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Authors names

Qinghao Zhang\textsuperscript{a,1}, Cuihong You\textsuperscript{a,1}, Fang Liu\textsuperscript{a}, Wendi Zhu\textsuperscript{a}, Shuqi Wang\textsuperscript{a}, Dizhi Xie\textsuperscript{a}, Óscar Monroig\textsuperscript{b}, Douglas R. Tocher\textsuperscript{b}, Yuanyou Li\textsuperscript{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 \textit{Siganus canaliculatus}

Affiliations and addresses of the authors

\textsuperscript{a} Marine Biology Institute & Guangdong Provincial Key Laboratory of Marine Biotechnology, Shantou University, Shantou, Guangdong, China

\textsuperscript{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

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

Long-chain (≥ C20) 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 C18 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 C18 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 facilitate 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 C18 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
Δ4, Δ5 or Δ6 specificity, respectively, or act in a bifunctional manner [5]. Δ4 Fad from vertebrates was first identified in rabbitfish Siganus canaliculatus by our group [6], followed by Senegalese sole (Solea senegalensis) [7], Mexican whitefish (Chirostoma estor) [8] and striped snakehead (Channa striata) [9], and separate Δ5 Fad have been identified in Atlantic salmon (Salmo salar) [10] and rainbow trout (Oncorhynchus mykiss) [11]. Bifunctional Δ5/Δ6 Fad were identified in zebrafish (Danio rerio) [12], rabbitfish [6] and Mexican whitefish [8], while Δ6 Fad have been found in many fish species [5,13]. In addition, elongase genes in teleosts include Elovl2, Elovl4 and Elovl5, among which Elovl5 genes have been identified in most teleosts studied to date [5]. Based on the presence and substrate specificity of the key enzymes involved in LC-PUFA biosynthesis, studies increasingly focused on the promoter of Fads where response elements for transcription factors were identified, such as sterol regulatory element binding proteins (Srebp), nuclear factor Y (NF-Y) and specificity protein 1 (Sp1) [14-16]. However, the mechanisms of transcriptional regulation for LC-PUFA biosynthesis are largely unexplored, especially in marine teleosts.

The Srebp family includes three members, Srebp1a and 1c, and Srebp2 in mammals and, among them, Srebp1 plays a major role in regulation of fatty acid synthesis [17]. Furthermore, Srebp1c can be activated by Liver X receptor (Lxr), a class I nuclear receptor that can be activated by oxysterols derived from cholesterol oxidation [18-20]. In mouse liver, it was established that Lxr and Srebp1 formed a functional pathway (called “Lxr-Srebp1 pathway”) that stimulates the production of
PUFA by transcriptional activation of Δ5 and Δ6 desaturase (encoded by *Fads1* and *Fads2* genes, respectively) and Elov15 [21]. In teleosts, the promoting effect of the Lxr-Srebp1 pathway on LC-PUFA biosynthesis has been implied in anadromous Atlantic salmon by increasing the expression of Δ5 and Δ6 *Fad* genes [16,22-24]. However, little is known about the presence or efficacy of Lxr-Srebp1 pathway in marine teleosts.

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

Rabbitfish *Siganus canaliculatus*, a true herbivore consuming algae and seagrass in nature, is widespread along the Indo-West Pacific coast and there is a commercial fishery. In aquaculture, rabbitfish can be domesticated to accept formulated feeds and, in recent years, rabbitfish farming has expanded rapidly in southeastern Asia, including China, due to its popularity in markets. It is noteworthy that *S. canaliculatus* was the first marine teleost demonstrated to have the ability of bioconverting C₁₈
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, Ppara, 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.
**Tissue collection**

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

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

Rabbitfish were fasted 24 h and then euthanized with 0.01 % 2-phenoxyethanol, followed by immersion in 70 % ethanol to sterilize the external surfaces. Primary hepatocytes were prepared from a pool of three livers as described previously [33].
Briefly, chopped livers were digested by 0.1 % collagenase/0.25 % hyaluronidase (Sigma-Aldrich, St. Louis, USA) followed by filtering through a 100 µm strainer. Isolated cells (viability ≥ 98 % evaluated by Trypan Blue dye exclusion) were seeded in 6-well plates at a density of $2 \times 10^6$ cells per well with 2 ml DMEM/F12 medium containing 20 % fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin, followed by incubation at 24 °C/4 % CO$_2$ for 24 h. The hepatocytes were incubated in FBS-free DMEM/F12 medium for 3 h prior to incubation with the following Lxr agonists: synthetic ligands T0901317 (1 and 2 µM) and GW3965 (2 and 4 µM), natural ligand 22(R)-hydroxycholesterol (22HC 5 and 10 µM), and treatment vehicles (DMSO for T091317 and GW3965, and ethanol (EtOH) for 22HC) on the basis of previous studies [34-36]. In addition, incubations without chemical supplement were included as control. After 6 h incubation, cells were lysed in wells and harvested for subsequent RNA extraction.

RNA isolation and cDNA synthesis

Total RNA from rabbitfish tissues was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA from rabbitfish primary hepatocytes was isolated and purified by RNAprep pure cell/bacteria kit (Tiangen Biotech, Beijing, China). The concentration and quality of total RNA preparations were confirmed by spectrophotometry (NanoDrop 2000, Thermo Scientific, USA) and agarose gel electrophoresis. For cloning partial sequences, one µg total RNA from liver was
subjected to cDNA synthesis using the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, USA). For semi-quantitative or quantitative analysis of target gene expression level, cDNA was synthesized from 1 µg or 500 ng of total RNA from rabbitfish tissues or primary hepatocytes, respectively, using FastQuant RT Kit (with gDNase) (Tiangen Biotech, Beijing, China).

Molecular cloning of rabbitfish Lxr and Srebp1 cDNAs

Partial cDNA fragments of Lxr and Srebp1 were obtained by polymerase chain reaction (PCR) using primer pair lxr-partial-F/R and srebp1-partial-F/R, respectively. Primers were designed on highly conserved regions of cDNA sequences based on alignments of human (Homo sapiens; NCBI accession: Lxra, AB307698.1; Srebp1, BC063281.1), mouse (Mus musculus; Lxra, AJ132601.1; Srebp1, BC056922.1), chicken (Gallus gallus; Lxr, AF492498.1; Srebp1, NM_204126.2), Atlantic salmon (Lxr, FJ470290.1; Srebp1, HM561860.1) and zebrafish (Lxr, BC092160.1; Srebp1, NM_001105129.1). The PCR was performed with initial denaturation at 95 °C for 5 min and 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, followed by a final extension at 72 °C for 10 min.

The full-length cDNA sequences of Lxr and Srebp1 were cloned by 3’ and 5’ rapid amplification of cDNA ends (RACE) PCR based on partial cDNA fragments using the GeneRacer® kit (Invitrogen, USA). According to the manufacturer’s 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 (Lxra, NCBI accession: AAA85856.1), mouse (Lxra, CAB51923.1), chicken (Lxra, NP_989873.1; Srebp1, NP_989457.2), Atlantic salmon (Lxra, NP_001138893.1; Srebp1, ADN28371.1) and zebrafish (Lxra, 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...
carp (*Ctenopharyngodon idella*) and Japanese sea bass (*Lateolabrax japonicus*).

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

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

The primer pairs used for semi-quantitative PCR were the same as used for qPCR (Table 1).

The expression level of *Lxr, Srebp1, Δ4 Fad* (GU594278.1), Δ6/Δ5 *Fad* (EF424276.2), *Elov5* (GU597350.1), *Ppara* (JF502070.1), *Pparβ* (JF502071.1) and *Pparγ* (JF502072.1) mRNA was determined by qPCR on a LightCycler® 480 thermocycler (Roche, Germany). All reactions were run in a total volume of 20 µl containing 10 µl LightCycler® 480 SYBR Green I Master (Roche, Germany), 0.4 µM each of gene specific primer pairs (Table 1), 20 ng cDNA templates (10 ng/µl) and ddH₂O in a white 96-well plate. The qPCR programs consisted of initial DNA 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 ddH₂O. 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 Lxra (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 Srebp1α 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 Srebp1α 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 Elov15 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 Ppara 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,
the expression of Srebp1 was higher in the liver of rabbitfish fed VO diets or reared at 10 ppt than that of fish fed FO diets or reared at 32 ppt ($P < 0.05$). There was no significant interaction between dietary lipid source and ambient salinity on the expression of Lxr or Srebp1 in rabbitfish liver.
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, Lxra and Lxrb, with overlapping or distinct functions [40]. However, rabbitfish possesses a unique form of Lxr that clustered with mammalian Lxra, and were Lxr orthologs of other fish species in the phylogenetic analysis. This suggested the gene loss of Lxrb 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 Lxra (Lxra-1 and Lxra-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.
that indirectly regulates fatty acid synthesis by driving Srebp1 transcription in mammals [20,21].

**Potential role and mechanism for Lxr and Srebp1 in regulation of LC-PUFA biosynthesis in rabbitfish liver**

As a ligand-activated nuclear receptor, Lxr can be activated by synthetic or natural agonists [18,39,47]. In the present study, agonists increased the expression of Lxr in rabbitfish hepatocytes, which can be explained by the positive autoregulation of Lxr that was shown to have LXREs in its own promoter in human and mice [48,49]. A similar autoregulatory situation has also been observed in recent studies performed in rainbow trout and Atlantic salmon [22,35,36]. Srebp1 was established as a target gene for Lxr in mammal and rodent systems [19,20]. Consistent with this, the expression of Srebp1 was increased by the Lxr agonists T0901317 and GW3965, whereas the 22HC produced no significant effects. In human myotubes, differential effects of T0901317 and 22HC were observed in regulation of genes involved in lipid metabolism, although both of them are effective agonists of Lxrα [50]. T0901317 and GW3965 were more effective activators for the Lxr of Atlantic salmon than 22HC [22]. Apparently, the effectiveness of Lxr agonists were model-specific, and thus in the present study, T0901317 was regarded as a potent activator for the expression of both Lxr and Srebp1 with maximal effect at minimum dose, which also indicated the conservation of Lxr-Srebp1 pathway in rabbitfish.
Fad and Elovl are key enzymes involved in LC-PUFA biosynthesis, and their gene expressions are commonly correlated to the LC-PUFA biosynthetic ability [4,5]. In mouse liver, the Lxr-Srebp1 pathway was demonstrated to be involved in LC-PUFA biosynthetic regulation through activating the expression of Δ5, Δ6 desaturase and Elovl5 [21]. In Atlantic salmon SHK-1 cells, T0901317 promoted the expression of Δ5 and Δ6 Fad but not Elovl5, accompanied by the increased expression of Lxr and Srebp1 [22]. As such, in the present study, the increased expression of Δ4 and Δ6/Δ5 Fad in rabbitfish hepatocytes incubated with T0901317 might be a result of activated Lxr-Srebp1 pathway, where Elovl5 might not be included.

Interestingly, the transcription of the Δ4 Fad was more sensitive to the activation of Lxr and Srebp1 than Δ6/Δ5 Fad in the present study. As the first vertebrate reported with Δ4 desaturation activity and the first marine teleost with Δ6/Δ5 desaturation activity, rabbitfish possesses two mechanisms for DHA biosynthesis from EPA. The first mechanism is the so-called “Sprecher pathway”, where EPA undergoes two successive elongation steps to produce 24:5n-3, followed by a second Δ6 desaturation step to produce 24:6n-3 that is chain-shorted to DHA by partial β-oxidation [51]. The second mechanism is the “Δ4 desaturation pathway” that requires a single elongation to 22:5n-3, which is then directly Δ4 desaturated to generate DHA [6]. Our recent study showed that there is coordination between the two pathways, in which miR-17 is involved through suppressing Δ4 Fad
post-transcriptionally rather than Δ6 Fad [33]. Likewise, the results presented herein further suggested the coordinating machinery at transcriptional level, possibly due to the distinct promoter structures between the two Fads. In addition, DHA is difficult to oxidize since its Δ4 bond must be removed by peroxisomal β-oxidation, as compared to EPA [52]. Herein, the higher sensitivity of Δ4 Fad towards the Lxr and Srebp1 activation may facilitate the bioconversion of EPA into DHA in rabbitfish liver.

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

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

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

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**Conflict of Interest**

The authors have declared that no competing interests exist.
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Figure legends

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

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

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

Fig. 4 Effects of the Lxr agonists T0901317, GW3965 and 22(R)-hydroxycholesterol (22HC) on the expression of *Lxr* and *Srebp1* in rabbitfish primary hepatocytes. The mRNA level of *Lxr* (a) and
Srebp1 (b) were determined by qPCR in rabbitfish primary hepatocytes incubated with T0901317 (1 µM and 2 µM), GW3965 (2 µM and 4 µM), 22HC (5 µM and 10 µM) and vehicle reagents (DMSO for T0901317, ethanol (EtOH) for 22HC) for 6 h, using 18S rRNA as a reference gene. Data are presented as the fold change from untreated cell control in means ± SEM (n = 3). Different superscripts above bars denote significant differences among doses (P < 0.05; ANOVA, Tukey's test).

Fig. 5 Effects of the Lxr agonist T0901317 on the expression of genes related to LC-PUFA biosynthesis in rabbitfish primary hepatocytes. The mRNA level of Δ4 Fad, Δ6/Δ5 Fad, Elovl5 and Ppar were determined by qPCR in rabbitfish primary hepatocytes incubated with T0901317 (1 µM and 2 µM) or vehicle DMSO for 6 h, using 18S rRNA as a reference gene. Data are presented as the fold change from untreated cell control in means ± SEM (n = 3). Different superscripts above bars denote significant differences among doses (P < 0.05; ANOVA, Tukey's test).

Fig. 6 Expression of Lxr and Srebp1 in the liver of rabbitfish fed VO and FO diets at 32 ppt and 10 ppt salinity. The mRNA level of Lxr and Srebp1 was determined by qPCR, using 18S rRNA as a reference gene. Data are presented as fold change relative to initial control samples in means ± SEM (n = 6). Two-way ANOVA results were presented in the upper right corner of the figure and significant levels were considered at P < 0.05. D, diet (FO, VO); S, salinity (10, 32 ppt); D×S, interaction; n.s., not significant.
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Table 1. Sequences of primers used for cloning rabbitfish *Lxr* and *Srebp1* cDNAs and for semi-quantitative PCR or qPCR.

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α The qPCR primers for *Lxr* and *Srebp1* were also used in the semi-quantitative PCR assays.