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PPARγ regulates *fabp4* expression to increase DHA content in golden pompano (*Trachinotus ovatus*) hepatocytes

Caixia Lei[1,2†], Bin Fan[1,3†], Jingjing Tian[2], Mengmeng Li[1,4] and Yuanyou Li[1*]

[1]*College of Marine Science, South China Agricultural University & Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou 510642, People's Republic of China*
[2]*Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510380, People's Republic of China*
[3]*Yangjiang Polytechnic & Yangjiang Haina Aquatic Co., Ltd, Yangjiang 529500, People's Republic of China*
[4]*College of Agronomy, Liaocheng University, Liaocheng 252000, People's Republic of China*

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Abstract

N-3 long-chain (≥C20) PUFA (LC-PUFA) are vital fatty acids for fish and humans. As a main source of *n*-3 LC-PUFA for human consumers, the *n*-3 LC-PUFA content of farmed fish is important. Previously, we identified fatty acid-binding protein *(fabp)-4* as a candidate gene for regulating the *n*-3 LC-PUFA content. Herein, we further assessed the role of *fabp4* in this process. First, a 2059 bp promoter sequence of *fabp4* in *Trachinotus ovatus* was cloned and, using progressive deletion, determined −2006 bp to −1521 bp to be the core promoter sequence. The *PPAR-γ* binding sites were predicted to occur in this region. A luciferase reporter assay showed that the promoter activity of *fabp4* decreased following mutation of the *PPARγ* binding site and that *PPARγ* increased the *fabp4* promoter activity in a dose-dependent manner, implying that *T. ovatus fabp4* is a target of *PPARγ*. The overexpression of *fabp4 or PPARγ* increased the DHA content in hepatocytes, whereas suppression of their expression diminished this effect, suggesting that both *fabp4* and *PPARγ* play an active role in regulating DHA content. Moreover, the inhibition of *fabp4* attenuated the increase in *PPARγ*-mediated DHA content, and the overexpression of *fabp4* alleviated this effect. Collectively, our findings indicated that *fabp4*, which is controlled by *PPARγ*, plays an important role in DHA content regulation. The new regulation axis can be considered a promising novel target for increasing the *n*-3 LC-PUFA content in *T. ovatus*.

Keywords: **fatty acid-binding protein 4: *n*-3 long-chain PUFA: Uptake and deposition: *Trachinotus ovatus***

N-3 long-chain (≥C20) PUFA (LC-PUFA) are fatty acids that contain more than two double bonds, with the first located three carbons from the methyl end group[1]. The most common LC-PUFA are EPA (20 : 5*n*-3, EPA) and DHA (22 : 6*n*-3, DHA). The *n*-3 LC-PUFA reportedly play numerous positive roles in a range of human pathologies, including obesity, CVD and cancer, and have beneficial effects on nervous system development[2]. Dietary intake of *n*-3 LC-PUFA is necessary for humans to satisfy certain physiological demands due to a limited ability to convert *α*-linolenic acid to *n*-3 LC-PUFA[3]. Fish, especially marine fish, serve as a main source of *n*-3 LC-PUFA for humans[4]. The *n*-3 LC-PUFA are also essential fatty acids for fish (particularly marine fish)[5], and presently, fishmeal and fish oil are almost indispensable as components of farmed marine fish feeds to compensate for the deficiency in *n*-3 LC-PUFA synthesis by the fish themselves[6]. The reliance on finite marine resources derived from capture fisheries is unsustainable; therefore, the development of methods to improve the *n*-3 LC-PUFA content in farmed fish is urgently required for both aquaculture and human consumers.

An effective way to increase the *n*-3 LC-PUFA content in fish is to enhance the uptake and deposition of dietary *n*-3 LC-PUFA. In fish, the processes of uptake and deposition of dietary fatty acids resemble those in mammals, that is mainly including the emulsification of fat and catabolism of emulsified fat[7]. Among them, fatty acid-binding proteins (FABP), which are involved in the intracellular transport of fatty acids, have been reported to be directly related to the fatty acids content. In human (*Homo sapiens*) Caco-2 cells, the knockdown of the *fabp1* gene resulted in a decrease in the uptake and accumulation of oleic acid[8]. Analogously, the gene expression of *fabp5* affects the DHA and EPA levels in rat (*Rattus norvegicus*) PC12 cells and *H. sapiens* hCMEC/D3[9–11]. *In vivo*, experimental mice (*Mus musculus*) showed decreased linoleic acid uptake and accumulation after the *fabp3* gene was knockout compared with the

Abbreviations: fabp, fatty acid-binding protein; LC-PUFA, long-chain PUFA.

* **Corresponding author:** Yuanyou Li, email yyli16@scau.edu.cn

† These authors contributed equally to this work.

wild-type *M. musculus*[12]. In fish, the gene expression of *fabps* has been shown to be associated with dietary fatty acids. For example, the *fabp3* mRNA level in the myosepta of Atlantic salmon (*Salmo salar*) fed vegetable oil was significantly lower compared with those fed fish oil[13]. In addition, the hepatic *fabp1* gene expression was considerably increased in Japanese seabass (*Lateolabrax japonicus*) fed arachidonic acid[14]. These studies suggest the potential feasibility of improving the *n*-3 LC-PUFA content of farmed fish by regulating the gene expression of *fabps*.

Arguably, the uptake and deposition of *n*-3 LC-PUFA in the muscle, that is the edible portion, are the focus in aquaculture nutritional studies, with the potential to improve the quality of meat. The liver, another important tissue is organisms, is widely involved in fatty acid synthesis[15] and is, therefore, rarely associated with fatty acid deposition. Indeed, studies on grass carp (*Ctenopharyngodon idella*)[16] and gibel carp (*Carassius auratus gibelio*)[17] have suggested that the liver has certain abilities to deposit fatty acid (mainly *n*-3 LC-PUFA) without having negative effects on growth performance and antioxidant markers. Besides, being the 'transit hub' of substance metabolism[1], the fatty acid levels of the liver and the whole body are inseparable. Therefore, liver *n*-3 LC-PUFA uptake and deposition are, arguably, very noteworthy, despite the paucity of studies of these processes.

Golden pompano (*Trachinotus ovatus*) is an important concerned marine fish by the Ministry of Agriculture of China for mariculture with a considerable market foreground. *T. ovatus* have been demonstrated to have little or no ability to biosynthesise *n*-3 LC-PUFA in our recent study[18]. Previously, we found that *T. ovatus* hepatic *n*-3 LC-PUFA content increased with increasing dietary *n*-3 LC-PUFA content within a certain range. Meanwhile, transcriptome analysis of the liver indicated that the *fabp4* gene expression was positively correlated with the hepatic *n*-3 LC-PUFA content; therefore, *fabp4* may be a candidate gene for regulating the *n*-3 LC-PUFA content[19]. Based on these findings, we aimed to validate and characterise the potential regulatory role and mechanism of *T. ovatus fabp4* on the *n*-3 LC-PUFA content. Our findings provide reference data for the reduction of fish oil and fishmeal supplement levels in marine fish feed.

Materials and methods

Animal study ethics

All procedures performed on fish were in accordance 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 Institutional Animal Care and Use Committee of South China Agricultural University (Guangdong, China).

Trachinotus ovatus *genomic DNA extraction and cloning of the 5′ flanking sequence of fatty acid-binding protein 4*

A live *T. ovatus* with unknown sex weighing approximately 500 g was randomly purchased from a local commercial market for the experiment. The fish was raised without food in a 320-litre aquarium for 4 h under natural conditions (dissolved oxygen was provided). Before sampling, the anaesthetic (2-phenoxyethanol; Sigma-Aldrich) was mixed with water in a 50-ml volumetric flask and then further diluted with water to a concentration of 0·01 % (w/v) in a bucket (17 litres) for the anaesthesia of the test fish. When the fish showed no response to external touch or stimuli and total loss of equilibrium, it was immediately removed from the bucket and the liver tissue was harvested. Genomic DNA was extracted from the liver tissue using a commercial kit (Beyotime Biotechnology Co. Ltd) according to the manufacturer's recommendation. The obtained DNA sample was electrophoresed on a 1 % (w/v) agarose gels to check its integrity, and the DNA was quantified using a spectrophotometer (NanoDrop 1000; Thermo Scientific). The *fabp4* gene sequence was referenced from our nonpublished whole-genome sequencing data of *T. ovatus*. Specific PCR primers (online Supplementary Table S1) were then designed to amplify the *fabp4* gene 5′ flanking sequence.

The PCR amplification mixture consisted of DNA, upstream primer, downstream primer, 2 × Taq PCR Mix (TianGen Biotech) and sterilised double-distilled water at 2 μl (50 ng), 2 μl, 2 μl, 25 μl and 19 μl, respectively. The amplification procedure comprised three steps: initial denaturation at 94°C for 3 min; 35 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 1 min; and final extension at 72°C for 5 min. The PCR products were then electrophoresed in a 1 % (w/v) agarose gel, and the bands of expected sizes were purified using a commercial kit (TianGen Biotech). The obtained fragments were then cloned into a pMD^{TM}19-T (TaKaRa) vector following the manufacturer's instructions. The ligation mixtures were transformed into *Escherichia coli* DH5*α* competent cells (TianGen Biotech). A plasmid preparation kit (TianGen Biotech) was used to purify the plasmids containing inserts of an expected size. The obtained fragment was then sequenced at Sangon Biotech.

Cell culture

The HEK 293T cell line was purchased from the Chinese Type Culture Collection (Shanghai, China). The medium for cell growth composed of Dulbecco's modified Eagle's medium/nutrient mixture F12 (Gibco), 10 % fetal bovine serum (Gibco), penicillin (100 U/ml; Beyotime Biotechnology Co. Ltd) and streptomycin (100 U/ml; Beyotime Biotechnology Co. Ltd). The cells were incubated at 37°C with 5 % CO_2, and the medium was changed every 2–3 d.

The *Epinephelus coioides* hepatocyte line was obtained from our laboratory, where it was successfully established without publication. The *Epinephelus coioides* hepatocyte line growth medium shared the same composition as that of HEK 293T cells. Differently, the fetal bovine serum used in *Epinephelus coioides* hepatocyte line culture was obtained from Clark (Clark Bioscience Co. Ltd). The cells were maintained at 28°C without CO_2.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from the cells using RNAiso Plus (TaKaRa) following the manufacturer's recommendation. Prior to this, the cells were collected using 0·25 % trypsin and centrifuged at 1500 rpm for 3 min to form pellets in 1·5 ml centrifuge tubes. The integrity of RNA samples was determined using 1 % (w/v) agarose gel electrophoresis, and the quantification of

RNA samples was detected using a spectrophotometer (NanoDrop 1000; Thermo Scientific). Prior to quantitative real-time PCR detection, 1 μg of total RNA was reverse-transcribed using a PrimeScript® RT reagent kit (TaKaRa). The gene transcript level was measured in triplicate (CFX 96 Real-Time PCR Detection System; Bio-Rad), and the amplification mixture contained 0·8 μl of each primer (10 μM) (online Supplementary Table S1), 1 μl (25 ng) of first-strand cDNA product, 10 μl of 2 × SYBR® Premix Ex TaqTMII (TaKaRa) and 7·4 μl sterilised double-distilled water. The amplification conditions began with 30 s at 95°C, followed by 40 cycles of 5 s at 95°C, 45 s at 62°C and 1 min at 72°C. The melting curve was used as a criterion to determine that a single product was amplified. The relative gene expression was normalised with that of the reference gene (*β-actin*). The comparative CT method ($2^{-\Delta\Delta Ct}$)[20] was introduced to calculate the results that expressed relative to the control group. In the quantitative real-time PCR of *fabp4* and *β-actin*, the PCR product sequences shared a close identity with that of *E. coioides*. Consequently, the basic expression levels of *fabp4 and β-actin* were detected. In contrast, the sequence of the *PPAR-γ* quantitative real-time PCR product shared a low identity with that of *E. coioides* and the basic expression level of *PPARγ* was not detected. For calculation purposes, we artificially set the expression level of the control group to 1.

Fatty acid composition analysis

The cellular lipids were extracted, and the fatty acid profile was assessed according to our previous methods[21,22]. The cells were digested and collected using 0·25 % trypsin after washing twice with PBS (pH = 7·3 (SD 0·1), Beyotime Biotechnology Co. Ltd). The cells were then mixed with chloroform/methanol (2:1, by vol) containing 0·01 % butylated hydroxytoluene with gentle shaking to extract the total lipids. Distilled water was added, then the mixture was centrifuged at 3000 rpm for 5 min and the supernatant was discarded. Subsequently, potassium hydroxide methanol (0·5 M) was used to saponify samples at 65°C, and hexane was then used to dissolve the samples. Finally, the fatty acid methyl esters in the upper layer were separated using centrifugation and analysed using GC (Agilent 7890B; Agilent Technologies). Individual methyl esters were identified by comparison with authentic standards (Sigma-Aldrich). The results were expressed as the percentages of each fatty acid in the total fatty acids.

Plasmid constructions and transient transfection

The complete open reading frame of *T. ovatus fabp4* was successfully cloned in our previous study[23] and amplified using 2 × Taq PCR Mix (TianGen Biotech) as described above. The pcDNA3.1 (+) vector (Invitrogen) was used to construct the recombinant plasmid which carries the *fabp4* gene (pcDNA3.1-fabp4). Another overexpression recombinant plasmid, pcDNA3.1-PPARγ was provided by Dr Meng Li of our research team. The accuracy of recombinant plasmid was verified using sequencing (Sangon Biotech Co. Ltd). The primers used are shown in online Supplementary Table S1.

A commercial endofree maxi plasmid kit (TianGen Biotech) was used to purify the pcDNA3.1-fabp4 and pcDNA3.1-PPARγ recombinant plasmids, and the X-tremeGene HP DNA transfection reagent (Roche) was used for cell transfection after the cells reached 70 % confluence. The culture medium was replaced with fresh medium without antibiotics prior to cell transfection. The ratio of X-tremeGene HP DNA transfection reagent (μl):DNA (μg) was 2:1.

According to the sequence from the *fabp4* gene 5′ flanking sequence cloning, corresponding primers (online Supplementary Table S1) were designed to obtain the accurate sequence of the full-length promoter fragment (D1) and another five progressive deletion fragments (D2–D6). These six fragments were amplified using 2 × Taq PCR Mix (TianGen Biotech) with genomic DNA as the template with PCR amplification conditions as described above. pGL4.10 (Promega) and pGL4.75 (Promega) were used as carrier plasmid and reference plasmid, respectively. Details regarding the construction of the recombinant plasmid were mentioned in our previous studies[24,25]. The commercial endofree maxi plasmid kit (TianGen Biotech) was used to extract the recombinant plasmids for further transfection. Previously, these recombinant plasmids were also sequenced (Sangon Biotech Co. Ltd) to ensure accuracy.

Site-directed mutagenesis of the potential regulator of fatty acid-binding protein 4

To further investigate the potential regulator of *fabp4*, site-directed mutagenesis was conducted based on overlap extension PCR. The site-directed mutant was designed from the deletion mutant D6 (pGL4.10-D6), which targets the transcription factor *PPARγ*. Two pairs of primers (F1 and R1, F2 and R2) were designed (online Supplementary Table S1). Primers F1 and R1 occur at either ends of the sequence, whereas F2 and R2 are positioned internally. The mutation points were introduced in the F2 and R2 primer pair. First, pGL 4.10-D6 was used as the template for PCR amplification in accordance with the patterns of 'F1 + R2' and 'F2 + R1' in a 50 μl amplification volume, containing 2 μl of each primer (10 μM), 50 ng (2 μl) of template, 25 μl of 2 × Pfu PCR Mix (TianGen Biotech) and 19 μl sterilised double-distilled water. After purification with a commercial kit (TianGen Biotech), the products were mixed at equal volumes. Subsequently, the mixed product was used as the template, and F1 and R1 were used as primers for amplification in a 50 μl volume, containing 2 μl of each primer (10 μM), 2 μl (50 ng) of template, 25 μl of 2 × Taq PCR Mix (TianGen Biotech) and 19 μl of sterilised double-distilled water. The Mastercycler® nexus gradient PCR system (Eppendorf) was programmed for touchdown PCR under the following conditions: 94°C for 3 min, 94°C for 30 s, 72°C for 30 s followed by 30 cycles of 1-min step: the initial annealing temperature of 72°C was decreased by 0·5°C per cycle to reach 57°C for the final cycle. The mixture was then subjected to 94°C for 30 s, 57°C for 30 s and 72°C for 1 min for further fifteen cycles. The PCR ended with an extra extension of 5 min at 72°C. After the amplification, electrophoresis of 10 μl samples in 1 % (w/v) agarose gels detected the unique PCR product. This was excised, isolated (TianGen Biotech), inserted into pMD™19-T (TaKaRa) and sequenced at Sangon Biotech. Purification of the site-directed mutant was performed using the above-mentioned endofree maxi plasmid kit (TianGen Biotech).

Dual-luciferase reporter assay

The dual-luciferase assay was performed after the HEK 293T cells reached 70 % confluence to determine the activity of a given promoter. The reporter firefly luciferase construct and reference plasmid pGL4.75 (Promega) were co-transfected into the cells at a ratio of 10 000:1 (w/w) using Lipofectamine® 2000 Reagent (Invitrogen) according to manufacturer's recommendation. Furthermore, empty pGL4.10 plasmids were used as the negative control. The plasmids were incubated with cells for 24 h; then, a fresh culture medium without plasmids was added to replace the original medium. After another 24 h (i.e. 48 h after transfection), luciferase assays were performed using a dual-luciferase reporter assay system (Promega). The results were expressed as the luminescence ratio of firefly:renilla luciferase. Twelve wells were included in one treatment group, and three dual-luciferase reporter assays were conducted independently.

Statistical analysis

All data are expressed as mean and standard deviations. The data from the core promoter region identification and luciferase assays after transfection with different concentrations of pcDNA3.1-PPAR*γ* were verified using a one-way ANOVA followed by Duncan's *post hoc* test using PASW Statistics version 18 (SPSS). All other data were tested by comparing groups using an independent-samples *t* test in PASW Statistics version 18 (SPSS). Tests for normality and homoscedasticity were performed prior to the formal statistical analysis.

Results

Cloning of the 5′ flanking sequence of fatty acid-binding protein 4 in Trachinotus ovatus *and activity analysis*

In this study, 2059 bp (from the initiation codon ATG) of the 5′ flanking sequence of the *fabp4* gene in *T. ovatus* was cloned and treated as the promoter candidate (Fig. 1). The first base of the initiation codon ATG was defined as +1 in the sequence. Five progressive deletion fragments were constructed, namely D1 (−2 bp to −2006 bp), D2 (−2 bp to −1521 bp), D3 (−2 bp to −1158 bp), D4 (−2 bp to −733 bp) and D5 (−2 bp to −241 bp). The dual-luciferase reporter analysis indicated that deletion of the fragment from −2006 bp to −1521 bp caused a decrease in promoter activity compared with that of the original promoter candidate (D1). No significant differences were found in promoter activity after the sequences between −1521 bp to −1158 bp and −1158 bp to −733 bp were deleted. However, the deletion of fragment from −733 bp to −241 bp resulted in an increase in the promoter activity. These results indicated that the core promoter region was located between −2006 bp and −1521 bp (Fig. 2).

Fatty acid-binding protein 4 promoted the uptake of DHA in hepatocytes

In our previous study on *T. ovatus*, we found that the hepatic mRNA level of *fabp4* increased with increasing *n*-3 LC-PUFA content in the diet and in the experimental fish liver (online Supplementary Fig. S1) and *fabp4* was identified as a candidate gene for the regulation of the *n*-3 LC-PUFA content[19]. Herein, we further verified the role of *fabp4* in regulating the *n*-3 LC-PUFA content *in vitro*. Compared with the control group, the DHA content increased significantly in the *fabp4* overexpression group and the content of DHA was decreased after the *fabp4* gene was suppressed by BMS309403 (inhibitor of *fabp4*; MedChemExpress) (Fig. 3). The changes in the other fatty acids are shown in online Supplementary Fig. S2.

Stimulation of PPARγ contributed to the uptake of DHA in hepatocytes

Previously, we found that the change in *PPARγ* gene expression was consistent with the change in hepatic *n*-3 LC-PUFA content (not published) in *T. ovatus* fed with *n*-3 LC-PUFA (0·64–2·30 %). Meanwhile, transcriptome analysis of *T. ovatus* liver also showed that *PPARγ* was a potential regulator of the *n*-3 LC-PUFA content[19]. In this study, the uptake of DHA was markedly enhanced after the *PPARγ* gene was up-regulated by overexpression. Conversely, an antagonist of *PPARγ*, GW9662 (Sigma-Aldrich) attenuated an increase in the DHA content by inducing *PPARγ* overexpression (Fig. 4). The changes in the other fatty acids are shown in online Supplementary Fig. S2.

PPARγ regulated the expression of *fatty acid-binding protein 4* at the transcriptional level

To identify possible transcription factor binding sites in the core promoter region of the *T. ovatus fabp4* gene, we used online software to search for the potential binding site (http://bioinfo.life.hust.edu.cn/hTFtarget#!/prediction). Many transcription factors, such as Forkhead box O1, CCAAT/enhancer binding protein and transcription factor 5 binding sites, were identified in the core promoter region of *T. ovatus fabp4* gene with high scores (Fig. 1). According to our previous study, *PPARγ* is a potential regulator of the *T. ovatus fabp4* gene and two *PPARγ* binding sites were predicted in the present study (online Supplementary Fig. S3). To determine whether *PPARγ* regulates *fabp4* gene expression, a site-directed mutant was constructed and transfected into HEK 293T cells for detection of luciferase activity. As shown in Fig. 5(a), the mutation of the *PPARγ* binding site (with the highest score) resulted in a significant decrease in luciferase activity. To further confirm the role of *PPARγ* in *fabp4* expression, HEK 293T cells were co-transfected with D1, together with different concentrations of pcDNA3.1-PPARγ. The results showed that pcDNA3.1-PPARγ increased the luciferase activity in a dose-dependent manner compared with the control group (Fig. 5(b)). These findings strongly suggest that *T. ovatus fabp4* may be a target of *PPARγ*.

PPARγ regulated the fatty acid-binding protein 4-mediated uptake of DHA in hepatocytes

As shown in Fig. 6, the simultaneous overexpression of *PPARγ* and *fabp4* increased the mRNA levels of *PPARγ* and *fabp4* significantly, by about 15-fold and 16-fold, respectively, compared with the empty plasmid group. An antagonist of *fabp4*, BMS309403 effectively reduced the expression of *fabp4* with no significant effect on the mRNA level of *PPARγ* (Fig. 6(a)).

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GAAGATAAATTTGACCATTTCTTTATTTTGCATGGCTGGTTTTGTTTTTTCTCAAATACAACTTTAACTCACCTGCCTTTATTTA
GTATTTTGGGTTGCTGGTGTTTTTAAAAGGCCAACTATAAGGTTTACTGTAATGTTTTGCTGAAACCATTGGGAGGCAGTACA
TGGCAGTCTGACATTTGGGACACAGATCCACTGATTGTTTTCTGCCAGAGTTTCAATAGAGCAATACTGCTAATCAATGAAAT
CTTTCAACACCCAACTCTTTTGCAGTTTTTGTTTTTAAGCAATGCAACAAATGTATCGCAAATATTCAAATGAATTAACAAAC
GTTTTGTAAAAAGATGTGTAAGGAATACATATGAATAAACTGAGAAATGTTTAACTTTGAATGAAAATCAGTTTCATCATATA
CTTTTTGTAAGGTCTGTTACATTTATCTGCAGTTGCGAACAGTTACTAACAGAGCCTTTATGGACTATTTTATCTGCTTTTCAC
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GTAATTCCCAACAGAACAAGGTGATGTCCTCATCAAATCTTCATATTTGAGGAGCTGGAAAAAGCAAATACTAGATATATGA
GTTTAAAAAGTTGCTAAACGATTATCATTGATCAAATTAATTATAAATTCATTTTCTGACCATCGATTAATCGACAAGTTCTGC
AGCTCTAGAATGGTCAAGAGTGTGTATGTGCAATTTTCTTCGTTAATACTTATTTTATTATCATGTTTACTATTAATGTAATTATT
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CACGTTTCTAAACTCCACTGGAGGGATGAACACTGTTCTTCCAAAGGATATTCCCTCATTTGGTGTTTCGATGAAGGTGGTG
GAGAAGGGTGTTCAACTGGGTTGAGATCTGGTGACTGCAAAGCCCATAGCATATCATTCACATCATTTCCATACTCATCAAA
CTATTCAGGGACCCATCGGGCACTGCCATGCTGGAAGAGACCACTTGTATCAGGATAGAAATGTTTCATCATAGGATGAAGG
TGATCATATCGCACTAATACACGAGCATCGCCGTCATGATGTCTTTCCCATAGATATAAATGTAGATGTCACTTTAGTCAGTTT
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TCAAAGTGACTGAGGTGTCGCCATCTTGATGCAAAATCAGAATCAACTGGGCCTGCTTAGCATCTTCATACATGCCACAGTC
CCATCCTGTGCATCTTTGTGGAAGCATGTTATAGTATTTGTTAACATTGTTCTTACAGAAAGAAAATGCAGCCACACGAACCA
AATCCTATCAGGTCACATTATTGCAGCACCCTATCACCAACCCAATTCACGCTGAAGTCAGCAGGCTCACACTTCCCTTCAAT
CAAACACACCATCAAAATCTGATTTGAAGACACGCCCCCTAATCCGCCCGTTTCCATGGCAACGTCAGCACCTGCTCATGAT
TGGTGGCCCTGGATGAGACGCGTGCTGTAGCAGGCTTTAAATATGAAGCTCGCTCCGGGTTTCTGCCTCTTGCGCTTCACCG
CTGAAACTGCTTGTTTTTCTGTTGTGTGTGTGTGTGTCAACACATCTGAGCGTCATCTTCACCACC**ATG**

Fig. 1. The 5′ flanking sequence of the *Trachinotus ovatus* fatty acid-binding protein *4 (fabp4)* gene and partial predicted binding sites of transcription factors (red: Forkhead box O1 (FoxO1); orange: CCAAT/enhancer binding protein (C/EBPα); green: PPARα; blue: PPARγ). Bold-faced bases indicate the initiation codon.

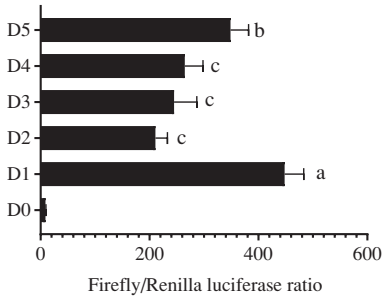

Fig. 2. Analysis of *Trachinotus ovatus* fatty acid-binding protein 4 (*fabp4*) gene promoter activity. D1–D5 indicates the five progressive deletion fragments, namely −2 bp to −2006 bp, −2 bp to −1521 bp, −2 bp to −1158 bp, −2 bp to −733 bp and −2 bp to −241 bp (The first base of the initiation codon ATG was defined as +1). D0 represents the negative control without the promoter region (pGL4.10-empty). Values are expressed as means and standard deviations. Means at a time without a common symbol are significantly different ($P \leq 0{\cdot}05$).

Meanwhile, the simultaneous overexpression of *PPARγ* and *fabp4* markedly enhanced the DHA content compared with the empty plasmid group. However, suppression of *fabp4* via BMS309403 attenuated the increase in the DHA content (Fig. 6(b)). Conversely, treating *Epinephelus coioides* hepatocyte line cells with GW9662 markedly attenuated the up-regulation of *PPARγ* induced by *PPARγ* overexpression, and *fabp4* was obviously increased through its overexpression (Fig. 6(c)). The DHA content was decreased by the suppression of *PPARγ*. However, the effect was restored by the overexpression of *fabp4* (Fig. 6(d)). The observed changes in the other fatty acids are shown in online Supplementary Fig. S2.

Discussion

In recent years, the *n*-3 LC-PUFA content in farmed fish has been redefined, resulting in fish being supplied with *n*-3 LC-PUFA levels that far exceed their biological requirements and with the aim of satisfying consumer demand[2]. Currently, this research area is receiving much focus, and two economic and effective approaches have been identified as potential avenues to increase the *n*-3 LC-PUFA content in farmed fish, namely the improvement of their biosynthesis ability and the enhancement of the uptake and deposition of dietary-limited *n*-3 LC-PUFA. Some regulators, such as microRNA, the hepatocyte nuclear factor 4*α*, and the sterol regulatory element-binding protein-1, have been demonstrated to be involved in the biosynthesis of *n*-3 LC-PUFA and can thus be used to further regulate the *n*-3 LC-PUFA content[24,26,27]. In comparison, the uptake and deposition of *n*-3 LC-PUFA have received less attention. In our previous study, *fabp4* was identified as a candidate gene involved in the uptake and deposition of *n*-3 LC-PUFA[19]. In the present study, we first cloned the promoter sequence of *fabp4* in *T. ovatus* and then evaluated the serial deletion constructs of the *fabp4* promoter in a dual-luciferase reporter assay, which showed that the core promoter was located between −2006 bp and −1521 bp upstream from the initiation codon. In mammals, the core promoter has generally been identified near the transcription initiation site[28,29]. For example, the core promoter of cattle (*Bos taurus*) *fabp4* was present in the region of 272 bp upstream from the initiation codon[30]. The distinctive location of the *T. ovatus fabp4* core promoter region indicated that there may be distinct regulatory mechanisms of *fabp4* between *T. ovatus* and mammals. Alternatively, the transcription initiation site of *T. ovatus fabp4* was far from the initiation codon; therefore, the core promoter was still near the transcription initiation site. This requires further investigation. In addition, the binding sites of transcription factors such as CCAAT/enhancer binding proteins, PPARγ, and Forkhead box O1 have been found in the core promoter region of *T. ovatus fabp4*, which is consistent with previous studies that showed that these transcription factors play a role in the transcriptional regulation of the *fabp4* gene[30–33].

Members of the FABP family, which consists of twelve members (FABP1–FABP12), are known to play important roles in fatty acid uptake[34,35]. In addition, a preference between the FABP subtype and fatty acid type has been suggested and is well studied in mammals[36]. In fish, studies have demonstrated that *fabps*

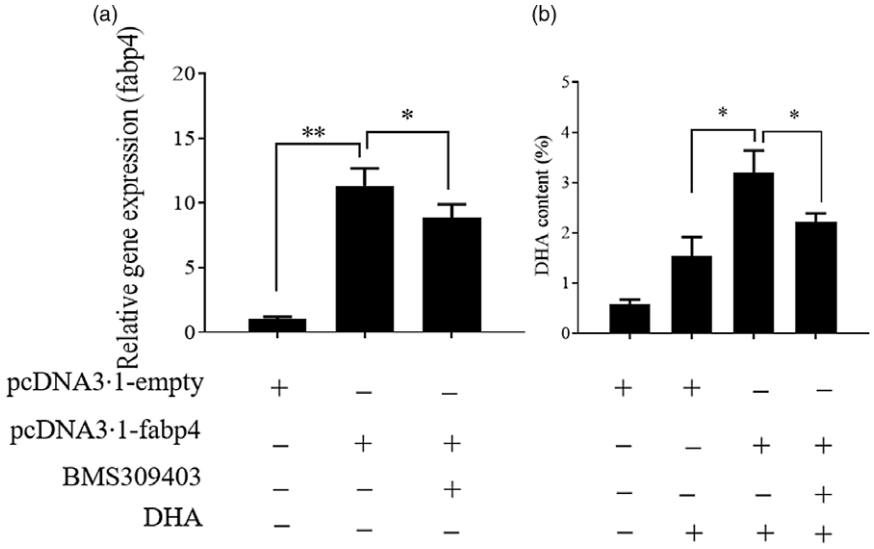

Fig. 3. Fatty acid-binding protein 4 (Fabp4) promotes the uptake of DHA. Values are expressed as means and standard deviations (*n* 3). The *Epinephelus coioides* hepatocyte line (ECHL) cells were transfected with pcDNA3.1-fabp4 or pcDNA3.1-empty plasmids for 12 h. Subsequently, 50 μM DHA and 75 μM BMS309403 were added as mentioned for another 24 h (BMS309403 was added 2 h prior to DHA) without recombinant plasmids. Cells were harvested for the detection of *fabp4* gene expression (a) DHA content (b). *, $P \leq 0{\cdot}05$; **, $P \leq 0{\cdot}01$.

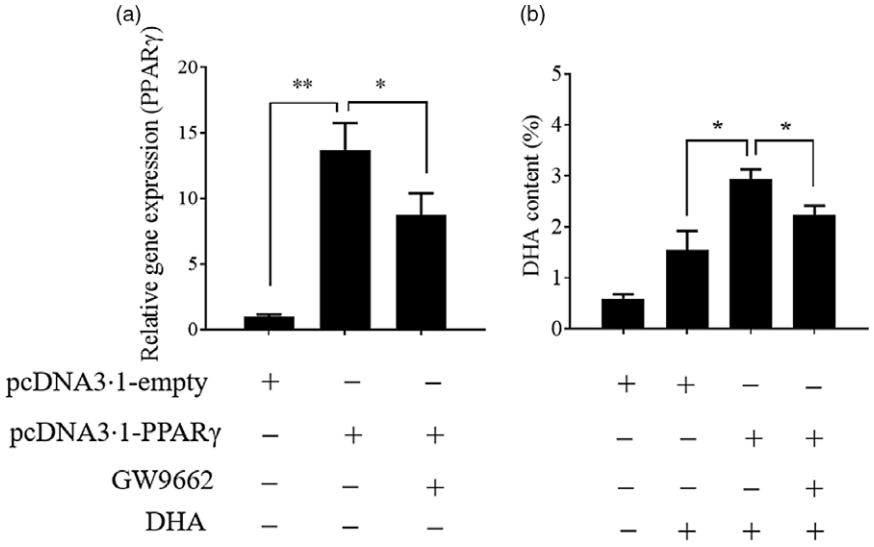

Fig. 4. Stimulation of PPARγ contributes to the uptake of DHA. Values are expressed as means and standard deviations (*n* 3). The *Epinephelus coioides* hepatocyte line (ECHL) cells were transfected with pcDNA3.1-empty or pcDNA3.1-PPARγ plasmids. After 12 h, the plasmids were removed and the cells were incubated with 50 μM DHA and 75 μM GW9662 as shown (GW9662 was added 2 h prior to DHA) for another 24 h. Cells were collected to measure *PPARγ* gene expression (a) and DHA content (b). *, $P \leq 0{\cdot}05$; **, $P \leq 0{\cdot}01$.

are associated with dietary fatty acids and that a similar fatty acid-selective property has been conserved[13,14], although the details regarding the FABP and its preferred fatty acid remain largely unclear. Recently, we identified *fabp4* as a candidate *fabp* gene involved in *n*-3 LC-PUFA uptake and deposition[19]. In mammals, *fabp4* is known to be involved in adipogenesis[37,38]. In the present study, the overexpression of *fabp4* triggered an increase in DHA content and the suppression of *fabp4* alleviated this effect; this provides direct evidence that *fabp4* contributes to the uptake and deposition of *n*-3 LC-PUFA. This result was similar to that of a previous study, in which the FABP4 protein from *H. sapiens* and *M. musculus* appeared to specifically bind to long-chain fatty acids (e.g. DHA) with high affinity[39].

As early as 1996, the FABP family was reported to be regulated mainly at the transcriptional level[40]. The transcription factor *PPARγ* is an important regulator for members of the FABP family both in mammals and in fish[41–43], and the influence of *PPARγ* on *fabp4* expression has been widely reported[33,44–46]. Analogously, in this study, the mutation of the *PPARγ* binding site significantly reduced the promoter activity of *fabp4*. Meanwhile, overexpression of *PPARγ* revealed a positive, dose-dependent effect on *fabp4* promoter activity, indicating that the *fabp4* gene was a target of *PPARγ* in *T. ovatus*.

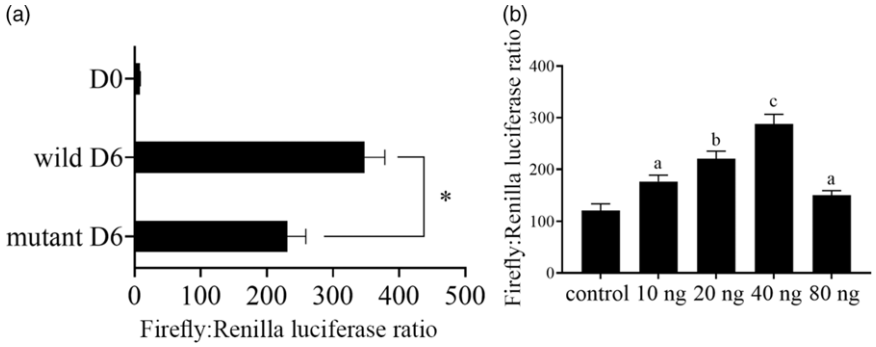

Fig. 5. Fatty acid-binding protein 4 (Fabp4) in *Trachinotus ovatus* is a target of PPARγ. Values are expressed as means and standard deviations. (a): Luciferase activity was detected in HEK 293T cells that were transfected with pGL4.10-empty (D0), wild-type D6 (wild D6) or mutant D6 for 24 h. *, $P \leq 0{\cdot}05$. (b): HEK 293T cells were co-transfected with the *fabp4* full-length promoter fragment (D1) and different concentrations of pcDNA3.1-PPARγ for 24 h. Means at a time point without a common symbol are significantly different ($P \leq 0{\cdot}05$).

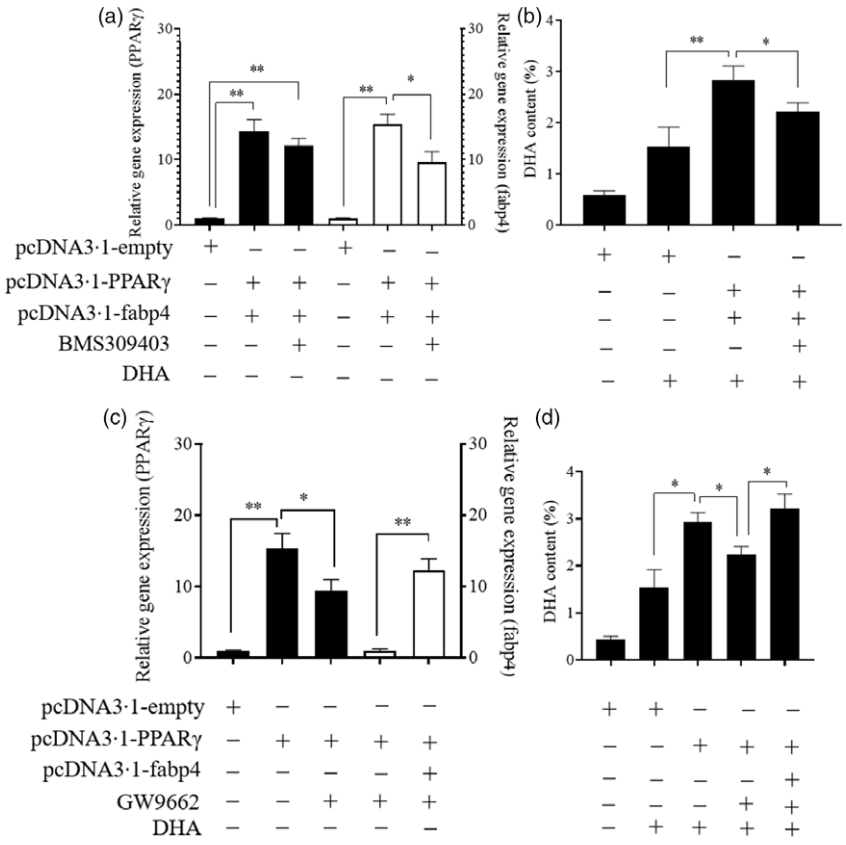

Fig. 6. PPARγ acts as a positive regulator in the fatty acid-binding protein 4 (fabp4)-mediated uptake of DHA. Values are expressed as means and standard deviations. *, $P \leq 0{\cdot}05$; **, $P \leq 0{\cdot}01$ (n 3). The *Epinephelus coioides* hepatocyte line (ECHL) cells were transfected with pcDNA3.1-empty alone or co-transfected with both pcDNA3.1-PPARγ and pcDNA3.1-fabp4. These plasmids were removed after 12 h and the cells were incubated with 75 μM BMS309403 and 50 μM DHA as shown for another 24 h (BMS309403 was added 2 h in advance). Cells were collected; the gene expression of *PPARγ* and *fabp4* (a) and the content of DHA (b) were detected; The ECHL cells were transfected with pcDNA3.1-empty or pcDNA3.1-PPARγ for 12 h and then treated with 75 μM GW9662 for another 12 h without the plasmids. Afterward, pcDNA3.1-fabp4 was transfected into cells, and 50 μM DHA was added as shown to incubate the cells for another 12 h. Cells were collected to assess *PPARγ* and *fabp4* mRNA levels (c) and DHA content was evaluated (d).

PPARγ is believed to be a master regulator of adipogenesis[47]. In *C. idella*, an increase in DHA content in the hepatopancreas is reportedly accompanied by the up-regulation of *PPARγ*[48]. Similarly, in this study, we found that *PPARγ* overexpression increased the DHA content and that the suppression of *PPARγ* mitigated this effect, thus indicating that *PPARγ* had a positive effect on DHA uptake and deposition. Our previous study indicated that *PPARγ* might be a key regulator of *n*-3 LC-PUFA uptake and deposition by modulating the genes (including *fabp4*) involved in fat emulsification and fatty acid transport[19]. In this study, the antagonist of *fabp4*, BMS309403 alleviated the increase in DHA content induced by *PPARγ* overexpression, indicating that *fabp4* was

required for DHA uptake and deposition evoked by *PPARγ*. Conversely, overexpression of *fabp4* reversed the decline in the DHA content caused by GW9662 (a common antagonist of *PPARγ)*. Collectively, the above findings demonstrate the functioning of *fabp4* in the uptake and deposition on DHA in *T. ovatus*, and that such functions are regulated by *PPARγ*. Some natural ligands of *PPARγ*, such as α-linolenic acid and linoleic acid[49,50], are common components of aquatic feeds. These ligands may further induce the expression of the *PPARγ* target gene *fabp4*, providing potential method to improve the *n*-3 LC-PUFA content of farmed fish in practical applications.

In the present study, we cloned the 5′ flanking sequence of *T. ovatus fabp4* and revealed and characterised its important role in the regulation of *n*-3 LC-PUFA content in *T. ovatus*. Targeting *fabp4* may, therefore, present an effective strategy for the regulation of the *n*-3 LC-PUFA content in this commercially important marine fish. Our findings also indicated that *fabp4*-mediated *n*-3 LC-PUFA uptake and deposition are probably regulated by *PPARγ* in *T. ovatus*. These results provide a new regulation axis that appears to be a key contributor to *n*-3 LC-PUFA content regulation in *T. ovatus*.

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The authors declare that there are no conflicts of interest.

Y. L., B. F. and C. L. conceived and designed the experiments. M. L. provided the experimental materials. Y. L., J. T. and C. L. co-wrote the paper.

Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114521000775

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