

Title

Ppar γ is Involved in the Transcriptional Regulation of Liver LC-PUFA Biosynthesis by Targeting the $\Delta 6\Delta 5$ Fatty Acyl Desaturase Gene in the Marine Teleost *Siganus canaliculatus*

Authors

Yuanyou Li^{a,b,*}, Ziyang Yin^{b#}, Yewei Dong^{a#}, Shuqi Wang^b, Óscar Monroig^c, Douglas R. Tocher^c, Cuihong You^{b*}

Addresses

^a*School of Marine Sciences, South China Agricultural University, Guangzhou 510642, China*

^b*Guangdong Provincial Key Laboratory of Marine Biotechnology, Shantou University, Shantou 515063, China*

^c*Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, UK*

[#] These authors contributed equally to the study

Keywords:

LC-PUFA, fatty acyl desaturase, transcriptional regulation, *Siganus canaliculatus*

*Correspondence to: Prof. Yuanyou Li, Ph.D. (E-mail: yyli16@scau.edu.cn)

Cuihong You, Ph.D. (E-mail: chyou@stu.edu.cn)

23 **Abbreviations**

- 24 ALA, α -linolenic acid (18:3n-3)
- 25 ARA, arachidonic acid (20:4n-6)
- 26 CPT-1, carnitine palmitoyl transferase-1
- 27 DHA, docosahexaenoic acid (22:6n-3)
- 28 EFA, essential fatty acid
- 29 EPA, eicosapentaenoic acid (20:5n-3)
- 30 Fads, fatty acyl desaturases
- 31 FABP, fatty acid binding protein
- 32 FO, fish oil
- 33 HEK 293T cell, human embryonic kidney 293T cell
- 34 HNF4 α , hepatocyte nuclear factor 4 α
- 35 LC-PUFA, long-chain polyunsaturated fatty acids
- 36 LPL, lipoprotein lipase
- 37 Lxr, liver X receptor
- 38 Ppar γ , peroxisome proliferator activated receptor γ
- 39 PUFA, polyunsaturated fatty acids
- 40 Q-PCR, quantitative polymerase chain reaction
- 41 SCHL, *Siganus canaliculatus* hepatocyte line
- 42 Srebp, sterol regulatory element binding protein
- 43 TF, transcription factor
- 44 TSS, transcription start site
- 45 VO, vegetable oil
- 46

Abstract

As the first marine teleost demonstrated to have the ability of long-chain polyunsaturated fatty acids (LC-PUFA) biosynthesis from C₁₈ PUFA precursors, the rabbitfish *Siganus canaliculatus* provides us a unique model for clarifying the regulatory mechanisms of LC-PUFA biosynthesis in teleosts aiming at the replacement of dietary fish oil (rich in LC-PUFA) with vegetable oils (rich in C₁₈ PUFA precursors but devoid of LC-PUFA). In the study of transcription regulation of gene encoding the $\Delta 6\Delta 5$ fatty acyl desaturase ($\Delta 6\Delta 5$ Fads), a rate-limiting enzyme catalyzing the first step of LC-PUFA biosynthesis in rabbitfish, a binding site for the transcription factor (TF), peroxisome proliferator-activated receptor γ (Ppar γ), was predicted in $\Delta 6\Delta 5$ *fads2* promoter by bioinformatics analysis, and thus the present study focused on the regulatory roles of Ppar γ on $\Delta 6\Delta 5$ *fads2*. First, the activity of the $\Delta 6\Delta 5$ *fads2* promoter was proved to be down-regulated by *ppar γ* overexpression and up-regulated by treatment of Ppar γ antagonist (GW9662), respectively, in HEK 293T cells with the dual luciferase reporter assay. Ppar γ was further confirmed to interact with the promoter by electrophoretic mobility shift assay. Moreover, in *S. canaliculatus* hepatocyte line (SCHL) cells, GW9662 decreased the expression of *ppar γ* together with increase of $\Delta 6\Delta 5$ *fads2* mRNA. Besides, $\Delta 6\Delta 5$ *fads2* expression was increased by *ppar γ* RNAi knock-down and reduced by its mRNA overexpression. Furthermore, knock-down of *ppar γ* induced a high conversion of 18:3n-3 to 18:4n-3 and 18:2n-6 to 18:3n-6, while *ppar γ* mRNA overexpression led to a lower conversion of that, and finally a significant decrease of 20:4n-6(ARA), 20:5n-3(EPA) and 22:6n-3(DHA) production. The results indicate that Ppar γ is involved in the transcriptional regulation of liver LC-PUFA biosynthesis by targeting $\Delta 6\Delta 5$ *fads2* in rabbitfish, which is the first report of Ppar γ involvement in the regulation of LC-PUFA biosynthesis in teleosts.

71 Introduction

72 Long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic acid
73 (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) are
74 physiologically essential fatty acids (EFA) for vertebrates, playing important roles in gene
75 regulation, signal transduction, lipogenesis and cell membrane fluidity (Benitez-santana et al., 2007,
76 Schmitz and Ecker, 2008, Uauy et al., 2001, Galli et al., 1994). From a human nutrition standpoint,
77 the so-called “omega-3” (n-3) LC-PUFA such as EPA and DHA have beneficial roles in a variety
78 of human pathologies and disorders (Lorente-Cebrián et al., 2013). Fish, especially marine species,
79 are major sources of n-3 LC-PUFA in the human diets. And, with over half of the production of
80 fish now deriving from aquaculture, research aiming to understand LC-PUFA metabolism of fish
81 farmed species has been driven to ensure the production of high quality (high n-3 LC-PUFA)
82 products (Tocher, 2003). Traditionally, inclusion of high levels of marine ingredients such as
83 fishmeal, but particularly fish oil (FO), in aquafeeds has delivered high n-3 LC-PUFA contents in
84 farmed fish. However, there currently exists a tendency in the farming industry of some species
85 such as the Atlantic salmon showing a reduction of n-3 LC-PUFA in the final product (Bell et al.,
86 2001). This is mostly due to the replacement of dietary FO by alternative oil sources such as
87 vegetable oils (VO) since the latter are devoid of LC-PUFA and rather contain high levels of
88 shorter-chain fatty acids (FA) such as the C_{18} polyunsaturated fatty acids (PUFA) linoleic acid (LA,
89 18:2n-6) and α -linolenic acid (ALA, 18:3n-3) (Sargent et al., 2002). This problem can aggravate in
90 fish species with limited capacity to convert C_{18} PUFA contained in dietary VO into LC-PUFA,
91 thus compromising not only its nutritional value for the human consumer but also the dietary
92 provision of essential nutrients for normal growth and development in captivity (Tocher, 2015).

93 The ability of a certain vertebrate species to convert C_{18} PUFA into C_{20-22} LC-PUFA varies
94 among species and depends upon the complement and function of two key enzymes, namely fatty
95 acyl desaturases (Fads) and elongation of very long-chain fatty acid (Elovl) proteins (Castro et al.,
96 2016). Unlike mammals, teleost fish appear to possess only one type of *fads*-like genes in their
97 genome, this being *fads2* (Castro et al., 2012). Consistent with the mammalian FADS2, Fads2
98 studied from many teleosts are also $\Delta 6$ desaturases but, interestingly, many teleost Fads2 have
99 acquired other desaturase capabilities such as the bifunctional $\Delta 6\Delta 5$ desaturases, monofunctional
100 $\Delta 5$ desaturases and $\Delta 4$ desaturases (Castro et al., 2016). Moreover, Elovl encoding genes with

relevant roles in the biosynthesis of LC-PUFA in teleosts include Elov12, Elov14 and Elov15, of which Elov14 and Elov15 are present in virtually all teleosts (Castro et al., 2016). Regulatory mechanisms controlling the expression of teleost *fads* and *elovl* have been investigated in order to understand the mechanisms underlying the response of fish to dietary components. With regards to *fads2*, transcriptional regulation by transcription factors (TF) such as sterol regulatory element binding proteins (Srebp), nuclear factor Y (NF-Y), liver X receptor (Lxr) and specificity protein 1 (Sp1) have been established for a range of species (Carmona-Antoñanzas et al., 2014, Geay et al., 2012, Xu et al., 2014, Zheng et al., 2009). Particular progress on elucidating the mechanisms involved in *fads2* regulation of teleosts has been made in rabbitfish *S. canaliculatus*, the first marine teleost demonstrated to have the ability of bioconverting C₁₈ PUFA to LC-PUFA (Li et al., 2010, Li et al., 2008, Monroig et al., 2012) and possessing two distinct Fads2 with $\Delta 4$ and $\Delta 6\Delta 5$ desaturase capabilities (Li et al., 2010). Thus, the expression of the rabbitfish *fads2* is controlled at transcriptional level by *Lxr* and *Srebp1*, regulating both $\Delta 4$ and $\Delta 6\Delta 5$ *fads2* in rabbitfish liver (Zhang et al., 2016a), and by hepatocyte nuclear factor alpha (HNF4 α) (Dong et al., 2016), specifically targeting $\Delta 4$ *fads2* expression. Moreover, unique post-transcriptional regulatory mechanisms have been further established, with miR-17 exhibiting its action directly on $\Delta 4$ *fads2* (Zhang et al., 2014), and miR-33, whose more indirect action involves regulation of *srebp1* (Zhang et al., 2016b).

Peroxisome proliferator-activated receptors (Ppar) play important roles in lipid metabolism (Desvergne et al., 2006, Poulsen et al., 2012, Tontonoz and Spiegelman, 2008) and studies in fish have confirmed that Ppar regulate genes of fatty acid oxidation and deposition among other pathways (Leaver et al., 2008). In general, PPAR can be activated by natural or artificial ligands, then heterodimerized with retinoid X receptor (RXR), and subsequently bound to PPAR response element (PPRE) in target genes (Adeghe et al., 2011). Ppar protein family consists of three isoforms including PPAR α , PPAR β (δ) and PPAR γ . In mammals, PPAR α is involved in stimulating fatty acid oxidation, PPAR β plays a role in the regulation of lipoprotein transport system (Desvergne et al., 2006), while PPAR γ regulates glucose and lipid homeostasis, and induces lipid accumulation (Gurnell, 2005, Tontonoz and Spiegelman, 2008). With regards to specific actions within the LC-PUFA biosynthesis, PPAR α , along with SREBP-1c, was demonstrated as an important TF in the feedback regulation of murine *Fads2* (Matsuzaka et al., 2002), whereas

PPAR γ was inferred as a possible target for Lxr (Zhang et al., 2016a). Nevertheless, the mechanisms of transcriptional regulation by Ppar γ on LC-PUFA biosynthesis in teleosts remain mostly unclear.

Recently, three Ppar genes including *ppara*, *ppar β* (δ) and *ppar γ* were cloned from rabbitfish, and the relatively high expression of *ppara* and *ppar γ* in liver and intestine, major sites for LC-PUFA biosynthesis in fish (You et al., 2017), suggested potential regulatory roles in these pathways. Supplementation of the Ppar agonist fenofibrate to rabbitfish primary hepatocytes increased the expression of *ppar γ* with depressed expression of $\Delta 6\Delta 5$ *fads2*, which indicated that Ppar γ may have a regulation role on $\Delta 6\Delta 5$ *fads2* (You et al., 2017). Moreover, preliminary investigations allowed us to identify a potential Ppar γ binding site on $\Delta 6\Delta 5$ *fads2* promoter. To further clarify the roles of Ppar γ in the regulation of genes encoding pivotal enzymes within rabbitfish LC-PUFA biosynthesis, the present study investigated the transcription regulation of *ppar γ* on $\Delta 6\Delta 5$ *fads2* expression. First, the promoter activity of $\Delta 6\Delta 5$ *fads2* was studied by overexpression *ppar γ* and supplementation of Ppar γ antagonist (GW9662) in HEK 293T cells. Second, the gene expression pattern of *ppar γ* and $\Delta 6\Delta 5$ *fads2* was measured in *S. canaliculatus* hepatocyte line (SCHL) by mRNA overexpression or inhibition of *ppar γ* . Furthermore, the capability of SCHL cells in bioconverting C₁₈ PUFA to LC-PUFA was evaluated under *ppar γ* overexpression or siRNA knock-down. These data will increase our knowledge of regulatory mechanisms of LC-PUFA biosynthesis in teleosts, and provide useful information for the successful replacement of VO in feeds for farmed fish.

Materials and methods

Cell lines and cell culture

Human embryonic kidney (HEK 293T) cells (Chinese Type Culture Collection, Shanghai, China) were grown at 37 °C with 5 % CO₂ concentration in high glucose Dulbecco's Modified Eagle Medium (DMEM) (GlutaMAX) (Gibco, Life Technologies, USA) supplemented with 10 % fetal bovine serum (FBS, Sijiqing Biological Engineering Material Company, Hangzhou, China). *S. canaliculatus* hepatocyte line (SCHL) recently established in our lab (Y et al., 2017) was grown at 28 °C using Dulbecco's modified Eagle's medium (DMEM)-F12 medium (Gibco) supplemented with 10% foetal bovine serum (FBS; Gibco) and 0.5 % rainbow trout *Oncorhynchus mykiss*

(Walbaum 1792) serum (Caisson Labs).

Ppar γ overexpression and detection of its influence on the $\Delta 6\Delta 5$ fads2 promoter activity

A potential Ppar γ binding site was predicted in the rabbitfish $\Delta 6\Delta 5$ fads2 promoter by using online software TRANSFAC® and JASPAR® (Fig. 1) (Dong et al., 2018). To confirm the effects of Ppar γ on the $\Delta 6\Delta 5$ fads2 promoter activity, the overexpression vector pcDNA3.1-ppar γ was constructed by cloning the rabbitfish ppar γ open reading frame (ORF) (GenBank: JF502072.1) DNA fragment into the pcDNA3.1 vector (Invitrogen).

The rabbitfish $\Delta 6\Delta 5$ fads2 promoter (2044 bp) was cloned from the $\Delta 6\Delta 5$ Fad mRNA of *S. canaliculatus* (GenBank: EF424276.2) (Dong et al., 2018). The promoter reporter vector was constructed with the $\Delta 6\Delta 5$ fads2 promoter fragment and pGL4.10, and the $\Delta 6\Delta 5$ fads2 Ppar γ binding site-directed mutant of the $\Delta 6\Delta 5$ fads2 promoter was constructed with the mutation site in the middle of the primer. The promoter reporter vector contained the Firefly luciferase gene. Subsequently, the overexpression vector pcDNA3.1-ppar γ was co-transfected with the $\Delta 6\Delta 5$ fads2 promoter reporter vector into HEK 293T cells seeded in 96-well cell culture plates by Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA), and the content in transfection complex per well was listed as following: pcDNA3.1-ppar γ (50 ng), $\Delta 6\Delta 5$ fads2 promoter (100ng) or $\Delta 6\Delta 5$ fads2 Ppar γ site-directed mutant, internal control vector pGL4.75 (0.02 ng) and Lipofectamine 2000 Reagent (0.25 μ L). The HEK 293T cell is a commercially available cell line commonly used for studying the promoter activity involving dual-luciferase reporter assays. After the cells were grown to about 80% confluency, the transfection assay was conducted. At 48 h post-transfection, the promoter activities were measured by Dual-Glo Luciferase Assay system (Promega, USA) according to manufacturer's instructions. Specifically, 75 μ l of Dual-Glo Luciferase Assay Reagent were added to each well, the plate was incubated at room temperature for 10 min. Then measure firefly luminescence on a Tecan microplate reader (Tecan, Switzerland), followed by the addition of Dual-Glo Stop & Glo Reagent to the plate and incubate at room temperature for 10 min. Finally, chemical luminescence intensity was detected in duplicate readings using a microplate reader (InfiniteM200 Pro, Tecan, Switzerland). The promoter activity was calculated by the ratio of luciferase to renilla intensity in each experiment well, and empty vector pGL4.10 was used as a negative control.

Effect of Ppar γ antagonist on the $\Delta 6\Delta 5$ fads2 promoter activity

To detect the effects of a Ppar γ antagonist (GW9662) on the $\Delta 6\Delta 5$ fads2 promoter activity, 100 ng of $\Delta 6\Delta 5$ fads2 promoter reporter vector and 0.02 ng of pGL4.75 were co-transfected into HEK 293T cells. The transfection, done as the detailed above for the ppar γ overexpression assay, lasted for 24 h, period after which the cell culture medium was replaced with DMEM + 10% FBS containing GW9662 at a final concentration of 20 μ M or an equivalent volume of DMSO (concentration did not exceed 0.1 %, v/v). Then, 48 h post-transfection, the promoter activity was measured as the detailed above for the ppar γ overexpression assay.

Electrophoretic Mobility Shift Assay (EMSA)

To further confirm the binding between Ppar γ and the $\Delta 6\Delta 5$ fads2 promoter, primers were designed according to the Ppar γ binding region in the $\Delta 6\Delta 5$ fads2 promoter and 5'-biotin labeled for production of EMSA probes (Table 1). The experimental probe was produced in a 100 μ L annealing reaction system including 40 μ L nuclease-free water, 40 μ L annealing buffer for DNA Oligos (5X) (Beyotime, China), 20 μ L primer F for EMSA (50 μ M), 20 μ L primer R for EMSA (50 μ M). The extraction of nuclear protein from the rabbitfish hepatocytes was conducted using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China) according to the manufacturer's instructions. For the binding reaction, 6 μ g of nuclear protein were incubated in a total volume of 10 μ L with binding buffer containing 2 μ L of biotin labeled probe (BP) (0.1 pM). Competing reaction and cold competing reaction were performed using a 100-fold excess of unlabeled probe (UP) and unlabeled mutant probe (UTP). The reaction was carried out in lane 1 (no proteins, 5' biotin labeled probe), lane 2 (rabbitfish hepatocyte nucleus proteins, 5' biotin labeled free probe), lane 3 (rabbitfish hepatocyte nucleus proteins, unlabeled competitor probe, 5' biotin labeled free probe), lane 4 (rabbitfish hepatocyte nucleus proteins, unlabeled mutant competitor probe, 5' biotin labeled free probe). Following addition of 2 μ L sample buffer, the protein-DNA complexes were resolved on a 4 % non-denaturing polyacrylamide gel in 0.5 \times TBE buffer at 380 mA for 1 h and then transferred to nylon membrane. Finally, biotin-labeled DNA was detected by chemiluminescence using the Chemiluminescent EMSA Kit (Beyotime, China) according to manufacturer's protocol.

Effect of Ppar γ antagonist on $\Delta 6\Delta 5$ fads2 gene expression in SCHL cells

To determine the effects of Ppar γ antagonist on $\Delta 6\Delta 5$ fads2 gene expression, the SCHL cells were seeded in 6-well plates at a density of 2×10^6 cells per well and cultured for 24 h. Then the medium was replaced with fresh DMEM + 10% FBS in addition of GW9662 with a final concentration of 20 μ M. The control group was treated with the same volume of medium without GW9662. All treatments were run in triplicate wells. After 48 h incubation, the cells were collected and lysed for RNA isolation under the instructions of TriPure RNA Isolation Reagent (Roche).

Effect of siRNA on ppar γ and $\Delta 6\Delta 5$ fads2 gene expression in SCHL cells

To further clarify the influence of Ppar γ on $\Delta 6\Delta 5$ fads2 regulation, an RNAi fragment targeting the rabbitfish Ppar γ was run by transfection into SCHL cells (Lipofectamine 2000, Invitrogen). The siRNA sequence included seq 1 (5'-CCUCCCAAACAGUCAGAUUTT-3') and seq 2 (5'-UUCUCCGAACGUGUCACGUTT-3'), seq 1 was 884 bp to ATG and set as experiment group, while seq 2 was negative control. The siRNA (0.2 nmol) was transfected into the SCHL cells after 24 h incubation, after 24 h transfection, the cells were collected and lysed for RNA and lipid extraction. The survival rate of the cells was more than 95 % over the course of the whole operation.

Influence of ppar γ mRNA overexpression on $\Delta 6\Delta 5$ fads2 gene expression in SCHL cells

The influence of Ppar γ on $\Delta 6\Delta 5$ fads2 expression was further established by running an mRNA overexpression assay performed by transfecting ppar γ mature transcripts into SCHL cells. mRNA transcription was performed on a linearized DNA template containing T7 promoter, ppar γ ORF (GenBank: JF502072.1) using mMACHINE T7 Ultra Kit (Ambion, Austin, TX, USA) according to the manufacturer to generate capped mRNA with a poly(A) tail. Plasmid pcDNA3.1-ppar γ was used as a template for a linearized DNA template above with T7 promoter primer and antisense primer of ppar γ containing termination codon in a pfu-PCR reaction (Table 1). Finally, the mRNA product was purified with MEGA clear TM Kit (Ambion), and stored in -80°C for further transfection into cell line. The method of rabbitfish SCHL cell culture was the

same with antagonist assay above. When the cells were grown to 80% confluence for 24 h, a transfection complex consisting of 2 μ g *ppary* mRNA and 6.25 μ L LipofectamineTM Messenger-MAXTM Reagent (Invitrogen) were transfected into cells within well. At 48 h after transfection, cells were lysed and harvested for RNA and lipid extraction (see subsequent section for methodological details). The survival rate of the cells was more than 90 % over the course of the entire operation.

Quantitative real-time PCR analysis

The expression of *ppary* and $\Delta 6\Delta 5$ *fads2* from experiments involving SCHL cells was analyzed by quantitative real-time PCR analysis (qPCR). Total RNA was extracted using TRIzol[®] Reagent (Invitrogen, USA). Total RNA (1 μ g) was reverse-transcribed into cDNA with FastKing RT Kit including gDNase treatment (Tiangen, China). Primers used for qPCR were listed in Table 1. The relative gene expression of *ppary* and $\Delta 6\Delta 5$ *fads2* was normalized with that of 18S *rRNA* (GenBank: AB276993) with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each qPCR (total volume of 20 μ L) consisted of 2 μ L diluted cDNA (10 ng/ μ L), 0.5 μ M of each primer and 10 μ L SYBR Green I Master (Roche), and reactions were carried out on a Lightcycler 480 system (Roche, Switzerland). No template controls (NTC) were run systematically in each plate as negative controls. The qPCR program consisted of an initial activation step at 95 $^{\circ}$ C for 5 min, followed by 35 cycles of 10 s at 95 $^{\circ}$ C, 20 s at 60 $^{\circ}$ C and 20 s at 72 $^{\circ}$ C. After the amplification, dissociation curves of 0.5 $^{\circ}$ C increments from 65 $^{\circ}$ C to 95 $^{\circ}$ C were carried out to confirm the amplification of a single product in each reaction.

Lipid extraction and fatty acid analysis

The cells were digested with Trypsin-EDTA (Invitrogen) and centrifuged at 4000 \times g for 5 min. Fatty acids were extracted from the cells precipitate by steeping in 2 ml chloroform/methanol (2:1 v/v), then 0.5 N methanolic KOH and 14 % boron trifluoride–methanol (Sigma-Aldrich, USA) were used to saponify the FA for fatty acid methyl ester (FAME) (Wijngaarden, 1967) derivative. Finally, FAME were separated and quantified by GC-2010 Plus gas chromatograph (Shimadzu, Japan) as we described before (Li et al., 2008).

Statistical analysis

All data for dual luciferase assays, gene expression and FA contents were presented as mean \pm SEM ($n=3$). The differences among the groups were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or Student's *t*-test (as indicated) using Origin 7.0. Differences were considered significant at $P < 0.05$ to all statistical tests performed.

Results

ppary overexpression decreased the activity of rabbitfish $\Delta 6\Delta 5$ *fads2* promoter

HEK 293T cells co-transfected with the overexpression vector pcDNA3.1-*ppary* and $\Delta 6\Delta 5$ *fads2* promoter showed a significant decrease of $\Delta 6\Delta 5$ *fads2* promoter activity, while $\Delta 6\Delta 5$ *fads2* promoter with *ppre* mutant had no response to *ppary* overexpression (Fig. 2). No response to the *ppary* overexpression was observed in the negative control (pGL4.10). This result was in agreement with our hypothesis that *ppre* site within the $\Delta 6\Delta 5$ *fads2* promoter is the target site for Ppar γ .

Ppar γ antagonist GW9662 increased the activity of rabbitfish $\Delta 6\Delta 5$ *fads2* promoter

To further confirm the action of Ppar γ on $\Delta 6\Delta 5$ *fads2* promoter, the effects of the Ppar γ antagonist GW9662 was tested. Thus, HEK 293T cells transfected with the rabbitfish $\Delta 6\Delta 5$ *fads2* promoter and treated with Ppar γ antagonist GW9662 showed a significant increase of the $\Delta 6\Delta 5$ *fads2* promoter activity. On the contrary, the *ppre* mutant and the control pGL4.10 exhibited no change when treated with GW9662 (Fig. 3). These data further suggested that Ppar γ is a negative regulator targeting the rabbitfish $\Delta 6\Delta 5$ *fads2* promoter.

Ppar γ interacted with the TF binding site in the $\Delta 6\Delta 5$ *fads2* promoter

Electrophoretic Mobility Shift Assay was performed with rabbitfish hepatocyte nucleus proteins (Fig. 4). No gel shift band was observed in lane 1 (no protein). Nevertheless, a gel shift band was observed in lane 2 containing rabbitfish hepatocyte nucleus proteins and 5' biotin labeled probe, while unlabeled competitor probe could compete with the binding reaction (lane 3) and unlabeled mutant competitor probe could not compete in the reaction (lane 4). The results confirmed the interaction between Ppar γ and the $\Delta 6\Delta 5$ *fads2* promoter binding region.

Ppar γ antagonist GW9662 down-regulated the expression of ppar γ and up-regulated that of $\Delta 6\Delta 5$ fads2

Using SCHL cells, the effect of Ppar γ antagonist GW9662 on the expression of ppar γ and $\Delta 6\Delta 5$ fads2 was investigated. The results showed that, compared to controls, the mRNA level of ppar γ was significantly decreased when SCHL cells were treated with GW9662, while that of $\Delta 6\Delta 5$ fads2 was significantly increased (Fig. 5a). These results indicated a negative regulatory role of Ppar γ on gene expression of $\Delta 6\Delta 5$ fads2 in rabbitfish hepatocytes.

siRNA of ppar γ induced the expression of $\Delta 6\Delta 5$ fads2 in SCHL cells

An experiment using RNAi of ppar γ was conducted to confirm the functional relationship between Ppar γ and $\Delta 6\Delta 5$ fads2 at transcription level. The results showed that, compared with the control group, siRNA of ppar γ significantly decreased the ppar γ mRNA level (70.2 ± 6.8 % decrease). Moreover, the levels of $\Delta 6\Delta 5$ fads2 mRNA were significantly increased (155 ± 14.6 %) (Fig. 5b). These results are consistent with the those with the Ppar γ antagonist assay indicating that Ppar γ is a negative regulator of rabbitfish $\Delta 6\Delta 5$ fads2.

ppar γ mRNA overexpression decreased the mRNA expression of $\Delta 6\Delta 5$ fads2 in SCHL cells

The inhibition of Ppar γ (GW9662 treatment and RNAi) was accompanied with an up-regulation of rabbitfish $\Delta 6\Delta 5$ fads2 expression in SCHL cells, and the influence of ppar γ overexpression to the possible target gene expression was identified by mRNA transfection into hepatocyte. Compared to the control group, ppar γ mRNA in SCHL cells overexpressing ppar γ increased between 400 to 500 fold, whereas the $\Delta 6\Delta 5$ fads2 mRNA decreased to 33.1 ± 14.0 % (Fig. 5c). The results clearly show that $\Delta 6\Delta 5$ fads2 transcription is down-regulated under ppar γ overexpression, thus proving its role as a negative regulator to $\Delta 6\Delta 5$ fads2.

Overexpression of ppar γ decreased while siRNA of ppar γ increased the enzymatic activity of $\Delta 6\Delta 5$ Fads in SCHL cells

The impact of Ppar γ on the desaturase activity was determined by analyzing the FA profiles of SCHL cells treated with ppar γ overexpression (Table 2). FA ratios of desaturation

products/substrates such as 18:3n-6/18:2n-6 and 18:4n-3/18:3n-3 was increased with knock-down of *ppary*, but decreased with overexpression of *ppary*, indicating a change of $\Delta 6\Delta 5$ Fad desaturation activity. Besides, the level of total LC-PUFA (Σ LC-PUFA) was significantly decreased in cells treated with overexpressing *ppary*, and the level of FA precursor 18:3n-3 (ALA) was significantly higher, compared with those in control. Overall, the FA results clearly indicated that Ppar γ impacted the LC-PUFA biosynthesis by reducing the desaturase capacity.

Discussion

The present study investigated the role of Ppar γ in the regulation of $\Delta 6\Delta 5$ *fads2*, a gene encoding a fatty acyl desaturase with key roles in the LC-PUFA biosynthesis in rabbitfish. Results from a set of experiments using HEK 293T cells showed that both *ppary* overexpression or a Ppar γ antagonist (GW9662) treatment affected the activity of the $\Delta 6\Delta 5$ *fads2* promoter, and EMSA confirmed that Ppar γ interacted with the promoter, suggesting a role for Ppar γ in $\Delta 6\Delta 5$ *fads2* regulation. Such role of Ppar γ as $\Delta 6\Delta 5$ *fads2* modulator was further confirmed at transcriptional level in SCHL cells using a varied range of methodological approaches including GW9662 treatment, *ppary* targeted siRNA, and *ppary* overexpression. Finally, the effects of Ppar γ on the LC-PUFA biosynthetic pathways were estimated by determining the changes produced in the FA profiles of SCHL cells overexpressing the rabbitfish *ppary*. Overall our data strongly suggested that Ppar γ acted as a negative regulator on $\Delta 6\Delta 5$ *fads2* expression in rabbitfish.

PPAR γ is a key inducer of differentiation, lipogenesis, and insulin sensitivity in white and brown adipocytes and is involved in lipid deposition in many other cell types (Poulsen et al., 2012). The previous study in human skeletal muscle tissue speculated that PPAR γ might be a positive regulator for carnitine palmitoyl transferase-1 (CPT-1), lipoprotein lipase (LPL) and fatty acid binding protein (FABP) (Lapsys et al., 2000). PPAR γ play an important role in modulating lipid accumulation (Auwerx, 1999), and is activated by many FA (Forman et al., 1996). In Atlantic salmon (*Salmo salar*), Ppar was demonstrated to be an important factor in mediating enzymatic response to fibrates (Ruyter et al., 1997). A previous study reported that troglitazone (a PPAR γ agonist) induced an increase in PPAR γ and a decrease in *FADS2* ($\Delta 6$ desaturase) expression in human skeletal muscle cells, speculating that there is a PPRE element in *FADS2* promoter (Wahl et al., 2002). However, another study demonstrated that the PPRE imparted PPAR α responsiveness

to the human *FADS2* promoter, but not PPAR γ (Tang et al., 2003). These data indicated that the effects of PPARs on human *FADS2* expression in skeletal muscle cells may similar to the situation as demonstrated in rabbitfish.

In rabbitfish, Ppar cDNAs were cloned and characterized their tissue distribution, PPAR α was widely expressed in tissues and was particularly abundant in the heart, brain and liver, PPAR β expression is considerably higher in the gills than in the other tissues, PPAR γ was predominantly expressed in the intestine, gills and liver (You et al., 2017), indicating that PPAR α and PPAR γ were potential candidates involved in the regulation of LC-PUFA biosynthesis in rabbitfish. In the present study, a potential Ppar γ binding site (ppre located at +51 bp to TSS) was found on the $\Delta 6\Delta 5$ *fads2* promoter. The $\Delta 6\Delta 5$ *fads2* promoter activity increased significantly after treated with GW9662, which was a potent and selective antagonist of PPAR γ and showed no effect on transcription on PPAR α and PPAR σ (Leesnitzer et al., 2002), and decreased significantly after treated with overexpression of *ppar γ* , while ppre mutant did not response to GW9662 and overexpression of *ppar γ* . Mutation of ppre resulted in significantly decreased transcriptional activity, which suggested that this TF binding site was important for maintaining $\Delta 6\Delta 5$ *fads2* promoter activity. In rat liver, PPAR γ , along with PPAR α , interacted with a PPRE located in the promoter of lipoprotein lipase (*lpl*) (Schoonjans et al., 1996). In rabbitfish primary hepatocytes, *ppara* expression was depressed in response to supplementation of Ppar γ -specific agonist 15-deoxy-D12,14-prostaglandin J2 (15d-J2), in agreement with the depression of rabbitfish $\Delta 6\Delta 5$ *fads2* expression (You et al., 2017). Hence, it was worth considering that whether there was PPAR α binding site on the $\Delta 6\Delta 5$ *fads2* promoter, the mechanism for concomitant activation of rabbitfish PPAR α and $\Delta 6\Delta 5$ Fads should be further investigated.

The studies in SCHL cells further confirmed that $\Delta 6\Delta 5$ *fads2* is regulated by Ppar γ since the expression of $\Delta 6\Delta 5$ *fads2* was regulated by each treatment of Ppar γ . Previous study in European seabass (*Dicentrarchus labrax*) demonstrated the concomitant increase of *ppar* and $\Delta 6$ *fads2* mRNA levels induced by dietary n-3 LC-UFA deficiency, suggested a potential role of Ppar members in the regulation of $\Delta 6$ *fads2* in this species (Vagner et al., 2009). However, when the Atlantic salmon SHK-1 cells were incubated with Ppar agonists (WY14643 and 2-bromopalmitate), there were no significant change in the expression of Ppar encoding genes (Carmona-Antoñanzas et al., 2014). In rabbitfish primary hepatocytes, the PPAR agonist

(Fenofibrate) induced *ppary* expression, and meanwhile suppressed the expression of $\Delta 6\Delta 5$ *fads2*, which suggested a possible regulation of Ppar γ on $\Delta 6\Delta 5$ *fads2* (You et al., 2017). These results suggested that PPAR γ may have different regulatory mechanism in different species. In the present study in SCHL cells, the Ppar γ antagonist GW9662 increased the expression of $\Delta 6\Delta 5$ *fads2*, coinciding with the suppression of Ppar γ antagonists, suggesting the role of $\Delta 6\Delta 5$ *fads2* as the downstream gene of Ppar γ . This was further confirmed by the function assay of Ppar γ on $\Delta 6\Delta 5$ *fads2* expression such as the *ppary* overexpression and knockdown in SCHL cells.

The regulation of $\Delta 6\Delta 5$ *fads2* by Ppar γ will eventually lead to altered FA profiles, particularly those that are desaturation substrates or products. Previously, a study on human skeletal muscle cells treated with troglitazone exhibited changes in unsaturated FA profiles despite the decrease of $\Delta 6$ *fads* mRNA levels (Wahl et al., 2002). In rabbitfish, functional characterization show that $\Delta 6/\Delta 5$ Fads could efficiently convert 18:3n-3 and 18:2n-6 to 18:4n-3 and 18:3n-6, respectively (Li et al., 2010). The ratio of C18:3n-6/C18:2n-6 could be an index of $\Delta 6$ Fads activity (Borkman et al., 1993). In the present study, remarkable changes of the FA profiles were detected in SCHL cells overexpressing and knock-down *ppary* mostly associated with decreased and increased levels of the direct $\Delta 6$ desaturation products such as 18:4n-3 and 18:3n-6, or further downstream products within the LC-PUFA biosynthetic pathways such as EPA and DHA. We showed that RNAi knockdown of *ppary* caused an increase in 18:4n-3/18:3n-3 and overexpression of *ppary* caused a decrease in 18:4n-3/18:3n-3, and 18:3n-6/18:2n-6 in rabbitfish hepatocyte cells, which indicates an increase and a decrease in $\Delta 6\Delta 5$ Fads enzymatic activity. And in *ppary* mRNA overexpression assay, the ratio of 18:4n-3/18:3n-3 was higher than the ratio of 18:3n-6/18:2n-6 in the control group, suggested that rabbitfish $\Delta 6\Delta 5$ Fads tend to convert n-3 PUFA. This result correspond to the previous study that dietary linoleic (18:2n-6) and α -linolenic acids (18:3n-3) may promote the expression of $\Delta 6$ desaturase, the promoting action of α -linolenic acid on $\Delta 6$ desaturase gene expression is stronger than that of linoleic acid in *S. canaliculatus* (Li et al., 2010). Moreover, there were significantly lower contents of EPA, DHA, ARA and higher contents of ALA in *ppary* mRNA overexpression group, whereas the contents of LA, showed no significant difference, when compared to control group, indicating that Ppar γ has a more significant effect on enzymatic activity of $\Delta 6\Delta 5$ Fads involved in n-3 pathway than that in n-6 pathway. As Ppar γ could be activated by fatty acids at physiological concentrations (Gearing et al.,

1994), this could be the underlying molecular mechanism whereby dietary lipids affect LC-PUFA synthesis through Ppar γ .

In summary, the present study demonstrated that Ppar γ negatively influences the biosynthesis of LC-PUFA by targeting $\Delta 6\Delta 5$ *fads* in rabbitfish liver, which was the first report of Ppar γ involved in regulation of LC-PUFA biosynthesis in teleosts, may contribute to the exploration of enhancing LC-PUFA biosynthesis in fish.

Acknowledgements

This work was financially supported by the Major International Joint Research Project from National Natural Science Foundation of China (No. 31110103913) and China Agriculture Research System (CARS-47).

References

- Adeghate, E., Adem, A., Hasan, M.Y., Tekes, K. & Kalasz, H. (2011). Suppl 2: Medicinal Chemistry and Actions of Dual and Pan PPAR Modulators. *Open Medicinal Chemistry Journal*, 5: 93-8.
- Auwerx, J. (1999). PPAR γ , the ultimate thrifty gene. *Diabetologia*, 42: 1033-1049.
- Bell, J., Mcevoy, J., Dr, Mcghee, F., Campbell, P. & Sargent, J. (2001). Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (*Salmo salar*) affects tissue lipid compositions and hepatocyte fatty acid metabolism. *Journal of Nutrition*, 131: 1535-1543.
- Benitez-santana, T., Masuda, R., Juarez, C.E., Ganuza, E., Valencia, A., Hernandezcruz, C.M. & Izquierdo, M.S. (2007). Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream Sparus aurata larvae. *Aquaculture*, 264: 408-417.
- Borkman, M., Storlien, L.H., Pan, D.A., Jenkins, A.B., Chisholm, D.J. & Campbell, L.V. (1993). The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids. *New England Journal of Medicine*, 328: 238-44.
- Carmona-Antoñanzas, G., Tocher, D.R., Martinez-Rubio, L. & Leaver, M.J. (2014). Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals. *Gene*, 534: 1-9.
- Castro, L.F.C., Óscar. Monroig, M.J. Leaver, J. Wilson, I. Cunha, D.R. Tocher (2012). Functional Desaturase Fads1 ($\Delta 5$) and Fads2 ($\Delta 6$) Orthologues Evolved before the Origin of Jawed Vertebrates. *Plos One*, 7: e31950.
- Castro, L.F.C., Tocher, D.R. & Monroig, O. (2016). Long-chain polyunsaturated fatty acid biosynthesis in chordates: Insights into the evolution of Fads and Elovl gene repertoire. *Progress in Lipid Research*, 62: 25.
- Desvergne, B., Michalik, L. & Wahli, W. (2006). Transcriptional Regulation of Metabolism. *Physiological Reviews*, 86: 465-514.
- Dong, Y., Wang, S., Chen, J., Zhang, Q., Liu, Y., You, C., Monroig, O., Tocher, D.R. & Li, Y. (2016). Hepatocyte Nuclear Factor 4 α (HNF4 α) Is a Transcription Factor of Vertebrate Fatty Acyl Desaturase Gene as Identified in Marine Teleost *Siganus canaliculatus*. *PloS one*, 11:

469 e0160361.

470 Dong, Y., Zhao, J., Chen, J., Wang, S., Liu, Y., Zhang, Q., You, C., Oacute, Monroig, S., Tocher, D.R.
 471 & Li, Y. (2018). Cloning and characterization of $\Delta 6/\Delta 5$ fatty acyl desaturase (Fad) gene
 472 promoter in the marine teleost *Siganus canaliculatus*. *Gene*, 647: 174–180.

473 Forman, B.M., Chen, J. & Evans, R.M. (1996). The peroxisome proliferator-activated receptors:
 474 ligands and activators. *Annals of the New York Academy of Sciences*, 804: 266.

475 Galli, C., Simopoulos, A.P. & Tremoli, E. (1994). Effects of fatty acids and lipids in health and disease.
 476 *World Revnutrdiet*, 76.

477 Gearing, K.L., Göttlicher, M., Widmark, E., Banner, C.D., Tollet, P., Strömstedt, M., Rafter, J.J., Berge,
 478 R.K. & Gustafsson, J.A. (1994). Fatty acid activation of the peroxisome proliferator activated
 479 receptor, a member of the nuclear receptor gene superfamily. *J Nutr*; 124: 1284S-1288S.

480 Geay, F., Zamboninoinfante, J., Reinhardt, R., Kuhl, H., Santigosa, E., Cahu, C. & Mazurais, D. (2012).
 481 Characteristics of fads2 gene expression and putative promoter in European sea bass
 482 (*Dicentrarchus labrax*): comparison with salmonid species and analysis of CpG methylation.
 483 *Marine Genomics*, 5: 7-13.

484 Gurnell, M. (2005). Peroxisome proliferator-activated receptor γ and the regulation of adipocyte
 485 function: lessons from human genetic studies. *Best Practice & Research Clinical*
 486 *Endocrinology & Metabolism*, 19: 501.

487 Lapsys, N.M., Kriketos, A.D., Limfraser, M., Poynten, A.M., Lowy, A., Furler, S.M., Chisholm, D.J. &
 488 Cooney, G.J. (2000). Expression of genes involved in lipid metabolism correlate with
 489 peroxisome proliferator-activated receptor gamma expression in human skeletal muscle. *J*
 490 *Clin Endocr Metab*, 85: 4293-7.

491 Leaver, M.J., Villeneuve, L.A., Obach, A., Jensen, L., Bron, J.E., Tocher, D.R. & Taggart, J.B. (2008).
 492 Functional genomics reveals increases in cholesterol biosynthetic genes and highly
 493 unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in
 494 Atlantic salmon (*Salmo salar*). *Bmc Genomics*, 9: 299.

495 Leesnitzer, L.M., Parks, D.J., Bledsoe, R.K., Cobb, J.E., Collins, J.L., Consler, T.G., Davis, R.G.,
 496 Hull-Ryde, E.A., Lenhard, J.M. & Patel, L. (2002). Functional consequences of cysteine
 497 modification in the ligand binding sites of peroxisome proliferator activated receptors by
 498 GW9662. *Biochemistry*, 41: 6640.

499 Li, Y., Hu, C., Zheng, Y., Xia, X., Xu, W., Wang, S., Chen, W., Sun, Z. & Huang, J. (2008). The effects
 500 of dietary fatty acids on liver fatty acid composition and Delta(6)-desaturase expression differ
 501 with ambient salinities in *Siganus canaliculatus*. *Comparative Biochemistry & Physiology*
 502 *Part B Biochemistry & Molecular Biology*, 151: 183-190.

503 Li, Y., Monroig, O., Zhang, L., Wang, S., Zheng, X., Dick, J.R., You, C. & Tocher, D.R. (2010).
 504 Vertebrate fatty acyl desaturase with $\Delta 4$ activity. *Proceedings of the National Academy of*
 505 *Sciences of the United States of America*, 107: 16840-16845.

506 Livak, K.J. & Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time
 507 quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 25: 402-408.

508 Lorente-Cebrián, S., Costa, A.G., Navas-Carretero, S., Zabala, M., Martínez, J.A. & Moreno-Aliaga,
 509 M.J. (2013). Role of omega-3 fatty acids in obesity, metabolic syndrome, and cardiovascular
 510 diseases: a review of the evidence. *Journal of Physiology & Biochemistry*, 69: 633-51.

511 Matsuzaka, T., Shimano, H., Yahagi, N., Amemiyakudo, M., Yoshikawa, T., Hastay, A.H., Tamura, Y.,
 512 Osuga, J., Okazaki, H. & Iizuka, Y. (2002). Dual regulation of mouse Delta(5)- and

513 Delta(6)-desaturase gene expression by SREBP-1 and PPARalpha. *Journal of Lipid Research*,
514 43: 107-14.

515 Monroig, , Wang, S., Zhang, L., You, C., Tocher, D.R. & Li, Y. (2012). Elongation of long-chain fatty
516 acids in rabbitfish *Siganus canaliculatus* : Cloning, functional characterisation and tissue
517 distribution of Elovl5- and Elovl4-like elongases. *Aquaculture*, 350-353: 63-70.

518 Poulsen, L.L.C., Siersbæk, M. & Mandrup, S. (2012). PPARs: Fatty acid sensors controlling
519 metabolism. *Seminars in Cell & Developmental Biology*, 23: 631.

520 Ruyter, B., Andersen, O., Dehli, A., Ostlund Farrants, A.K., Gjøen, T. & Thomassen, M.S. (1997).
521 Peroxisome proliferator activated receptors in Atlantic salmon (*Salmo salar*): effects on PPAR
522 transcription and acyl-CoA oxidase activity in hepatocytes by peroxisome proliferators and
523 fatty acids. *Biochimica et biophysica acta*, 1348: 331-338.

524 Sargent, J.R., Tocher, D.R. & Bell, J.G. (2002). The Lipids. *Elsevier*, 3: 181-257.

525 Schmitz, G. & Ecker, J. (2008). The opposing effects of n-3 and n-6 fatty acids. *Progress in lipid*
526 *research*, 47: 147.

527 Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A.M., Heyman, R.A., Briggs, M., Deeb, S., Staels, B.
528 & Auwerx, J. (1996). PPARα and PPARγ activators direct a distinct tissue-specific
529 transcriptional response via a PPRE in the lipoprotein lipase gene. *Embo Journal*, 15:
530 5336-5348.

531 Tang, C., Cho, H.P., Nakamura, M.T. & Clarke, S.D. (2003). Regulation of human delta-6 desaturase
532 gene transcription: identification of a functional direct repeat-1 element. *Journal of Lipid*
533 *Research*, 44: 686-95.

534 Tocher, D.R. (2003). Metabolism and Functions of Lipids and Fatty Acids in Teleost Fish. *Reviews in*
535 *Fisheries Science*, 11: 107-184.

536 Tocher, D.R. (2015). Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective.
537 *Aquaculture*, 449: 94-107.

538 Tontonoz, P. & Spiegelman, B.M. (2008). Fat and Beyond: The Diverse Biology of PPARγ. *Annual*
539 *Review of Biochemistry*, 77: 289-312.

540 Uauy, R., Hoffman, D.R., Peirano, P., Birch, D.G. & Birch, E.E. (2001). Essential fatty acids in visual
541 and brain development. *Lipids*, 36: 885-895.

542 Vagner, M., Robin, J.H., Zamboninoinfante, J.L., Tocher, D.R. & Personle, R.J. (2009). Ontogenic
543 effects of early feeding of sea bass (*Dicentrarchus labrax*) larvae with a range of dietary n-3
544 highly unsaturated fatty acid levels on the functioning of polyunsaturated fatty acid
545 desaturation pathways. *Brit J Nutr*, 101: 1452.

546 Wahl, H.G., Kausch, C., Machicao, F., Rett, K., Stumvoll, M. & Häring, H.U. (2002). Troglitazone
547 Downregulates Δ-6 Desaturase Gene Expression in Human Skeletal Muscle Cell Cultures.
548 *Diabetes*, 51: 1060-1065.

549 Wijngaarden, L.V. (1967). On the growth of small cavitation bubbles by convective diffusion.
550 *International Journal of Heat & Mass Transfer*, 10: 127-134.

551 Xu, H., Dong, X., Ai, Q., Mai, K., Xu, W., Zhang, Y. & Zuo, R. (2014). Regulation of tissue LC-PUFA
552 contents, Δ6 fatty acyl desaturase (FADS2) gene expression and the methylation of the
553 putative FADS2 gene promoter by different dietary fatty acid profiles in Japanese seabass
554 (*Lateolabrax japonicus*). *Plos One*, 9: e87726.

555 Y. Liu, Q. Zhang, Y. Dong, S. Wang, C. You, Y. Li, Y. Li. (2017). Establishment of a hepatocyte line for
556 studying biosynthesis of long-chain polyunsaturated fatty acids from a marine teleost, the

- 557 white-spotted spinefoot *Siganus canaliculatus*. *Journal of fish biology*.
- 558 You, C., Jiang, D., Zhang, Q., Xie, D., Wang, S., Dong, Y. & Li, Y. (2017). Cloning and expression
559 characterization of peroxisome proliferator-activated receptors (PPARs) with their agonists,
560 dietary lipids, and ambient salinity in rabbitfish *Siganus canaliculatus*. *Comparative*
561 *Biochemistry & Physiology Part B Biochemistry & Molecular Biology*, 206: 54-64.
- 562 Zhang, Q., Xie, D., Wang, S., You, C., Monroig, O., Tocher, D.R. & Li, Y. (2014). miR-17 is involved
563 in the regulation of LC-PUFA biosynthesis in vertebrates: Effects on liver expression of a fatty
564 acyl desaturase in the marine teleost *Siganus canaliculatus*. *Biochim Biophys Acta*, 1841:
565 934-943.
- 566 Zhang, Q., You, C., Liu, F., Zhu, W., Wang, S., Xie, D., Monroig, O., Tocher, D.R. & Li, Y. (2016a).
567 Cloning and Characterization of Lxr and Srebp1, and Their Potential Roles in Regulation of
568 LC-PUFA Biosynthesis in Rabbitfish *Siganus canaliculatus*. *Lipids*, 51: 1-13.
- 569 Zhang, Q., You, C., Wang, S., Dong, Y., Monroig, O., Tocher, D.R. & Li, Y. (2016b). The miR-33 gene
570 is identified in a marine teleost: a potential role in regulation of LC-PUFA biosynthesis in
571 *Siganus canaliculatus*. *Scientific reports*, 6: 32909.
- 572 Zheng, X., Leaver, M.J. & Tocher, D.R. (2009). Long-chain polyunsaturated fatty acid synthesis in fish:
573 Comparative analysis of Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.)
574 Delta6 fatty acyl desaturase gene promoters. *Comparative Biochemistry & Physiology Part B*
575 *Biochemistry & Molecular Biology*, 154: 255.

578 **Table 1.**

579 Primers used for gene clone, EMSA, qPCR or vector construction.

Aim	Gene/vector name	Primers/oligo nucleotides	Nucleotide sequence
Vector reconstruction	pcDNA3.1- <i>pparγ</i>	<i>pparγ</i> -F	5'-CCCGAATTCATGTGTCCTCCCTGTCGCC-3'
		<i>pparγ</i> -R	5'-CCCTCTAGATCCCACTTGTTCCCTCCTTGC-3'
<i>pparγ</i> mRNA construction	<i>pparγ</i>	T7 promoter primer	5'-TAATACGACTCACTATAGGG-3'
		<i>pparγ</i> -R	5'-CCCTCTAGATCCCACTTGTTCCCTCCTTGC-3'
EMSA probes	<i>pparγ</i>	BPF (5'-biotin labeled)	5'-GGAGCACGGTCAACGTGACCATAGGAA-3'
		BPR (5'-biotin labeled)	5'-TTCCTATGGTCACGTTGACCGTGCTCC-3'
		UPF	5'-GGAGCACGGTCAACGTGACCATAGGAA-3'
		UPR	5'-TTCCTATGGTCACGTTGACCGTGCTCC-3'
		UTPF	5'-GGAGCACTTTCGACGGGAAAATAGGAA-3'
		UTPR	5'-TTCCTATTTTCCCGTCGAAAGTGCTCC-3'
qPCR	<i>pparγ</i>	qPCR- <i>pparγ</i> -F	5'-CTGCTGGCTGAGTTCTCGTCT-3'
		qPCR- <i>pparγ</i> -R	5'-ATGACAAAAGGCGCGTTATCTC-3'
	$\Delta 6\Delta 5$ <i>fads2</i>	qPCR- $\Delta 6\Delta 5$ <i>fads2</i> -F	5'-TCACTGGAACCTGCCCACAT-3'
		qPCR- $\Delta 6\Delta 5$ <i>fads2</i> -R	5'-TTCATTCTCAGACAGTGCAAACAG-3'
	18S <i>rRNA</i>	qPCR-18S-F	5'-CGCCGAGAAGACGATCAAAC-3'
		qPCR-18S-R	5'-TGATCCTTCCGCAGGTTAC-3'

580

581

Table 2.

Fatty acid composition (% total fatty acids) of *Siganus canaliculatus* hepatocyte line (SCHL) cells transfected with *ppary* siRNA or *ppary* mRNA.

Fatty acid	Knock-down of <i>ppary</i>		Overexpression of <i>ppary</i>	
	Negative control	<i>ppary</i> siRNA	Control	<i>ppary</i> mRNA
14:0	0.43±0.06	0.50±0.02	1.79±0.15	1.86±0.66
16:0	16.81±0.77	15.17±0.47	16.43±0.07	18.83±0.83
18:0	13.65±0.51	13.57±0.93	18.38±0.61	23.22±0.59
22:0	1.55±0.12	1.54±0.16	1.94±0.23	1.44±0.24
16:1n-7	4.54±0.19	5.08±0.55	2.42±0.13	2.67±0.23
18:1n-9	31.87±0.73	31.38±2.12	23.93±0.55	23.37±1.77
20:1n-9	0.72±0.04	0.71±0.04	0.95±0.19	0.51±0.09
24:1	0.32±0.13	0.33±0.02	1.45±0.09	1.29±0.08
18:2n-6(LA)	3.77±0.11	3.78±0.17	2.65±0.07	2.32±0.44
20:2n-6	0.90±0.08	0.82±0.08	0.38±0.03	0.90±0.34
18:3n-6	0.59±0.17 ^b	1.53±0.35 ^a	1.09±0.17 ^a	0.49±0.15 ^b
18:3n-3(ALA)	0.62±0.21	0.65±0.04	0.72±0.21 ^b	2.81±0.99 ^a
18:4n-3	0.32±0.05 ^b	0.88±0.18 ^a	0.51±0.07 ^a	0.29±0.06 ^b
20:4n-6(ARA)	4.84±0.51	4.73±0.73	7.50±0.16 ^a	5.98±0.34 ^b
20:5n-3(EPA)	3.17±0.10	3.22±0.25	3.50±0.09 ^a	2.87±0.21 ^b
22:6n-3(DHA)	16.09±1.33	16.12±1.09	16.35±0.15 ^a	11.09±1.80 ^b
ΣSFA	32.45±0.15	30.78±1.36	38.53±0.31	45.35±1.63
ΣMUFA	37.45±0.77	37.49±2.51	28.76±0.44	27.83±2.00
ΣPUFA	30.28±0.69	31.73±1.17	32.71±0.44	26.76±2.45
ΣLC-PUFA	24.09±0.79	24.07±1.64	27.35±0.40 ^a	19.94±1.27 ^b
18:3n-6/18:2n-6	0.16±0.04 ^b	0.41±0.10 ^a	0.41±0.07 ^a	0.21±0.02 ^b
18:4n-3/18:3n-3	0.54±0.16 ^b	1.36±0.31 ^a	0.75±0.26 ^a	0.12±0.06 ^b

Notes: Values are means ± SEM from three treatments ($n = 3$). In the treatment of Knock-down of *ppary* or overexpression of *ppary*, different superscripts in the same rows indicate significant difference at $P < 0.05$ by Student's *t*-test. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, fatty acids with 2 or more double bonds; LC-PUFA, sum of ARA, EPA and DHA

Figure Legends

Fig. 1. The nucleotide sequence and predicted binding sites for Ppar γ in the core region of the rabbitfish $\Delta 6\Delta 5$ *fads2* promoter. Numbers are given relative to the first base of the transcription start site (TSS). Potential transcription binding motif for Ppar γ is marked in grey (Dong et al., 2018).

Fig. 2. Effects of *Siganus canaliculatus* ppar γ overexpression on activity of $\Delta 6\Delta 5$ *fads2* promoter. HEK 293T cells were co-transfected with the $\Delta 6\Delta 5$ *fads2* promoter vector and the overexpression vector pcDNA3.1-ppar γ or the empty vector pcDNA3.1 (control). The negative control (pGL4.10) was an empty vector with no promoter sequence upstream in the reporter gene. $\Delta 6\Delta 5$ *fads2*-mutant was $\Delta 6\Delta 5$ *fads2* - Ppar γ site-directed mutant. Y-axis is the Firefly/Renilla luciferase ratio, while x-axis stands for different reporter vector. Data are means \pm SEM ($n = 3$) and asterisks represent significant differences (Student's *t*-test; $P < 0.05$).

Fig. 3. Effects of the Ppar γ antagonist GW9662 on activity of the $\Delta 6\Delta 5$ *fads2* promoter. HEK 293T cells transfected with the $\Delta 6\Delta 5$ *fads2* promoter were treated with or without (control) GW9662. The negative control (pGL4.10) was an empty vector with no promoter sequence upstream in the reporter gene. $\Delta 6\Delta 5$ *fads2*-mutant was $\Delta 6\Delta 5$ *fads2*-Ppar γ site-directed mutant. Y-axis is the Firefly/Renilla luciferase ratio, while x-axis stands for different reporter vector. Data are shown as means \pm SEM ($n = 3$) and asterisks represent significant differences (Student's *t*-test; $P < 0.05$).

Fig.4. Electrophoretic mobility shift assay (EMSA) of TF binding site in the $\Delta 6\Delta 5$ *fads2* promoter with rabbitfish hepatocytes nucleus proteins. Lane 1, negative control; Lane 2, nucleus proteins reactions; Lane 3, unlabeled probe competing reactions; Lane 4, unlabeled mutant probe competing reactions. BP, biotin labeled probe; UP, unlabeled probe; UTP, unlabeled mutant probe.

Fig.5. Q-PCR analyses of ppar γ and $\Delta 6\Delta 5$ *fads2* expression in *Siganus canaliculatus* hepatocyte line (SCHL) cells, (a) treated with the Ppar γ antagonist GW9662, (b) transfected with ppar γ

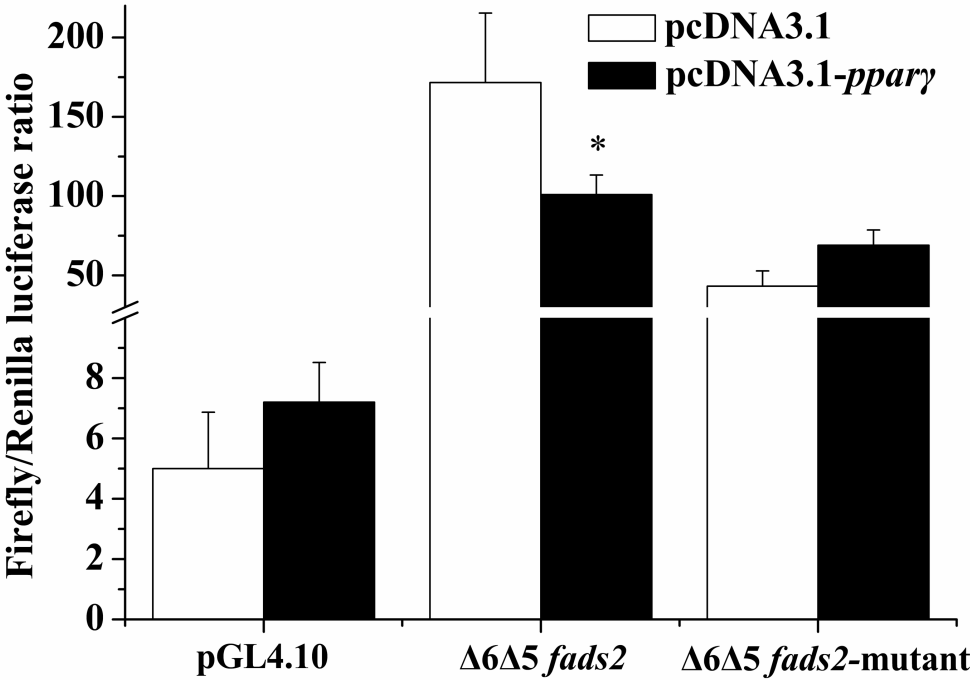
siRNA, (c) transfected with *ppary* mRNA. Relative expression of the target genes in SCHL cells was quantified for each transcript and was normalized with the expression of *18S rRNA* by $2^{-\Delta\Delta C_t}$ method. Results are means \pm SEM ($n=3$) and asterisks indicate significant differences of gene expression between the control and the GW9662 treatment (Student's *t*-test; $P < 0.05$).

Figures

Fig. 1.

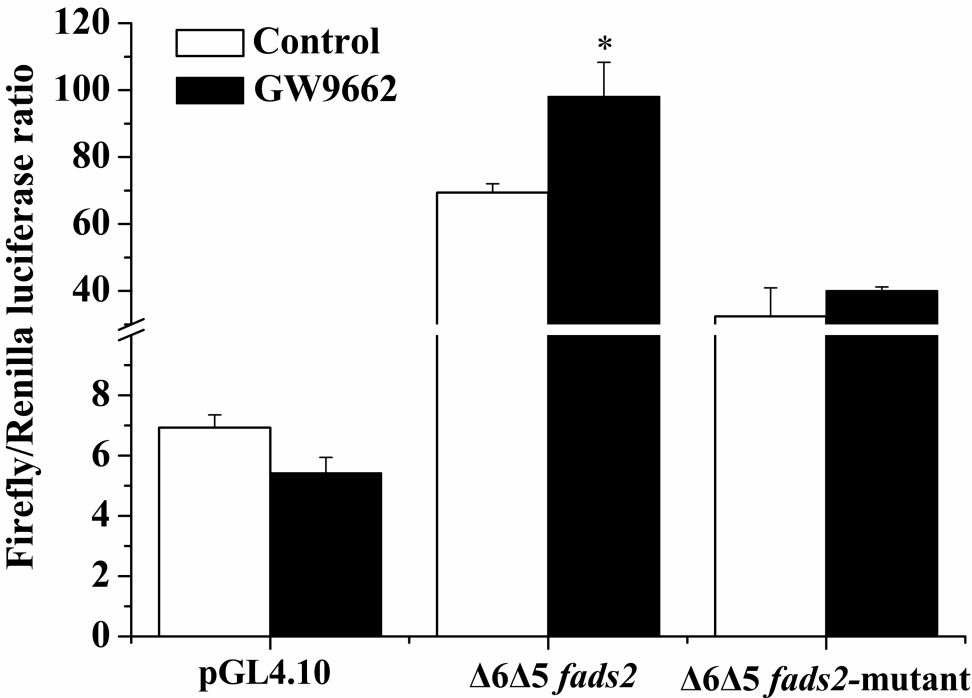
-18 TATCAGTGGGTGAATCCC ^{TSS+1} TGAACCTATTTGAGGAGGATGAGGATGTGAGGAGGTGAACT +41
Pparγ
+42 CGAATGTGGACGG AGCACGGTCAACGTGACCATAGG AAAAGCAGACAACGTTTGCAAAT +100

631 Fig. 2.



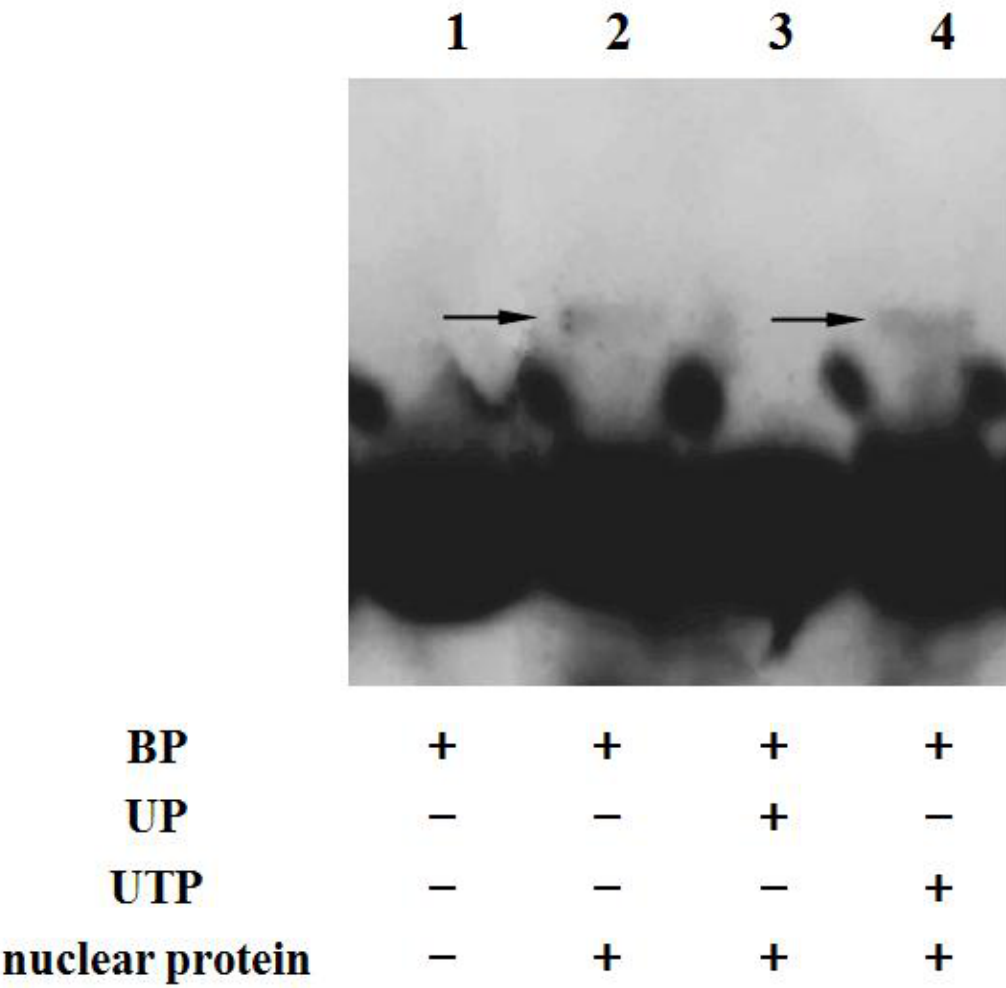
632

633



635

636



638

639

640 Fig. 5.

