diet (FO and VO) at both salinities (10 and 32 ppt). The feeding trial lasted for 8-weeks in an indoor aquarium system at NAMBS. During the trial, oxygen-saturation was maintained by aeration, temperature was kept at 22 ± 3 °C and photoperiod was set at a 12 h light: 12 h dark cycle.

180

181 **Tissue collection**

182 The liver of one fish in seawater during the initial acclimation period was collected
183 for RNA isolation followed by cDNA cloning of *Lxr* and *Srebp1*. In addition, tissues
184 including heart, liver, spleen, gill, muscle, eyes, intestine and brain were sampled
185 from a further three individuals for determination of tissue distribution of *Lxr* and
186 *Srebp1* mRNA. To determine the effects of diet and salinity on gene expression, livers
187 were collected from three fish in seawater at the beginning of the feeding trial as
188 initial control samples, and at the end of the trial, livers were collected from two fish
189 per tank (six fish per dietary treatment per salinity). Tissue samples were immediately
190 frozen in liquid nitrogen and subsequently stored at -80 ºC before analysis. All
191 procedures performed on fish conformed with the National Institutes of Health guide
192 for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978)
193 and approved by the Animal Care and Use Committee of Shantou University
194 (Guangdong, China). All surgery was performed under 0.01 % 2-phenoxyethanol
195 anesthesia, and all efforts were made to minimize suffering of fish.

196

197 **Rabbitfish primary hepatocyte isolation and incubation with Lxr agonists**

198 Rabbitfish were fasted 24 h and then euthanized with 0.01 % 2-phenoxyethanol,
199 followed by immersion in 70 % ethanol to sterilize the external surfaces. Primary
200 hepatocytes were prepared from a pool of three livers as described previously [33].

201 Briefly, chopped livers were digested by 0.1 % collagenase/0.25 % hyaluronidase
202 (Sigma-Aldrich, St. Louis, USA) followed by filtering through a 100 μm strainer.
203 Isolated cells (viability ≥ 98 % evaluated by Trypan Blue dye exclusion) were seeded
204 in 6-well plates at a density of 2×10^6 cells per well with 2 ml DMEM/F12 medium
205 containing 20 % fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml
206 streptomycin, followed by incubation at 24 ˚C/4 % CO_2 for 24 h. The hepatocytes
207 were incubated in FBS-free DMEM/F12 medium for 3 h prior to incubation with the
208 following Lxr agonists: synthetic ligands T0901317 (1 and 2 μM) and GW3965 (2
209 and 4 μM), natural ligand 22(R)-hydroxycholesterol (22HC 5 and 10 μM), and
210 treatment vehicles (DMSO for T091317 and GW3965, and ethanol (EtOH) for 22HC)
211 on the basis of previous studies [34-36]. In addition, incubations without chemical
212 supplement were included as control. After 6 h incubation, cells were lysed in wells
213 and harvested for subsequent RNA extraction.
214
215 **RNA isolation and cDNA synthesis**
216 Total RNA from rabbitfish tissues was extracted by Trizol reagent (Invitrogen,
217 Carlsbad, CA, USA) and total RNA from rabbitfish primary hepatocytes was isolated
218 and purified by RNAprep pure cell/bacteria kit (Tiangen Biotech, Beijing, China).
219 The concentration and quality of total RNA preparations were confirmed by
220 spectrophotometry (NanoDrop 2000, Thermo Scientific, USA) and agarose gel
221 electrophoresis. For cloning partial sequences, one μg total RNA from liver was

222 subjected to cDNA synthesis using the Cloned AMV First-Strand cDNA Synthesis
223 Kit (Invitrogen, USA). For semi-quantitative or quantitative analysis of target gene
224 expression level, cDNA was synthesized from 1 μg or 500 ng of total RNA from
225 rabbitfish tissues or primary hepatocytes, respectively, using FastQuant RT Kit (with
226 gDNase) (Tiangen Biotech, Beijing, China).
227
228 **Molecular cloning of rabbitfish *Lxr* and *Srebp1* cDNAs**
229 Partial cDNA fragments of *Lxr* and *Srebp1* were obtained by polymerase chain
230 reaction (PCR) using primer pair lxr-partial-F/R and srebp1-partial-F/R, respectively.
231 Primers were designed on highly conserved regions of cDNA sequences based on
232 alignments of human (*Homo sapiens*; NCBI accession: *Lxrα*, AB307698.1; *Srebp1*,
233 BC063281.1), mouse (*Mus musculus*; *Lxrα*, AJ132601.1; *Srebp1*, BC056922.1),
234 chicken (*Gallus gallus*; *Lxr*, AF492498.1; *Srebp1*, NM_204126.2), Atlantic salmon
235 (*Lxr*, FJ470290.1; *Srebp1*, HM561860.1) and zebrafish (*Lxr*, BC092160.1; *Srebp1*,
236 NM_001105129.1). The PCR was performed with initial denaturation at 95 °C for 5
237 min and 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, followed by a
238 final extension at 72 °C for 10 min.
239 The full-length cDNA sequences of *Lxr* and *Srebp1* were cloned by 3' and 5'
240 rapid amplification of cDNA ends (RACE) PCR based on partial cDNA fragments
241 using the GeneRacer® kit (Invitrogen, USA). According to the manufacturer's
242 instructions, specific primers lxr-5'-F1/F2 and srebp1-5'-F1/F2 were designed to

amplify the 5' ends of *Lxr* and *Srebp1* cDNAs while lxr-3'-R1/R2 and srebp1-3'-R1/R2 were prepared for the 3' ends of the two cDNAs, respectively. The multiple amplicons from PCR and RACE were assembled and the full-length cDNA sequences of rabbitfish *Lxr* and *Srebp1* were further generated by "end to end" PCR. All primer sequences are shown in Table 1.

Sequence and phylogenetic analysis

ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to align the deduced amino acid (aa) sequences of the newly cloned rabbitfish *Lxr* and *Srebp1* cDNA with their corresponding orthologs from other species, including human (Lxrα, NCBI accession: AAA85856.1), mouse (Lxrα, CAB51923.1), chicken (Lxrα, NP_989873.1; Srebp1, NP_989457.2), Atlantic salmon (Lxrα, NP_001138893.1; Srebp1, ADN28371.1) and zebrafish (Lxrα, NP_001017545.1; Srebp1, NP_001098599.1). The aa sequences for Srebp1 isoforms of human (Srebp1a, P36956-1; Srebp1c, P36956-3) and mouse (Srebp1a, Q9WTN3-1; Srebp1c, Q9WTN3-3) were acquired from the UniProtKB database.

Mega5 [37] and the neighbor joining method were used to construct phylogenetic trees based on the aa sequences of Lxr and Srebp proteins from rabbitfish and other species including human, mouse, chicken, zebrafish, Atlantic salmon, carp (*Cyprinus carpio*), Japanese flounder (*Paralichthys olivaceus*), large yellow croaker (*Larimichthys crocea*), rainbow trout, tongue sole (*Cynoglossus semilaevis*), grass

264 carp (*Ctenopharyngodon idella*) and Japanese sea bass (*Lateolabrax japonicus*).

265

266 **Semi-quantitative PCR and Real-time qPCR assays**

267 Semi-quantitative PCR was conducted on tissue cDNA samples from three rabbitfish
268 to determine the tissue distribution of *Lxr* and *Srebp1* mRNA, with *18S rRNA*
269 (AB276993) as an internal control to check the efficiency of cDNA synthesis and
270 cDNA integrity. To confirm the absence of genomic DNA contamination,
271 non-template controls (NTC), consisting of reactions without reverse transcriptase,
272 were also run. Semi-quantitative PCR was carried out with an initial denaturing step
273 at 94 °C for 5 min, followed by 28 cycles of denaturation at 94 °C for 30 s, annealing
274 at 60 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min.
275 The primer pairs used for semi-quantitative PCR were the same as used for qPCR
276 (Table 1).

277 The expression level of *Lxr*, *Srebp1*, Δ4 *Fad* (GU594278.1), Δ6/Δ5 *Fad*
278 (EF424276.2), *Elovl5* (GU597350.1), *Pparα* (JF502070.1), *Pparβ* (JF502071.1) and
279 *Pparγ* (JF502072.1) mRNA was determined by qPCR on a LightCycler® 480
280 thermocycler (Roche, Germany). All reactions were run in a total volume of 20 μl
281 containing 10 μl LightCycler® 480 SYBR Green I Master (Roche, Germany), 0.4 μM
282 each of gene specific primer pairs (Table 1), 20 ng cDNA templates (10 ng/μl) and
283 ddH_2O in a white 96-well plate. The qPCR programs consisted of initial DNA
284 denaturation at 94 °C for 5 min and 45 cycles of 95 °C for 10 s, 60 °C for 20 s, 72 °C

20 s, followed by a melting curve to confirm the amplification of a single product in each reaction. The amplification efficiency of primers was also determined by serial dilution of cDNA template in ddH_2O. The relative RNA level of genes in each sample was normalized to *18S rRNA* expression and calculated by the comparative threshold cycle (Ct) method as fold change relative to initial control samples [38]. Triplicates of each reaction were conducted for each sample.

Statistical analysis

All data were presented as means ± SEM (n = 6 individuals for gene expression in liver of rabbitfish fed VO or FO diets at 10 ppt or 32 ppt salinity; n = 3 technical replicates for cell studies). In cell studies, differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test at a significance of $P < 0.05$. A two-way ANOVA was used to analyze the differences of gene expression in rabbitfish liver, using FO and VO diets as the first independent variable, and the two salinity treatments (32 ppt and 10 ppt) as the second independent variable, and significant levels were considered at $P < 0.05$. All statistical analyses were performed using OriginPro 7.5 software (OriginLab Corporation, USA).

Results

Sequence characteristics of rabbitfish *Lxr* and *Srebp1* cDNAs

The full-length rabbitfish *Lxr* cDNA was 3055 bp containing a 5' untranslated region (UTR) of 407 bp, a 3' UTR of 1259 bp and an open reading frame (ORF) of 1389 bp encoding a protein of 462 aa. The nucleotide and deduced aa sequences were submitted to NCBI with accession numbers JF502074.1 and AFH35110.1, respectively. Rabbitfish Lxr has a typical structure of Lxr family proteins, including a DNA binding domain (DBD), ligand binding domain (LBD), activation function domains (AF-1/2) and a D (hinge) region (Fig. 1a). The full-length of *Srebp1* cDNA was 3952 bp containing 5' and 3' UTRs of 201 bp and 239 bp, respectively, and an ORF of 3513 bp encoding a 1171 aa protein. The nucleotide and aa sequences were submitted to NCBI with accession numbers JF502069.1 and AFH35105.1, respectively. The deduced rabbitfish Srebp1 protein showed the conserved basic helix-loop-helix (bHLH) domain, which is essential for DNA binding as transcription factors (Fig. 1b and supplementary Fig. S1).

Multiple sequence alignment and phylogenetic analysis

Alignment analysis of the deduced rabbitfish proteins with their orthologs in other species showed that rabbitfish Lxr shared high sequence identity with zebrafish (92 %) and salmon (85 %) and 74 -77 % identity with Lxrα from human and mouse, and Lxr from chicken (Fig. 1a). Similarly, phylogenetic analysis revealed that the Lxr proteins

of fish species were all clustered with mammalian Lxrα (Fig. 2a).

As for Srebp1, results showed that the rabbitfish Srebp1 shared 77 % and 72 % identity with those of Atlantic salmon and zebrafish, respectively, and 54 - 56 % identity with Srebp1a from human, mouse and chicken, and 54 % identity with Srebp1c from human and mouse (Fig. 1b). The N-terminal protein sequence alignment showed that the rabbitfish Srebp1 aa sequence was more similar to mammalian Srebp1a isoform (Fig. 1b). Phylogenetic analysis showed rabbitfish Srebp1 clustered with vertebrate Srebp1 rather than Srebp2, and closest to Srebp1 of *L. japonicus* (Fig. 2b).

Tissue distribution of *Lxr* and *Srebp1* mRNA in rabbitfish

The abundance of *Lxr* and *Srebp1* mRNA in different tissues was analyzed. Rabbitfish *Lxr* mRNA was detected in all studied tissues with lowest abundance in muscle and relatively high level in spleen and heart, and then tissues regarded as having high LC-PUFA biosynthetic ability including liver, intestine, eyes and brain (Fig. 3). Relatively high expression level of *Srebp1* was observed in eyes, intestine and brain, followed by heart, spleen, gill, and liver whereas the abundance of *Srebp1* mRNA in muscle was too low to be detected (Fig. 3).

Effects of Lxr agonists on the expression of *Lxr*, *Srebp1* and other genes related to LC-PUFA biosynthesis in rabbitfish primary hepatocytes

First, three Lxr agonists were tested for their effects on activating the expression of *Lxr* and *Srebp1* in rabbitfish hepatocytes. Results showed that the expression level of *Lxr* and *Srebp1* mRNA was increased 1.2 to 1.4-fold and ~9-fold, respectively, by T0901317, whereas 22HC promoted the expression of *Lxr* (~1.3-1.4 fold) and GW3965 promoted the expression of *Srebp1* (~6-13 fold), separately (Fig. 4). Based on minimum activating doses, T0901317 was determined as the most potent activator for *Lxr* and *Srebp1* expression in rabbitfish liver. Subsequently, the expression level of other genes involved in LC-PUFA biosynthesis was determined in hepatocytes incubated with T0901317. Results showed that Δ4 *Fad* was induced by 1 μM T0901317 (~1.7-fold), whereas 2 μM T0901317 activated the transcription of both Δ4 (~1.7-fold) and Δ6/Δ5 *Fad* (~2-fold), but decreased the expression level of *Elovl5* as compared to 1 μM T0901317 (Fig. 5). As for Ppar subtypes, T0901317 treatment increased the expression of *Pparγ* at 1 μM, with no significant effect on the expression level of *Pparα* and *β* (Fig. 5).

Effects of dietary PUFA and ambient salinity on the expression of *Lxr* and *Srebp1* in rabbitfish liver

The expression pattern of *Lxr* and *Srebp1* in rabbitfish liver was different in response to dietary lipid source and ambient salinity (Fig. 6). Rabbitfish fed FO diets displayed higher expression level of *Lxr* than fish fed VO diets (P <0.05), while ambient salinity produced no significant change on the expression of *Lxr* in rabbitfish liver. In addition,

367 the expression of *Srebp1* was higher in the liver of rabbitfish fed VO diets or reared at
368 10 ppt than that of fish fed FO diets or reared at 32 ppt (P <0.05). There was no
369 significant interaction between dietary lipid source and ambient salinity on the
370 expression of *Lxr* or *Srebp1* in rabbitfish liver.
371

Discussion

Comparison of *Lxr* and *Srebp1* sequences between rabbitfish and other species

As a class I nuclear receptor, Lxr possesses two important functional regions, LBD and DBD, that are associated with ligand binding and downstream target interaction [39]. Herein, the LBD and DBD of rabbitfish Lxr showed high identity with those of Lxr orthologs from other species, which indicated similar roles in ligand recognition and downstream target activation. In mammals, there are two isoforms of Lxr, Lxrα and Lxrβ, with overlapping or distinct functions [40]. However, rabbitfish possesses a unique form of Lxr that clustered with mammalian Lxrα, and were Lxr orthologs of other fish species in the phylogenetic analysis. This suggested the gene loss of Lxrβ in teleosts, which was consistent with observations in zebrafish, rainbow trout and salmon [34,41].

The Srebps subfamily is encoded by two distinct genes, designated *Srebp1* and *Srebp2*, in vertebrates [17,23], and the cDNA characterized in the present study was homologous to mammalian *Srebp1*. In mammals, the transcription products of *Srebp1* consist of two isoforms, *Srebp1a* and *1c*, which differ only in the first exon as a result of alternative splicing [42]. However, this is not the case in rabbitfish, although it cannot be excluded that the cDNA used for cloning was synthesized from liver RNA. Specifically, the rabbitfish Srebp1 was closely related to mammal Srebp1a rather than Srebp1c, which was also observed in zebrafish, salmon and Japanese sea bass [23,43]. In mammals, the relatively long N-terminal AF-1 domain confers the Srebp1a with

potent transactivation activity while Srebp1c with a short AF-1 domain requires further post-translational modification to achieve complete activity [44,45]. Thus, the high similarity of rabbitfish Srebp1 to mammal Srebp1a ensures its efficacy in transactivation.

Tissue distribution of *Lxr* and *Srebp1* mRNA implies their relevance in LC-PUFA biosynthesis in rabbitfish

The tissue expression pattern of *Lxr* varies among species [39,41,46]. In the present study, rabbitfish *Lxr* displayed high expression level in spleen, which is consistent with the observation in rainbow trout, but low in muscle, similar to the finding in salmon [41]. In yellow catfish (*Pelteobagrus fulvidraco*), transcript variants of *Lxrα* (*Lxrα-1* and *Lxrα-2*) were identified with different tissue expression patterns [46]. Thus, the varied tissue distribution profile of *Lxr* mRNA among fish species may be due to the presence of multiple *Lxr* isoforms. In addition, the ubiquitous expression pattern of *Lxr* in rabbitfish tissues implied its pleiotropic effects in physiology.

Srebp1 play major role in the regulation of fatty acid synthesis in mammals [17]. In the present study, *Srebp1* mRNA displayed high abundance in eyes, brain and intestine, which are tissues with high level of Δ4 and Δ6 *Fad* mRNA and likewise potent LC-PUFA biosynthetic activity [31,33]. Thus, Srebp1 might be implicated in the regulation of LC-PUFA biosynthesis in rabbitfish. However, *Srebp1* mRNA is lacking in rabbitfish muscle, which can be attributed to the reduced expression of *Lxr*

414 that indirectly regulates fatty acid synthesis by driving *Srebp1* transcription in
415 mammals [20,21].
416
417 **Potential role and mechanism for Lxr and Srebp1 in regulation of LC-PUFA**
418 **biosynthesis in rabbitfish liver**
419 As a ligand-activated nuclear receptor, Lxr can be activated by synthetic or natural
420 agonists [18,39,47]. In the present study, agonists increased the expression of *Lxr* in
421 rabbitfish hepatocytes, which can be explained by the positive autoregulation of Lxr
422 that was shown to have LXREs in its own promoter in human and mice [48,49]. A
423 similar autoregulatory situation has also been observed in recent studies performed in
424 rainbow trout and Atlantic salmon [22,35,36]. Srebp1 was established as a target gene
425 for Lxr in mammal and rodent systems [19,20]. Consistent with this, the expression of
426 *Srebp1* was increased by the Lxr agonists T0901317 and GW3965, whereas the 22HC
427 produced no significant effects. In human myotubes, differential effects of T0901317
428 and 22HC were observed in regulation of genes involved in lipid metabolism,
429 although both of them are effective agonists of Lxrα [50]. T0901317 and GW3965
430 were more effective activators for the Lxr of Atlantic salmon than 22HC [22].
431 Apparently, the effectiveness of Lxr agonists were model-specific, and thus in the
432 present study, T0901317 was regarded as a potent activator for the expression of both
433 Lxr and Srebp1 with maximal effect at minimum dose, which also indicated the
434 conservation of Lxr-Srebp1 pathway in rabbitfish.

435 Fad and Elovl are key enzymes involved in LC-PUFA biosynthesis, and their
436 gene expressions are commonly correlated to the LC-PUFA biosynthetic ability [4,5].
437 In mouse liver, the Lxr-Srebp1 pathway was demonstrated to be involved in
438 LC-PUFA biosynthetic regulation through activating the expression of Δ5, Δ6
439 desaturase and Elovl5 [21]. In Atlantic salmon SHK-1 cells, T0901317 promoted the
440 expression of Δ5 and Δ6 *Fad* but not *Elovl5*, accompanied by the increased
441 expression of *Lxr* and *Srebp1* [22]. As such, in the present study, the increased
442 expression of Δ4 and Δ6/Δ5 *Fad* in rabbitfish hepatocytes incubated with T0901317
443 might be a result of activated Lxr-Srebp1 pathway, where *Elovl5* might not be
444 included.

445 Interestingly, the transcription of the Δ4 *Fad* was more sensitive to the activation
446 of Lxr and Srebp1 than Δ6/Δ5 *Fad* in the present study. As the first vertebrate
447 reported with Δ4 desaturation activity and the first marine teleost with Δ6/Δ5
448 desaturation activity, rabbitfish possesses two mechanisms for DHA biosynthesis
449 from EPA. The first mechanism is the so-called "Sprecher pathway", where EPA
450 undergoes two successive elongation steps to produce 24:5n-3, followed by a second
451 Δ6 desaturation step to produce 24:6n-3 that is chain-shorted to DHA by partial
452 β-oxidation [51]. The second mechanism is the "Δ4 desaturation pathway" that
453 requires a single elongation to 22:5n-3, which is then directly Δ4 desaturated to
454 generate DHA [6]. Our recent study showed that there is coordination between the
455 two pathways, in which miR-17 is involved through suppressing Δ4 *Fad*

456 post-transcriptionally rather than Δ6 *Fad* [33]. Likewise, the results presented herein
457 further suggested the coordinating machinery at transcriptional level, possibly due to
458 the distinct promoter structures between the two *Fads*. In addition, DHA is difficult to
459 oxidize since its Δ4 bond must be removed by peroxisomal β-oxidation, as compared
460 to EPA [52]. Herein, the higher sensitivity of Δ4 *Fad* towards the Lxr and Srebp1
461 activation may facilitate the bioconversion of EPA into DHA in rabbitfish liver.

462 Dietary PUFA and ambient salinity were demonstrated as important factors that
463 influence LC-PUFA biosynthesis and Fad expression in rabbitfish liver [31,32], which
464 provided the physiological context to further determine the role of Lxr and Srebp1 in
465 the regulation of LC-PUFA biosynthesis. Results showed that the liver expression
466 pattern of *Srebp1* was correlated with that of Δ4 and Δ6/Δ5 *Fad* reported previously,
467 with higher mRNA level in liver of rabbitfish fed VO diets or reared at 10 ppt than
468 that of fish fed FO diets or reared at 32 ppt [31,32]. In combination with the *in vitro*
469 results, there is good reason to speculate that Srebp1 is a critical transcription factor
470 for the expression of Δ4 and Δ6/Δ5 *Fad* in rabbitfish liver. In contrast to *Srebp1*, *Lxr*
471 displayed higher expression level in liver of rabbitfish fed FO diets than that of fish
472 fed VO diets, while ambient salinity produced no significant effects on the expression
473 of *Lxr*. Similarly, the asynchronous expression of *Lxr* and *Srebp1* in circadian
474 rhythmicity has been observed in the liver of Atlantic salmon [24]. As such, Lxr was
475 not the only regulator for *Srebp1* expression in rabbitfish liver physiologically and the

476 extent to which Lxr impacts on LC-PUFA biosynthesis in rabbitfish liver should be
477 further investigated.

478 In mammals, LC-PUFA inhibited the transcription of *Srebp1c* [53], accelerated
479 *Srebp1* mRNA decay [54] and suppressed proteolytic release of Srebp1 protein [55].
480 Similarly, the mRNA level of *Srebp1* was lower in the liver of sea bass fed FO
481 (LC-PUFA-rich) diets than that of fish fed VO diets containing low LC-PUFA level
482 [56]. In addition, FO is also rich in cholesterol, the precursor of oxysterols that are
483 endogenous ligands for Lxr, which is absent in VO [3]. Thus in the present study, the
484 different expression pattern of *Lxr* and *Srebp1* in the liver of rabbitfish fed VO or FO
485 diets can be explained by their specific responses to dietary nutrients such as
486 LC-PUFA and cholesterol. Furthermore, these results suggested the complexity of the
487 molecular mechanisms of transcriptional regulation of *Lxr* and *Srebp1* in LC-PUFA
488 biosynthesis of teleosts, which requires further investigation.

489 Ppar interact with Lxr in regulation of lipid metabolism in a tissue- and
490 species-dependent manner [22,35,36,57]. In the present study, only the expression of
491 *Pparγ* was up-regulated by Lxr activation. In rodent adipocytes, Lxrα activated the
492 transcription of *Pparγ* and induced adipogenesis [58], but this was not the case for
493 *Pparγ* in rainbow trout adipocytes [36]. In SHK cells of Atlantic salmon, the
494 activation of Lxr did not affect the expression of any of the *Ppar* subtypes [22]. Hence,
495 the mechanism for the promotion of *Pparγ* by Lxr and its significance in LC-PUFA
496 biosynthesis should be further investigated.

In the present study, the cDNAs encoding *Lxr* and *Srebp1* were cloned and characterized from rabbitfish *S. canaliculatus*. Furthermore, our results implied their roles in LC-PUFA biosynthesis, probably by activating the expression of Δ4 and Δ6/Δ5 *Fad* in liver. In return, the expression of *Lxr* and *Srebp1* in liver was also affected by dietary lipid sources. Additionally, *Pparγ* was inferred as a possible target for Lxr although the mechanism and significance remained to be clarified. These results increased our understanding of the regulatory mechanisms for LC-PUFA biosynthesis in rabbitfish and teleosts, which will contribute to optimizing the LC-PUFA biosynthetic pathway in marine teleosts and developing more efficient, low-cost aquaculture diets based on vegetable lipid sources.

Acknowledgements

This work was financially supported by the Major International Joint Research Project from the National Natural Science Foundation of China (NSFC) (31110103913), NSFC General Projects (No. 41276179) and Youth Projects (Nos. 31202011, 31202012).

Conflict of Interest

The authors have declared that no competing interests exist.

References

1. Tocher DR (2010) Fatty acid requirements in ontogeny of marine and freshwater fish. Aquac Res 41(5):717-732
2. Tocher DR, Agaba M, Hastings N, Teale AJ (2003) Biochemical and molecular studies of the fatty acid desaturation pathway in fish. Paper presented at the The Big Fish Bang, Browman, H., Skiftesvik, A.B.
3. Turchini GM, Torstensen BE, Ng W (2009) Fish oil replacement in finfish nutrition. Rev Aquacult 1(1):10-57
4. Cook HW, McMaster CR (2004) In: Vance DE, Vance JE (eds) Fatty acid desaturation and chain elongation in eukaryotes, 4th edn. Elsevier, Amsterdam
5. Castro LF, Tocher DR, Monroig O (2016) Long-chain polyunsaturated fatty acid biosynthesis in chordates: Insights into the evolution of Fads and Elovl gene repertoire. Prog Lipid Res 62:25-40
6. Li Y, Monroig O, Zhang L, Wang S, Zheng X, Dick JR, You C, Tocher DR (2010) Vertebrate fatty acyl desaturase with Δ4 activity. Proc Natl Acad Sci USA 107(39):16840-16845
7. Morais S, Castanheira F, Martinez-Rubio L, Conceicao LE, Tocher DR (2012) Long chain polyunsaturated fatty acid synthesis in a marine vertebrate: ontogenetic and nutritional regulation of a fatty acyl desaturase with Δ4 activity. Biochim Biophys Acta 1821(4):660-671
8. Fonseca-Madrigal J, Navarro JC, Hontoria F, Tocher DR, Martinez-Palacios CA, Monroig O (2014) Diversification of substrate specificities in teleostei Fads2: characterization of Δ4 and Δ6/Δ5 desaturases of *Chirostoma estor*. J Lipid Res 55(7):1408-1419
9. Kuah MK, Jaya-Ram A, Shu-Chien AC (2015) The capacity for long-chain polyunsaturated

539 fatty acid synthesis in a carnivorous vertebrate: Functional characterisation and nutritional
540 regulation of a Fads2 fatty acyl desaturase with Δ4 activity and an Elovl5 elongase in striped
541 snakehead (*Channa striata*). Biochim Biophys Acta 1851(3):248-260
542 10. Hastings N, Agaba MK, Tocher DR, Zheng X, Dickson CA, Dick JR, Teale AJ (2004)
543 Molecular cloning and functional characterization of fatty acyl desaturase and elongase cDNAs
544 involved in the production of eicosapentaenoic and docosahexaenoic acids from α-linolenic acid
545 in Atlantic salmon (*Salmo salar*). Mar Biotechnol 6(5):463-474
546 11. Abdul HN, Carmona-Antonanzas G, Monroig O, Tocher DR, Turchini GM, Donald JA (2016)
547 Isolation and Functional Characterisation of a fads2 in Rainbow Trout (*Oncorhynchus mykiss*)
548 with Δ5 Desaturase Activity. PLoS One 11(3):e150770
549 12. Hastings N, Agaba M, Tocher DR, Leaver MJ, Dick JR, Sargent JR, Teale AJ (2001) A
550 vertebrate fatty acid desaturase with Δ5 and Δ6 activities. Proc Natl Acad Sci USA
551 98(25):14304-14309
552 13. Vagner M, Santigosa E (2011) Characterization and modulation of gene expression and
553 enzymatic activity of delta-6 desaturase in teleosts: A review. Aquaculture 315(1-2):131-143
554 14. Xu H, Dong X, Ai Q, Mai K, Xu W, Zhang Y, Zuo R (2014) Regulation of tissue LC-PUFA
555 contents, Δ6 fatty acyl desaturase (FADS2) gene expression and the methylation of the putative
556 FADS2 gene promoter by different dietary fatty acid profiles in Japanese seabass (*Lateolabrax*
557 *japonicus*). PLoS One 9(1):e87726
558 15. Geay F, Zambonino-Infante J, Reinhardt R, Kuhl H, Santigosa E, Cahu C, Mazurais D (2012)
559 Characteristics of *fads2* gene expression and putative promoter in European sea bass
560 (*Dicentrarchus labrax*): comparison with salmonid species and analysis of CpG methylation.

561 Mar Genomics 5:7-13
562 16. Zheng X, Leaver MJ, Tocher DR (2009) Long-chain polyunsaturated fatty acid synthesis in fish:
563 Comparative analysis of Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.)
564 Δ6 fatty acyl desaturase gene promoters. Comp Biochem Physiol B Biochem Mol Biol
565 154(3):255-263
566 17. Horton JD, Goldstein JL, Brown MS (2002) SREBPs: activators of the complete program of
567 cholesterol and fatty acid synthesis in the liver. J Clin Invest 109(9):1125-1131
568 18. Aranda A, Pascual A (2001) Nuclear hormone receptors and gene expression. Physiol Rev
569 81(3):1269-1304
570 19. Yoshikawa T, Shimano H, Amemiya-Kudo M, Yahagi N, Hasty AH, Matsuzaka T, Okazaki H,
571 Tamura Y, Iizuka Y, Ohashi K, Osuga J, Harada K, Gotoda T, Kimura S, Ishibashi S, Yamada
572 N (2001) Identification of liver X receptor-retinoid X receptor as an activator of the sterol
573 regulatory element-binding protein 1c gene promoter. Mol Cell Biol 21(9):2991-3000
574 20. Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS,
575 Goldstein JL, Mangelsdorf DJ (2000) Regulation of mouse sterol regulatory element-binding
576 protein-1c gene (SREBP-1c) by oxysterol receptors, LXRα and LXRβ. Genes Dev
577 14(22):2819-2830
578 21. Qin Y, Dalen KT, Gustafsson JA, Nebb HI (2009) Regulation of hepatic fatty acid elongase 5
579 by LXRα-SREBP-1c. Biochim Biophys Acta 1791(2):140-147
580 22. Carmona-Antonanzas G, Tocher DR, Martinez-Rubio L, Leaver MJ (2014) Conservation of
581 lipid metabolic gene transcriptional regulatory networks in fish and mammals. Gene 534(1):1-9
582 23. Minghetti M, Leaver MJ, Tocher DR (2011) Transcriptional control mechanisms of genes of

583 lipid and fatty acid metabolism in the Atlantic salmon (*Salmo salar* L.) established cell line,
584 SHK-1. Biochim Biophys Acta 1811(3):194-202
585 24. Betancor MB, McStay E, Minghetti M, Migaud H, Tocher DR, Davie A (2014) Daily rhythms
586 in expression of genes of hepatic lipid metabolism in Atlantic salmon (*Salmo salar* L.). PLoS
587 One 9(9):e106739
588 25. Jump DB, Tripathy S, Depner CM (2013) Fatty acid-regulated transcription factors in the liver.
589 Annu Rev Nutr 33:249-269
590 26. Desvergne B, Michalik L, Wahli W (2006) Transcriptional regulation of metabolism. Physiol
591 Rev 86(2):465-514
592 27. Tontonoz P, Spiegelman BM (2008) Fat and beyond: the diverse biology of PPARγ. Annu Rev
593 Biochem 77:289-312
594 28. Yoshikawa T, Ide T, Shimano H, Yahagi N, Amemiya-Kudo M, Matsuzaka T, Yatoh S,
595 Kitamine T, Okazaki H, Tamura Y, Sekiya M, Takahashi A, Hasty AH, Sato R, Sone H, Osuga
596 J, Ishibashi S, Yamada N (2003) Cross-talk between peroxisome proliferator-activated receptor
597 (PPAR) α and liver X receptor (LXR) in nutritional regulation of fatty acid metabolism. I.
598 PPARs suppress sterol regulatory element binding protein-1c promoter through inhibition of
599 LXR signaling. Mol Endocrinol 17(7):1240-1254
600 29. Ide T, Shimano H, Yoshikawa T, Yahagi N, Amemiya-Kudo M, Matsuzaka T, Nakakuki M,
601 Yatoh S, Iizuka Y, Tomita S, Ohashi K, Takahashi A, Sone H, Gotoda T, Osuga J, Ishibashi S,
602 Yamada N (2003) Cross-talk between peroxisome proliferator-activated receptor (PPAR) α and
603 liver X receptor (LXR) in nutritional regulation of fatty acid metabolism. II. LXRs suppress
604 lipid degradation gene promoters through inhibition of PPAR signaling. Mol Endocrinol

605 17(7):1255-1267
606 30. Monroig O, Wang S, Zhang L, You C, Tocher DR, Li Y (2012) Elongation of long-chain fatty
607 acids in rabbitfish *Siganus canaliculatus*: Cloning, functional characterisation and tissue
608 distribution of Elovl5- and Elovl4-like elongases. Aquaculture 350:63-70
609 31. Li Y, Hu C, Zheng Y, Xia X, Xu W, Wang S, Chen W, Sun Z, Huang J (2008) The effects of
610 dietary fatty acids on liver fatty acid composition and Δ^6-desaturase expression differ with
611 ambient salinities in *Siganus canaliculatus*. Comp Biochem Physiol B Biochem Mol Biol
612 151(2):183-190
613 32. Xie D, Wang S, You C, Chen F, Tocher DR, Li Y (2015) Characteristics of LC-PUFA
614 biosynthesis in marine herbivorous teleost *Siganus canaliculatus* under different ambient
615 salinities. Aquacult Nutr 21(5):541-551
616 33. Zhang Q, Xie D, Wang S, You C, Monroig O, Tocher DR, Li Y (2014) miR-17 is involved in
617 the regulation of LC-PUFA biosynthesis in vertebrates: effects on liver expression of a fatty
618 acyl desaturase in the marine teleost *Siganus canaliculatus*. Biochim Biophys Acta
619 1841(7):934-943
620 34. Archer A, Lauter G, Hauptmann G, Mode A, Gustafsson J (2008) Transcriptional activity and
621 developmental expression of liver X receptor (*lxr*) in Zebrafish. Dev Dynam 237(4):1090-1098
622 35. Cruz-Garcia L, Sanchez-Gurmaches J, Gutierrez J, Navarro I (2011) Regulation of LXR by fatty
623 acids, insulin, growth hormone and tumor necrosis factor-α in rainbow trout myocytes. Comp
624 Biochem Physiol A Mol Integr Physiol 160(2):125-136
625 36. Cruz-Garcia L, Sanchez-Gurmaches J, Gutierrez J, Navarro I (2012) Role of LXR in trout
626 adipocytes: target genes, hormonal regulation, adipocyte differentiation and relation to lipolysis.

627 Comp Biochem Physiol A Mol Integr Physiol 163(1):120-126
628 37. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular
629 evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum
630 parsimony methods. Mol Biol Evol 28(10):2731-2739
631 38. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time
632 quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods 25(4):402-408
633 39. Alberti S, Steffensen KR, Gustafsson JA (2000) Structural characterisation of the mouse nuclear
634 oxysterol receptor genes LXRα and LXRβ. Gene 243(1-2):93-103
635 40. Zhang Y, Mangelsdorf DJ (2002) LuXuRies of lipid homeostasis: the unity of nuclear hormone
636 receptors, transcription regulation, and cholesterol sensing. Mol Interv 2(2):78-87
637 41. Cruz-Garcia L, Minghetti M, Navarro I, Tocher DR (2009) Molecular cloning, tissue expression
638 and regulation of liver X receptor (LXR) transcription factors of Atlantic salmon (*Salmo salar*)
639 and rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol B Biochem Mol Biol
640 153(1):81-88
641 42. Hua X, Wu J, Goldstein JL, Brown MS, Hobbs HH (1995) Structure of the human gene
642 encoding sterol regulatory element binding protein-1 (SREBF1) and localization of SREBF1
643 and SREBF2 to chromosomes 17p11.2 and 22q13. Genomics 25(3):667-673
644 43. Dong X, Xu H, Mai K, Xu W, Zhang Y, Ai Q (2015) Cloning and characterization of SREBP-1
645 and PPARα in Japanese seabass *Lateolabrax japonicus*, and their gene expressions in response
646 to different dietary fatty acid profiles. Comp Biochem Physiol B Biochem Mol Biol 180:48-56
647 44. Pai JT, Guryev O, Brown MS, Goldstein JL (1998) Differential stimulation of cholesterol and
648 unsaturated fatty acid biosynthesis in cells expressing individual nuclear sterol regulatory

element-binding proteins. J Biol Chem 273(40):26138-26148

45. Hannah VC, Ou J, Luong A, Goldstein JL, Brown MS (2001) Unsaturated fatty acids down-regulate SREBP isoforms 1a and 1c by two mechanisms in HEK-293 cells. J Biol Chem 276(6):4365-4372

46. Pan YX, Luo Z, Zhuo MQ, Hu W, Wu K, Shi X, Xu YH (2016) Liver X Receptor (LXR) in yellow catfish *Pelteobagrus fulvidraco*: Molecular characterization, mRNA tissue expression and transcriptional regulation by insulin *in vivo* and *in vitro*. Comp Biochem Physiol B Biochem Mol Biol 191:13-19

47. Peet DJ, Janowski BA, Mangelsdorf DJ (1998) The LXRs: a new class of oxysterol receptors. Curr Opin Genet Dev 8(5):571-575

48. Laffitte BA, Joseph SB, Walczak R, Pei L, Wilpitz DC, Collins JL, Tontonoz P (2001) Autoregulation of the human liver X receptor alpha promoter. Mol Cell Biol 21(22):7558-7568

49. Whitney KD, Watson MA, Goodwin B, Galardi CM, Maglich JM, Wilson JG, Willson TM, Collins JL, Kliewer SA (2001) Liver X receptor (LXR) regulation of the LXRα gene in human macrophages. J Biol Chem 276(47):43509-43515

50. Kase ET, Andersen B, Nebb HI, Rustan AC, Thoresen GH (2006) 22-Hydroxycholesterols regulate lipid metabolism differently than T0901317 in human myotubes. Biochim Biophys Acta 1761(12):1515-1522

51. Sprecher H (2000) Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochim Biophys Acta 1486(2-3):219-231

52. Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. Rev Fish Sci 11(2):107-184

671 53. Ou J, Tu H, Shan B, Luk A, DeBose-Boyd RA, Bashmakov Y, Goldstein JL, Brown MS (2001)
672 Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c
673 (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. Proc Natl Acad Sci
674 USA 98(11):6027-6032
675 54. Xu J, Teran-Garcia M, Park JH, Nakamura MT, Clarke SD (2001) Polyunsaturated fatty acids
676 suppress hepatic sterol regulatory element-binding protein-1 expression by accelerating
677 transcript decay. J Biol Chem 276(13):9800-9807
678 55. Xu J, Cho H, O'Malley S, Park JH, Clarke SD (2002) Dietary polyunsaturated fats regulate rat
679 liver sterol regulatory element binding proteins-1 and -2 in three distinct stages and by different
680 mechanisms. J Nutr 132(11):3333-3339
681 56. Geay F, Santigosa ICE, Corporeau C, Boudry P, Dreano Y, Corcos L, Bodin N, Vandeputte M,
682 Zambonino-Infante JL, Mazurais D, Cahu CL (2010) Regulation of FADS2 expression and
683 activity in European sea bass (*Dicentrarchus labrax*, L.) fed a vegetable diet. Comp Biochem
684 Physiol B Biochem Mol Biol 156(4):237-243
685 57. Colin S, Bourguignon E, Boullay AB, Tousaint JJ, Huet S, Caira F, Staels B, Lestavel S,
686 Lobaccaro JM, Delerive P (2008) Intestine-specific regulation of PPARα gene transcription by
687 liver X receptors. Endocrinology 149(10):5128-5135
688 58. Seo JB, Moon HM, Kim WS, Lee YS, Jeong HW, Yoo EJ, Ham J, Kang H, Park MG,
689 Steffensen KR, Stulnig TM, Gustafsson JA, Park SD, Kim JB (2004) Activated liver X
690 receptors stimulate adipocyte differentiation through induction of peroxisome
691 proliferator-activated receptor γ expression. Mol Cell Biol 24(8):3430-3444
692

693 **Figure legends**

694 **Fig. 1** Alignment of the deduced amino acid sequence of rabbitfish *S. canaliculatus* Lxr (**a**) and
695 N-terminus of Srebp1 (**b**) with their orthologs in human (*H. sapiens*), mouse (*M. musculus*),
696 chicken (*G. gallus*), zebrafish (*D. rerio*) and Atlantic salmon (*S. salar*). Identical amino acids (aa)
697 are shaded black and similar aa are shaded grey. (**a**) Protein structural domains of Lxr are marked
698 under the arrow line: AF-1 indicates N-terminal ligand-independent activation function (AF)
699 domain; DBD for DNA binding domain; D region for peptides linking LBD and DBD; LBD for
700 ligand binding domain containing the AF-2 region that is framed out in grey. (**b**) Exon regions of
701 Srebp proteins are indicated by arrow lines.

702

703 **Fig. 2** Phylogenetic analysis of Lxr and Srebp. The deduced amino acid sequences of Lxr (**a**) and
704 Srebp (**b**) proteins from rabbitfish and other organisms are used to construct the phylogenetic tree
705 by the neighbor joining method with Mega5. The horizontal branch length is proportional to aa
706 substitution rate per site. The numbers represent the frequencies with which the tree topology
707 presented was replicated after 1000 iterations.

708

709 **Fig. 3** Tissue distribution of *Lxr* and *Srebp1* mRNA in *S. canaliculatus* examined by
710 semi-quantitative PCR. H, heart; L, liver; S, spleen; G, gill; M, muscle; E, eyes; I, intestine; B,
711 brain; NTC: non-template control.

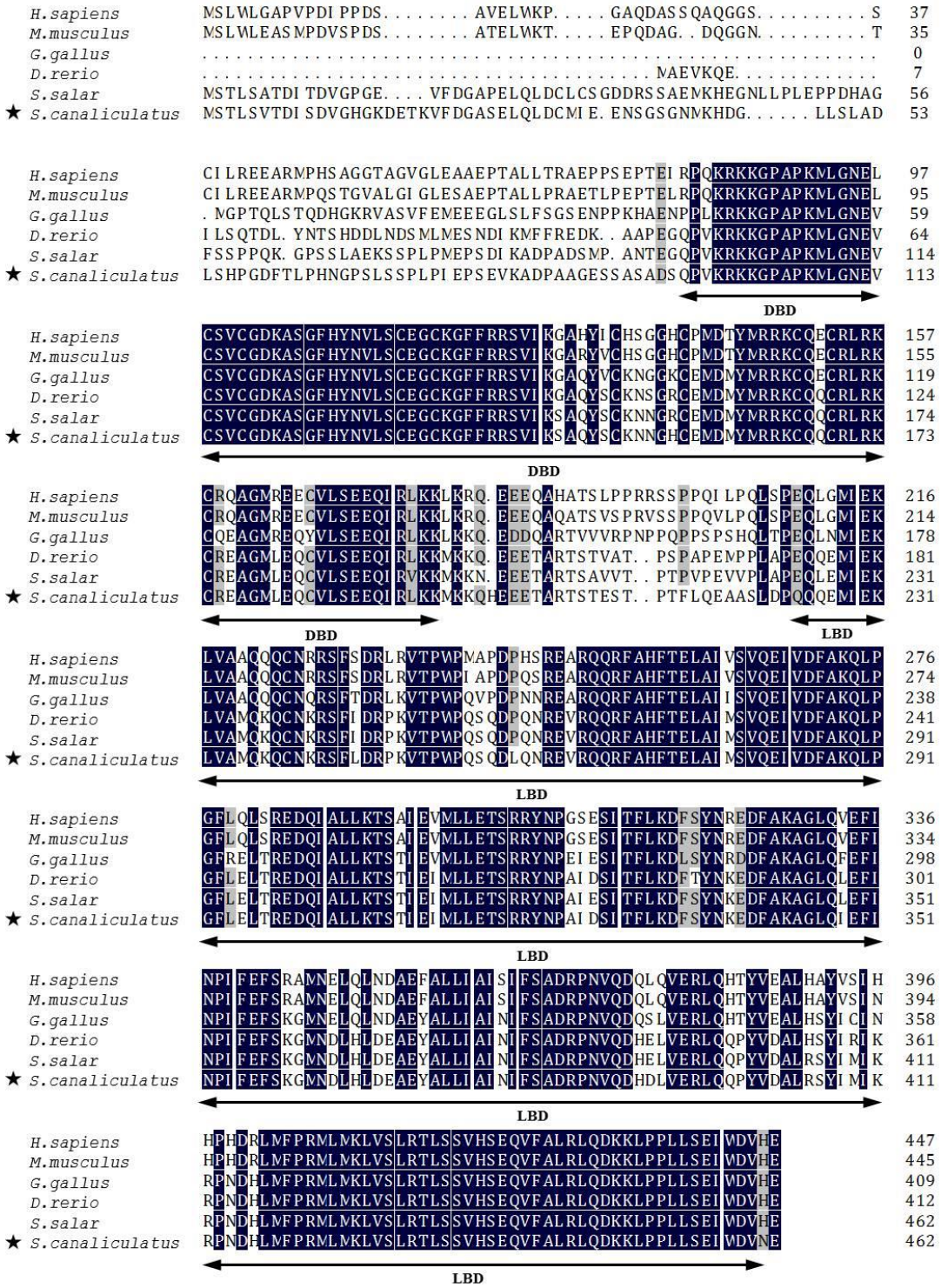
712

713 **Fig. 4** Effects of the Lxr agonists T0901317, GW3965 and 22(R)-hydroxycholesterol (22HC) on the
714 expression of *Lxr* and *Srebp1* in rabbitfish primary hepatocytes. The mRNA level of *Lxr* (**a**) and

715 *Srebp1* (**b**) were determined by qPCR in rabbitfish primary hepatocytes incubated with T0901317
716 (1 μM and 2 μM), GW3965 (2 μM and 4 μM), 22HC (5 μM and 10 μM) and vehicle reagents
717 (DMSO for T0901317, ethanol (EtOH) for 22HC) for 6 h, using *18S rRNA* as a reference gene.
718 Data are presented as the fold change from untreated cell control in means ± SEM (n = 3). Different
719 superscripts above bars denote significant differences among doses ($P < 0.05$; ANOVA, Tukey's
720 test).
721
722 **Fig. 5** Effects of the Lxr agonist T0901317 on the expression of genes related to LC-PUFA
723 biosynthesis in rabbitfish primary hepatocytes. The mRNA level of Δ4 *Fad*, Δ6/Δ5 *Fad*, *Elovl5* and
724 *Ppar* were determined by qPCR in rabbitfish primary hepatocytes incubated with T0901317 (1 μM
725 and 2 μM) or vehicle DMSO for 6 h, using *18S rRNA* as a reference gene. Data are presented as the
726 fold change from untreated cell control in means ± SEM (n = 3). Different superscripts above bars
727 denote significant differences among doses ($P < 0.05$; ANOVA, Tukey's test).
728
729 **Fig. 6** Expression of *Lxr* and *Srebp1* in the liver of rabbitfish fed VO and FO diets at 32 ppt and 10
730 ppt salinity. The mRNA level of *Lxr* and *Srebp1* was determined by qPCR, using *18S rRNA* as a
731 reference gene. Data are presented as fold change relative to initial control samples in means ±
732 SEM (n = 6). Two-way ANOVA results were presented in the upper right corner of the figure and
733 significant levels were considered at $P < 0.05$. D, diet (FO, VO); S, salinity (10, 32 ppt); D×S,
734 interaction; n.s., not significant.
735
736

Fig. 1

(A)


H.sapiens
M.musculus
G.gallus
D.rerio
S.salar
★ S.canaliculatus
DBD
LBD

(B)

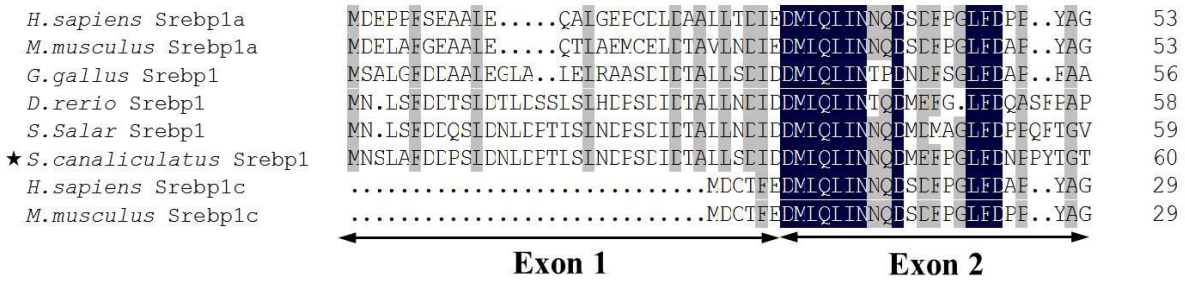
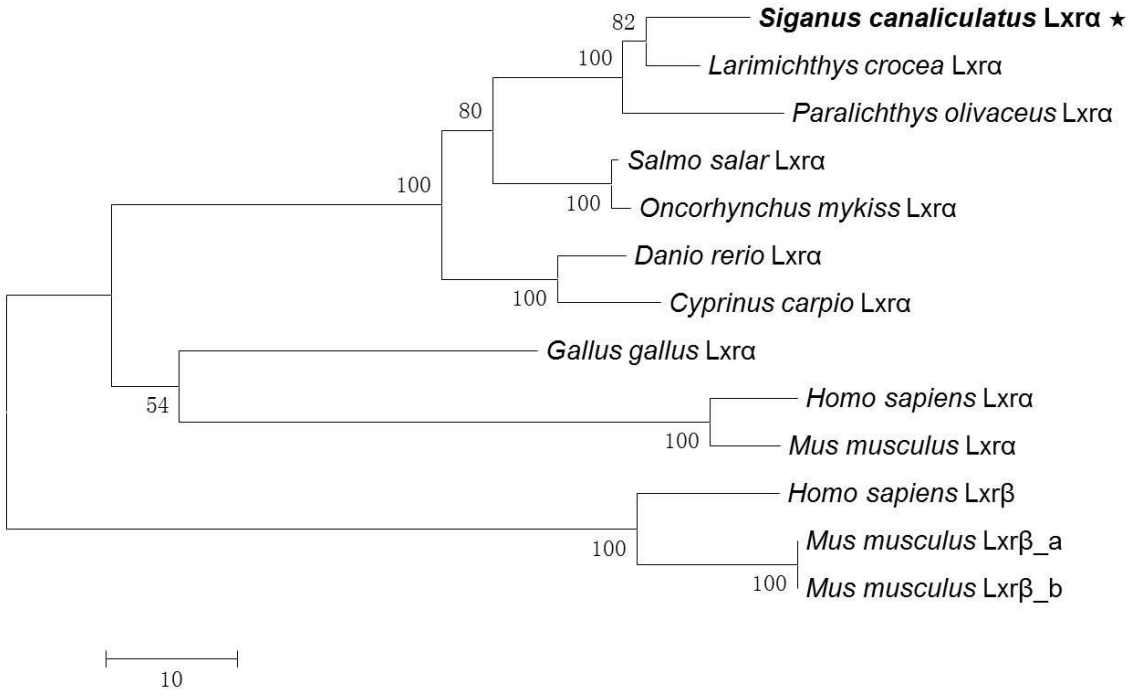

H.sapiens Srebp1a
M.musculus Srebp1a
G.gallus Srebp1
D.rerio Srebp1
S.Salar Srebp1
★S.canaliculatus Srebp1
H.sapiens Srebp1c
M.musculus Srebp1c
Exon 1
Exon 2

Fig. 2

(A)


82
100
80
100
100
100
54
100
100
100
Siganus canaliculatus Lxrα ★
Larimichthys crocea Lxrα
Paralichthys olivaceus Lxrα
Salmo salar Lxrα
Oncorhynchus mykiss Lxrα
Danio rerio Lxrα
Cyprinus carpio Lxrα
Gallus gallus Lxrα
Homo sapiens Lxrα
Mus musculus Lxrα
Homo sapiens Lxrβ
Mus musculus Lxrβ_a
Mus musculus Lxrβ_b
10

(B)

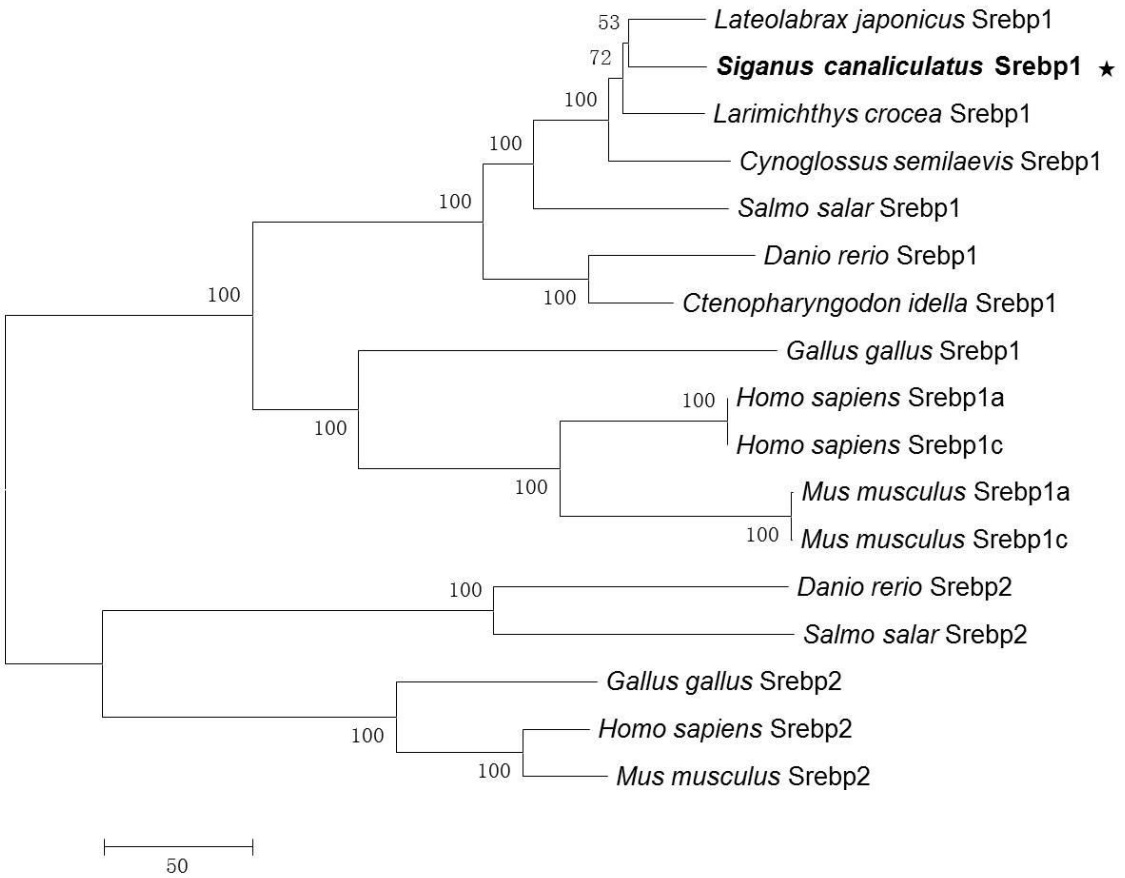

53
72
100
100
100
100
100
100
100
100
100
Lateolabrax japonicus Srebp1
Siganus canaliculatus Srebp1 ★
Larimichthys crocea Srebp1
Cynoglossus semilaevis Srebp1
Salmo salar Srebp1
Danio rerio Srebp1
Ctenopharyngodon idella Srebp1
Gallus gallus Srebp1
Homo sapiens Srebp1a
Homo sapiens Srebp1c
Mus musculus Srebp1a
Mus musculus Srebp1c
Danio rerio Srebp2
Salmo salar Srebp2
Gallus gallus Srebp2
Homo sapiens Srebp2
Mus musculus Srebp2
50

Fig. 3

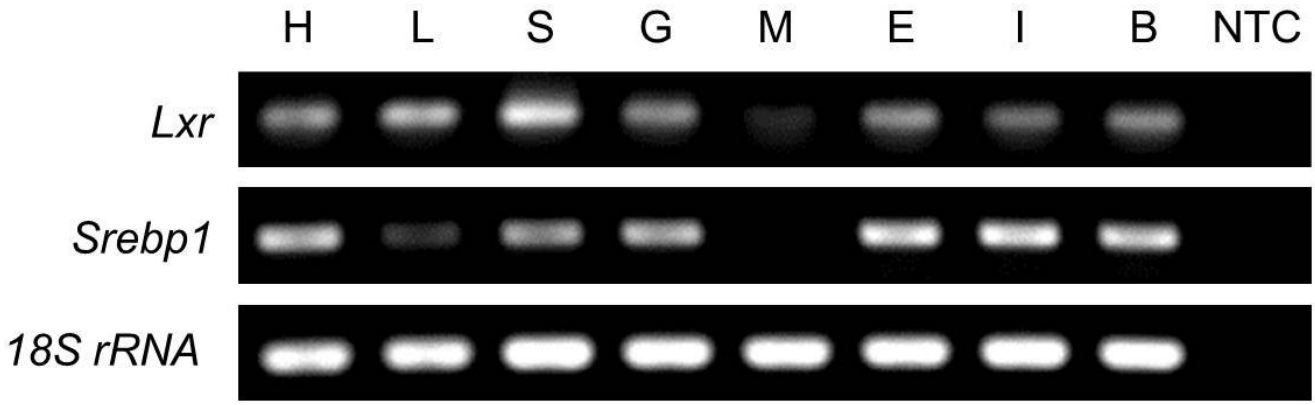


Fig. 4

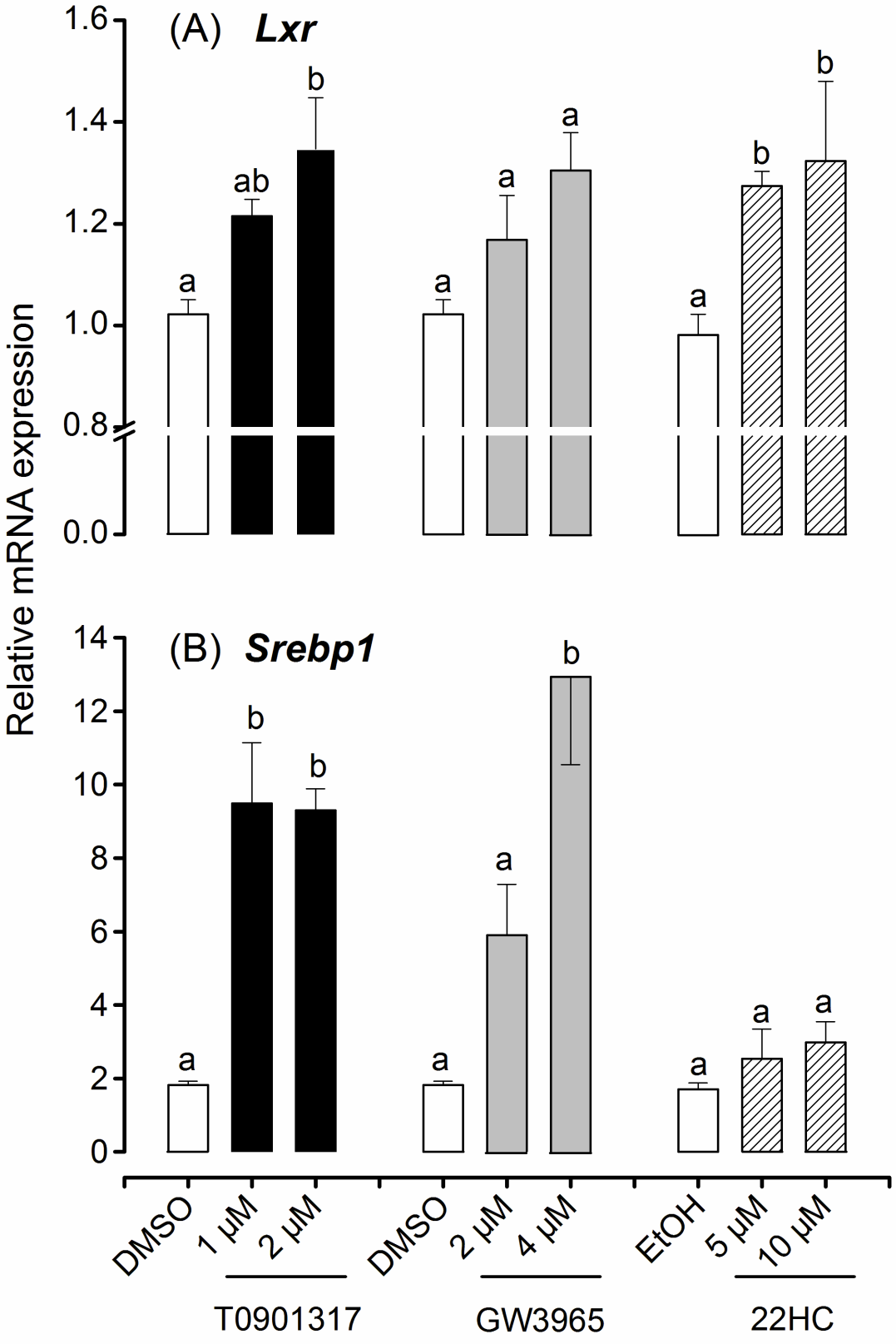

(A) Lxr
(B) Srebp1
Relative mRNA expression
1.6
1.4
1.2
1.0
0.8
0.0
14
12
10
8
6
4
2
0
a
ab
b
DMSO
1 μM
2 μM
2 μM
4 μM
EtOH
5 μM
10 μM
T0901317
GW3965
22HC

Fig. 5

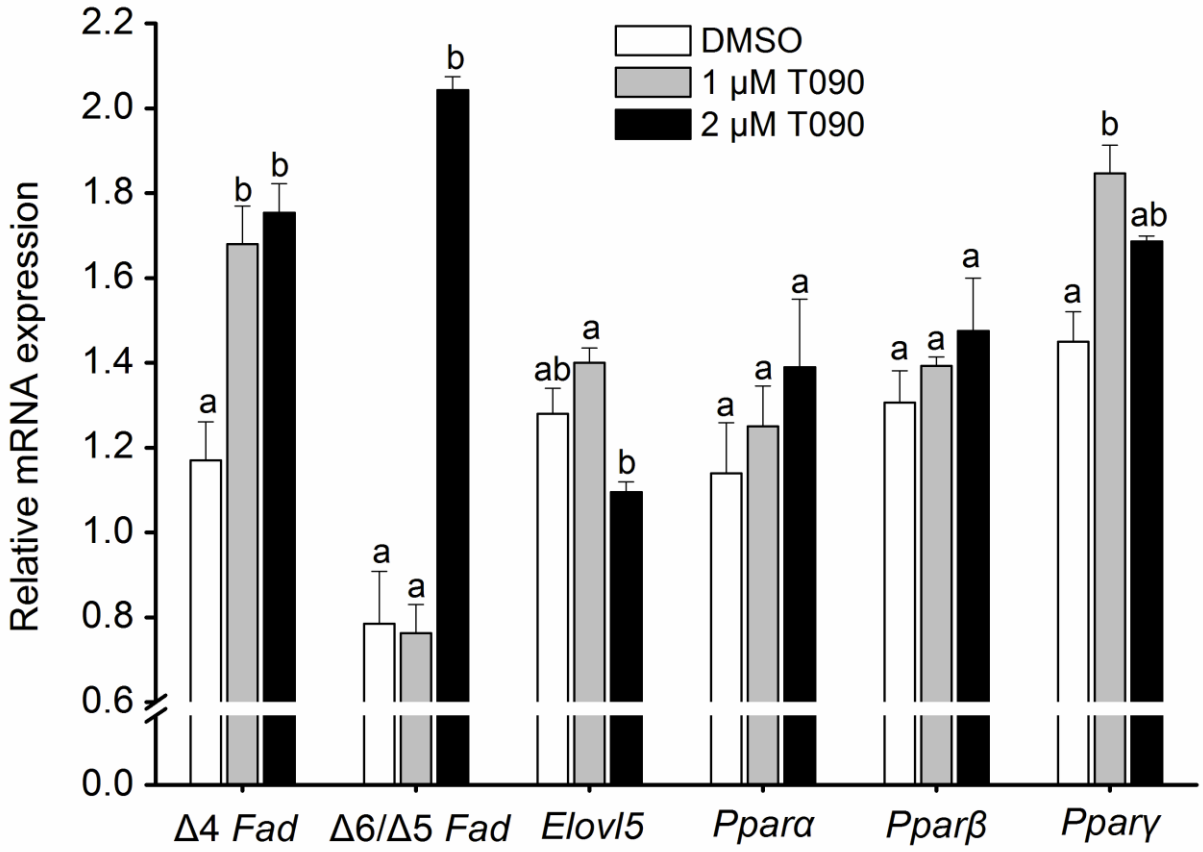
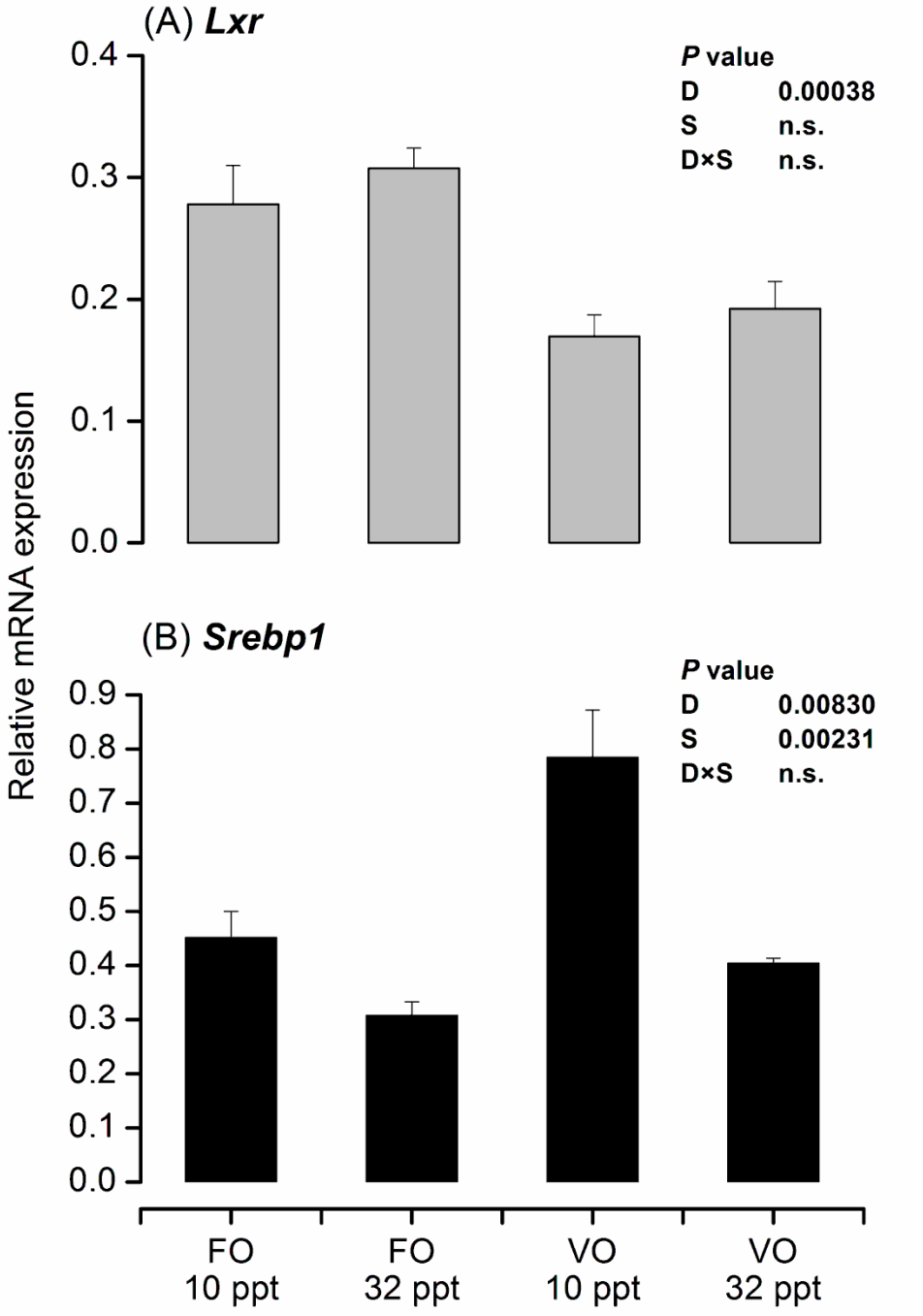

DMSO
1 μM T090
2 μM T090
Relative mRNA expression
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.0
a
b
b
a
a
b
ab
a
b
a
a
a
a
a
a
a
b
ab
Δ4 Fad
Δ6/Δ5 Fad
Elovl5
Pparα
Pparβ
Pparγ

Fig. 6


(A) Lxr
P value
D 0.00038
S n.s.
D×S n.s.
0.4
0.3
0.2
0.1
0.0
Relative mRNA expression
(B) Srebp1
P value
D 0.00830
S 0.00231
D×S n.s.
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
FO 10 ppt
FO 32 ppt
VO 10 ppt
VO 32 ppt

737 **Tables**

738

739

740

741

742

743

744

745

746

747

748

749

750

751

752

753

754

755

756

757

Table 1 Sequences of primers used for cloning rabbitfish *Lxr* and *Srebp1* cDNAs and for semi-quantitative PCR or qPCR.

Aim	Transcript	Forward primer	Primer sequence (5' - 3')	Reverse primer	Primer sequence (5' - 3')
RT-PCR	*Lxr*	lxr-partial-F	TYCAYTACAAYGTGCTGAGCTG	lxr-partial-R	CATBAGCATSCGDGGRAACAT
	Srebp1	srebp1-partial-F	GAGCAAGTCTCTGAAGGATCTGGT	srebp1-partial-R	CCTCATCCACACAAAGAAGCGGTG
RACE PCR	*Lxr*	lxr-5'-F1	CAGTGGCCGTTGTTTTGCAGGAGTAC	lxr-3'-R1	GCAGATCGACCAAATGTGCAGGATC A
		lxr-5'-F2	GCGTAAGCGGCACTGCTGACATTTC	lxr-3'-R2	GTCTCGGCGGTACAATCCTGCTATCG
	Srebp1	srebp1-5'-F1	TGGCTGTGACCCTCACTCCAAGT	srebp1-5'-R1	CATGGGACTATCCGGTTCCGAATC
		srebp1-5'-F2	AGAGTCTGTTGCCCAAGGCGTGT	srebp1-5'-R2	TGGGCAGCTCACTCTTCACATCAG
Full length clone	*Lxr*	lxr-full-F	AGGGCAACTGTGCTCTCATCC	lxr-full-R	TTGGCAGTCTGAGGTCATGGAC
	Srebp1	srebp1-full-F	GCGGTTTCAGGTACACTGGATT	srebp1-full-R	CCCTTCTGCCTAGCTGTTGGT
qPCR[a]	*Lxr*	lxr-qPCR-F	CTTTGCCCTTCGCCTCCAG	lxr-qPCR-R	TGTTCCTGCCAGACGCCAC
	Srebp1	srebp1-qPCR-F	CGGAGCCAAGACAGAGGAGTG	srebp1-qPCR-R	GTCTCCCAGCTTCTCCAAGGTAC
	Δ4 *Fad*	Δ4 fad-qPCR-F	TCACTGGAACCTGCCCACAT	Δ4 fad-qPCR-R	TTCATTCTCAGACAGTGCAAACAG
	Δ6 *Fad*	Δ6 fad-qPCR-F	TGCACCTAACAGGAGTCACGTT	Δ6 fad-qPCR-R	GCTCAGCACAGGATTGAGTACG
	Elovl5	elovl5-qPCR-F	CACAGCTTCAGCTGGTCCAG	elovl5-qPCR-R	TGCGTTTCTTATAAGTCTGAATGTAG
	Pparα	pparα-qPCR-F	CAAGGCTGAGAGCAAGATGGT	pparα-qPCR-R	AAGGCGTCCTGCTGGTCTTTC
	Pparβ	pparβ-qPCR-F	TGCTAGCAGAGGAACTGAACCTTG	pparβ-qPCR-R	AACGGCGAGGTGCTGCTT
	Pparγ	pparγ-qPCR-F	CTGCTGGCTGAGTTCTCGTCT	pparγ-qPCR-R	ATGACAAAAGGCGCGTTATCTC
	18S rRNA	18S rRNA-qPCR-F	CGCCGAGAAGACGATCAAAC	18S rRNA-qPCR-R	TGATCCTTCCGCAGGTTCAC

[a] The qPCR primers for *Lxr* and *Srebp1* were also used in the semi-quantitative PCR assays

758