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Research Article

Tissue Distribution and Nutritional Regulation of Fatty Acid-Binding Proteins (fabps) in Two Marine Teleosts, Turbot (Scophthalmus maximus), and Tiger Puffer (Takifugu rubripes)

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Fatty acid-binding proteins (fabps) play important roles in lipid homeostasis. In the present study, 7 fabp isoforms, namely, fabp1, fabp2, fabp3, fabp4, fabp6, fabp7, and fabp10, in two marine teleosts, were characterized. In general, turbot and tiger puffer fabp genes showed high identity to their orthologs in other fish species and mammals, but tiger puffer Fabp6 shared the lowest identity to its known orthologs in zebrafish and human. The tissue distribution patterns of fabps were generally in accordance with their function features. However, tiger puffer fabps, in particular Fabp1, Fabp2, Fabp6 and Fabp7, may have functions distinct from other teleosts, as indicated by the phylogenetic tree and tissue distribution patterns. In both species, high dietary lipid levels downregulated the expression of fabp2, fabp3, fabp6, and fabp7a but tended to upregulate the fabp1 expression. Starvation downregulated the expression of most fabps in both fish species, but the downregulation of fabp expression in turbot was much more drastic and earlier compared to tiger puffer. Long-term (30-day) starvation increased the fabp7 expression in tiger puffer and tended to increase the fabp6 expression in turbot. Results of this study contribute to fish fabp physiology and its nutritional regulation.

1. Introduction

Fatty acid-binding proteins (fabps) belong to the conserved multigene family of intracellular lipid binding proteins (iLBPs) that are individual genes arising from an ancestral iLBP gene through gene duplication and diversification [1]. They play important roles in lipid uptake and transport in various tissues which are highly active in fatty acid metabolism and thus play important roles in the overall lipid homeostasis [2]. Fatty acids are the major ligands of fabps, and each fabp has specific ligands [3, 4].

Mammalian fabps have been well-studied, but many relevant mechanisms remain unclear, such as the function difference among different fabps as well as among different species [5]. In contrast, less relevant information has been available in fish. Nevertheless, fish fabps are attracting more and more research interest, considering fish, unlike mammals, preferring lipids to carbohydrates as their main source of energy [6–9]. In our previous studies with Japanese seabass (Lateolabrax japonicus), several fabp isoforms, i.e., fabp1, fabp2, fabp3, fabp4, and fabp7, have been cloned and characterized [10]. fabps from other fish species, such as zebrafish (Danio rerio) [11], orange-spotted grouper (Epinephelus coioides) [12], Japanese rice fish (Oryzias latipes; medaka) [7], three-spined stickleback (Gasterosteus aculeatus) [7], Atlantic salmon (Salmo salar) [8], goldfish

(Carassius auratus) [13], golden pompano (Trachinotus ovatus) [14, 15], and javelin goby (Synechogobius hasta) [16], have also been cloned and characterized.

The present study was aimed at characterizing the fabp genes in two important aquaculture species turbot (Scophthalmus maximus) and tiger puffer (Takifugu rubripes). These two fish species are also becoming important model fish species for lipid studies due to their special lipid storage patterns, namely, turbot and tiger puffer store lipid predominantly in the subcutaneous adipose tissue and liver, respectively [17]. Besides the sequence feature and tissue distribution, the present study was also aimed at investigating the fabp expression in response to different nutritional status, namely, different dietary lipid levels and different starvation periods, considering that both feeding ration and dietary lipid level have been used in the aquaculture industry to regulate the fish growth and fillet quality. Results of this study will shed light on the fish fabp physiology and its regulation.

2. Materials and Methods

2.1. Feeding Trial and Sampling

2.1.1. Turbot Trial and Sampling. For the study on tissue distribution of fabp expression, samples of 13 tissues, namely, eye (E), brain (B), heart (H), gill (G), muscle (M), liver (L), kidney (K), skin (Sk), and subcutaneous adipose tissue around the fin (Su), spleen (Sp), stomach (St), pyloric caecum (P), and intestine (I), were collected from 30 juvenile turbot (approximately 20 g, 10 fish in a pool) reared in the tanks in Huanghai Aquaculture Co., Ltd. (Yantai, Shandong Province, China). Anterior intestine (AI), middle intestine (MI), and hind intestine (HI) were sampled separately. The fish were treated with eugenol and then immediately dissected. Tissue samples were stored at -76°C before use.

For the nutritional experiment, a 9-week feeding trial with juvenile turbot (average initial body weight, 26 g) was conducted, followed by a one-month starvation period [18]. Three experimental diets were designed in the feeding trial (Table 1). A diet with a suitable lipid level (8.0%) for this fish species was used as the control diet (Diet LL). Fish oil was added into the control diet to obtain the other two experimental diets with a moderately (12.0%, Diet ML) or extremely high lipid level (16.0%, Diet HL). The diets were made according to the standard procedures in our laboratory and stored at -20°C before use.

The feeding trial was conducted in a flow-through seawater system in Tianyaun Aquaculture Co. Ltd. (Yantai, China), and the experimental fish were purchased from that farm. Before the start of the feeding trial, fish were reared in polyethylene tanks and fed a commercial diet for 7 days to acclimate to the experimental conditions. At the onset of the feeding trial, fish were randomly distributed into 18 polyethylene tanks (500 L). Each diet was randomly fed to sextuplicate tanks, each of which was stocked with 35 fish. During the feeding trial, fish were hand-fed to apparent satiation twice daily (7:30 and 17:30). After each feeding, the tanks were cleaned daily by siphoning out residual feeds and feces. At the end of the feeding trial, after the experimental fish were fasted for 24 h, liver samples from six randomly selected fish per tank were collected.

After the termination of the feeding trial, 20 fish from each tank of the control group were randomly selected and subjected to a following starvation experiment. In the following starvation period, liver samples from six randomly selected fish per tank were collected at 3 sampling time points, namely, day 10 (S10), 20 (S20), and 30 (S30), respectively [the end of the feeding period was designated as day 0 (S0)]. All the collected tissue samples were immediately frozen in liquid nitrogen and then stored at -76°C before use. During the whole experiment, the temperature ranged from 13.2 to 15.2°C; salinity, 27-29 g L^{-1}; pH, 7.5-8.0; and dissolved oxygen, 6-8 mg L^{-1}.

2.1.2. Tiger Puffer Trial and Sampling. For the study on tissue distribution of fabp expression, samples of 8 tissues, namely, liver (L), muscle (M), brain (B), intestine (I), heart (H), eye (E), skin (Sk), and spleen (Sp), were collected from 15 juvenile tiger puffer (approximately 20 g, 5 fish in a pool) reared in the tanks in Huanghai Aquaculture Co., Ltd. (Yantai, China). The tissues were sampled similarly to the turbot trial.

For the nutritional experiment, a 9-week feeding trial with juvenile tiger puffer (average initial body weight, 19.5 g) was conducted, followed by a one-month starvation period. Three experimental diets very similar to the turbot trial were used in this feeding trial considering the similar lipid requirement levels between turbot and tiger puffer (Table 1) [19, 20].

The feeding trial was conducted in Haidu Aquaculture Co., Ltd. (Tangshan City, Hebei Province, China), and the experiment fish were purchased from that farm. Before the start of the feeding trial, fish were reared in indoor cement tanks (6.2 m × 6.2 m × 1.8 m) and fed a commercial diet for 14 days to acclimate to the experimental conditions. During the acclimation period, the lower teeth of fish were cut away to prevent cannibalism. At the onset of the feeding trial, fish were randomly distributed into 18 net cages (1.4 m × 1.4 m × 1.0 m) placed in the cement tanks. Placing net cages in a large water body was beneficial to keeping a low water temperature that the fish need. Each diet was randomly fed to sextuplicate net cages, each of which was stocked with 50 fish. Fish were hand-fed to apparent satiation twice daily (6:00 and 18:00). Eighty percent of the rearing water was changed after each feeding. At the end of the feeding trial, after the experimental fish were fasted for 24 h, liver samples from six randomly selected fish per net cage were collected.

After the termination of the feeding trial, 30 fishes from each net cages of the control group were randomly selected and subjected to a following starvation experiment. In the following starvation period, three randomly selected fish per net cages were sampled at 5 sampling time points, namely, day 1 (S1), 4 (S4), 9 (S9), 16 (S16), and 31 (S31), respectively [the end of the feeding period was designated as day 0 (S0)]. During the whole experiment, the water

Table 1: Formulation and proximate composition of the experimental diets (% dry matter basis).

Ingredients	Turbot			Tiger puffer		
	Control	MHL	EHL	Control	MHL	EHL
Fish meal	40.0	40.0	40.0	40.0	40.0	40.0
Soy protein concentrate	4.00	4.00	4.00	6.00	6.00	6.00
Soybean meal	7.00	7.00	7.00	8.00	8.00	8.00
Wheat meal	18.0	18.0	18.0	18.5	18.5	18.5
Casein	9.00	9.00	9.00	6.00	6.00	6.00
Brewer's yeast	5.68	5.68	5.68	6.00	6.00	6.00
α-Starch	9.60	5.60	1.60	8.00	4.00	0.00
Mineral premix[1]	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix[1]	0.20	0.20	0.20	0.20	0.20	0.20
Monocalcium phosphate	1.00	1.00	1.00	1.00	1.00	1.00
L-Ascorbyl-2-polyphosphate	0.20	0.20	0.20	0.20	0.20	0.20
Choline chloride	0.20	0.20	0.20	0.20	0.20	0.20
Attractant (betaine)	0.30	0.30	0.30	0.30	0.30	0.30
Ethoxyquin	0.02	0.02	0.02	0.02	0.02	0.02
Mold inhibitor[2]	0.10	0.10	0.10	0.10	0.10	0.10
Soya lecithin	1.00	1.00	1.00	1.00	1.00	1.00
Fish oil	3.20	7.20	11.2	4.00	8.00	12.0
Proximate composition						
Moisture	9.29	8.61	6.78	8.33	7.90	6.54
Crude protein	50.7	51.1	51.4	50.6	50.8	51.1
Crude lipid	7.70	11.7	15.7	8.05	12.0	16.4
Ash	9.78	9.56	9.52	9.58	9.64	9.67

[1]Vitamin premix and mineral premix, designed for marine fish, were purchased from Qingdao Master Biotech Co., Ltd., Qingdao, China. [2]Contained 50% calcium propionic acid and 50% fumaric acid.

temperature ranged from 20 to 23°C; salinity, 22-30; pH, 7.4-8.2; and dissolved oxygen, 5-7 mg L^{-1}.

Before all sampling processes, fish were anesthetized with eugenol, and efforts were taken to minimize the suffering of the experimental fish. All sampling protocols, as well as fish rearing practices, were reviewed and approved by the Animal Care and Use Committee of Yellow Sea Fisheries Research Institute.

2.2. RNA Extraction, cDNA Synthesis, and Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR) Analysis. The total RNA in liver samples was extracted using RNAiso Plus (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China) and reversely transcribed with Evo M-MLV RT Mix Kit with gDNA Clean for qPCR (Accurate Biotechnology (Hunan) Co., Ltd., Hunan, China) according to the user manual.

Real-time fluorescent quantitative PCR (qRT-PCR) was used to assay the relatively quantitative mRNA expression of fabps in different fish tissues, as well as the gene expression under different nutritional statuses. The complete CDS of all the fabps studied were available in the GenBank database. Specific primers were designed using Primer 5.0 according to the fabps sequences in GenBank and synthesized by TsingKe Biological Technology Co., Ltd. (Qingdao, China) (Table 2). According to our previous screening, B2M, β-actin, and EF1α were used as the reference genes for the turbot study, and β-actin and RPL19 were used as the reference genes for the tiger puffer study [21]. The amplification efficiency for all primers, which was estimated by standard curves based on a 6-step 4-fold dilution series of target template, was within 95~105%, and the coefficients of linear regression (R^2) were ⊠0.99. SYBR Green Premix Pro Taq HS qPCR Kit II (Accurate Biotechnology (Hunan) Co., Ltd., Hunan, China) and a quantitative thermal cycler (Roche LightCycler 96, Basel, Switzerland) were used for the real-time qPCR. The reaction system consists of 2 μL cDNA template, 10 μL SYBR Green Pro Taq HS Premix II, 0.8 μL forward primer (10 μM), 0.8 L reverse primer (10 μM), and 6.4 μL sterilized water. The program was as follows: 95°C for 30 s followed by 40 cycles of "95°C for 5 s, 57°C for 30 s, and 72°C for 30 s." Melting curve analysis (6.4°C increment/min from 65°C to 97°C) was performed after the amplification phase for confirmation of the sole product. Each sample was run in triplicate. The mRNA levels were expressed according to the $2^{-\Delta\Delta CT}$ method [22].

2.3. Bioinformatics Analysis and Statistical Analysis. The multiple-sequence alignments of amino acids were performed with BioEdit. Similarity analysis of the fabps cDNA sequences was done with ClustalW (http://www.ncbi.nlm.nih.gov/blast/). The deduced amino acid sequences were analyzed with ExPASy to compute pI/MW (http://web.expasy.org/compute_pi/). The SMART program (http://

Table 2: Sequences of the PCR primers used in this work.

Primer	Sequences (5′-3′)	GenBank reference	PL (bp)
Sm-fabp1-qF	GCCTGAAGGGAATCGTGTCT	XM_035640764.1	105
Sm-fabp1-qR	ACATGCGTTTGCTCGTCCTC		
Sm-fabp2-qF	GAGGAATCGTGGTGCACAGT	XM_035620492.1	165
Sm-fabp2-qR	CGGTCGTCAACTTCTGGGAG		
Sm-fabp3-qF	GCTTGGTGTGGGATTTGCT	XM_035621538.1	175
Sm-fabp3-qR	AACCTTCCTGTCATCGGCG		
Sm-fabp4a-qF	ATGAAAGCAGTCGGTGTGG	XM_035621375.1	129
Sm-fabp4a-qR	CTCGGTGGTTTTGAAGGTG		
Sm-fabp4b-qF	GGACGACAGACCAAGACCAC	XM_035635918.1	92
Sm-fabp4b-qR	TCTCGTTCCAGTGTTGTGGC		
Sm-fabp6-qF	CGACATGGAGACCATCGGAG	XM_035635561.1	96
Sm-fabp6-qR	GAGGTGTGGTGGTAGTTGGG		
Sm-fabp7a-qF	ACGTCAAATCCACCTTCACC	XM_035610205.1	110
Sm-fabp7a-qR	CATCACCATCTTCCCATCCT		
Sm-fabp10a-qF	TATCACCTCCAAGACCCCTG	XM_035605050.1	128
Sm-fabp10a-qR	AGACCATTTTTCCACCCTCC		
Sm-RPL19-F	AGGATGGTCTTATCATTCGC	XM_035614206.1	206
Sm-RPL19-R	CTTCTTTGCCTCCCTGTAGC		
Sm-B2M-F	CTGGCTGTTTTCGTCTGCT	DQ848854.1	97
Sm-B2M-R	GGGTGTTGTCCTTTCCGTT		
Sm-β-actin-F	GTAGGTGATGAAGCCCAGAGCA	MT023044.1	204
Sm-β-actin-R	CTGGGTCATCTTCTCCCTGT		
Sm-EF1α-F	TATTAACATCGTGGTCATTGG	KU057926.1	153
Sm-EF1α-R	CAGGCGTACTTGAAGGAG		
Tr-fabp1-qF	CCATCGGTCTCCCTGATGAAG	XM_003974807.3	121
Tr-fabp1-qR	TTGACCGTTACCTTCGGTCC		
Tr-fabp2-qF	GGAGACACGCTGAAGGGAAAA	XM_003976003.3	113
Tr-fabp2-qR	TCCACGCCCTCGTAGTTGTAA		
Tr-fabp3-qF	GGTCAACGACAAGAGCCTCA	XM_003968832.3	72
Tr-fabp3-qR	GCCTTTTCATAATGGCGCGT		
Tr-fabp6-qF	ATGGGAACGACTTCACCTGG	XM_003970396.3	84
Tr-fabp6-qR	CAGCTCACATTCCTGACCGA		
Tr-fabp7-qF	CGTGACCAGACCGACCGTC	XM_003971476.3	113
Tr-fabp7-qR	CATCCAACTCCTCCCCCAG		
Tr-fabp10a-qF	CTGTGACCAACTCCTTTACCAT	XM_003965635.3	150
Tr-fabp10a-qR	TCTCTCCACCTTTGAGCTCCTG		
Tr-β-actin-F	GAGAGGGAAATCGTGCGTGA	XM_003964421.3	186
Tr-β-actin-R	GAAGGATGGCTGGAAGAGGG		
Tr-RPL19-F	AAGGGTCGTCAATCTGCGGG	XM_003971891.3	136
Tr-RPL19-R	TGGGAGGGATGAACTCTGGG		

PL: product length; Sm: Scophthalmus maximus; Tr: Takifugu rubripes.

smart.embl-heidelberg.de/) and PROSITE program (http://kr.expasy.org/prosite/) were used to predict the functional sites or domains in the amino acid sequence. The phylogenetic analysis was carried out based on the amino acid sequences using the neighbour-joining method, and the phylogenetic tree was constructed using MEGA 4.1.

All the gene expression data were subjected to one-way analysis of variance (one-way ANOVA) in SPSS 16.0 (SPSS Inc., Chicago, USA) for Windows. Differences between means were tested by Tukey's multiple range test. The level of significance was chosen at $P < 0.05$. The results were presented as the means ± standard error. The mRNA expression of fabps in various fish tissues was presented only as means, because of the large difference in intragroup variation among different tissues (sometimes in different orders of magnitudes among different tissues).

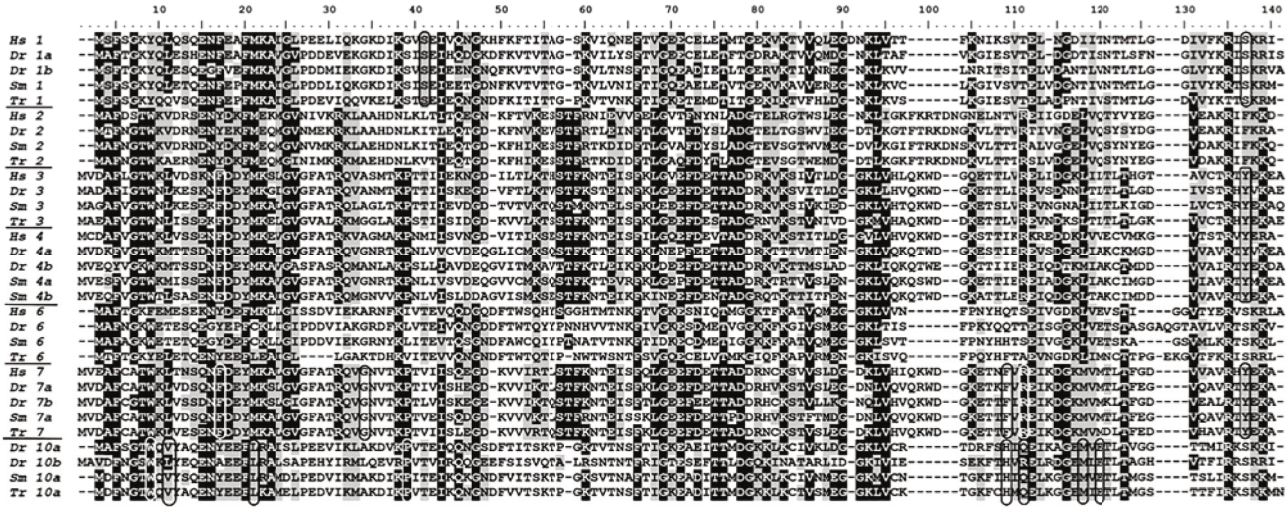

FIGURE 1: Comparison of the deduced fabp amino acid sequences from turbot (Scophthalmus maximus), tiger puffer (Takifugu rubripes), zebrafish, and human. The amino acid sequences were aligned using ClustalX. Identical residues are shaded black and similar residues are shaded grey. Gaps (-) were introduced to maximize the alignment. The boxes indicate the amino acid residues which play important roles in ligand binding. Hs: Homo sapiens; Dr: Danio rerio; Sm: Scophthalmus maximus; Tr: Takifugu rubripes; 1: Fabp1; 2: Fabp2; 3: Fabp3; 4: Fabp4; 6: Fabp6; 7: Fabp7; 10: Fabp10.

3. Results and Discussion

3.1. Sequence Analysis. The complete coding sequence of fabp1, 2, 3, 4a, 4b, 6, 7a, and 10a in turbot encodes a polypeptide of 127, 132, 133, 134, 133, 127, 132, and 126 amino acids, respectively, and that of fabp1, 2, 3, 6, 7, and 10a in tiger puffer encodes a polypeptide of 127, 132, 133, 125, 132, and 127 amino acids, respectively (Figure 1). The predicted molecular weight and theoretical isoelectric point were in the range of 14.0~15.2 kDa and 4.96~8.38, respectively. The low-molecular mass characteristic of fabps from both turbot and tiger puffer was similar to what was observed in other species [10].

No signal peptide sequences and transmembrane domains were predicted in all these fabps, confirming that they all are intracellular proteins. Conserved domains of lipocalin superfamily, i.e., the lipocalin/cytosolic fatty-acid binding protein family, were observed in all the deduced protein sequences. The predicted three-dimensional structure of these fabps showed a 10-stranded antiparallel β-barrel and an N-terminal helix-turn-helix motif, a highly conserved structure of the fabp family [23].

3.2. Multiple Sequence Alignment and Phylogenetic Analysis. The fabps of turbot and tiger puffer generally shared high identity to their known orthologs in zebrafish (62.7%-87.9% and 44.3-82.5%, respectively) and human (56.1-80.3% and 48.4-85%, respectively). Tiger puffer Fabp6 shared the lowest identity to its known orthologs in zebrafish (44.3%) and human (48.4%). The fabps from turbot and tiger puffer were also highly conserved in the putative amino acid residues playing important roles in ligand binding (see amino acid residues in boxes in Figure 1). These features suggested that the fabps from turbot and tiger puffer may share similar functions to their orthologs in zebrafish and human.

The phylogenetic tree of fabps was consistent to the reported amino acid sequence-based grouping of fabps (Figure 2), i.e., (1) Fabp1 and Fabp6; (2) Fabp2; and (3) Fabp3, Fabp4, Fabp5, Fabp7, Fabp9, and Mp2 [23]. It has been hypothesized that the large fabp subfamilies have diverged from a common progenitor before the fish/mammalian divergence but the individual members of the subfamilies branched out after the fish/mammalian divergence [24]. Nevertheless, Fabp6 and Fabp7 of tiger puffer clustered to their human orthologs than to their orthologs in other teleosts (Figure 2). This indicates the possible conserved functions of Fabp6 and Fabp7 between tiger puffer and human, but possible diversified functions between tiger puffer and other teleosts.

The duplication of fabps genes in fish, which putatively arose owing to the teleost-specific whole genome duplication, has been reported in previous studies [9, 15, 25]. This was typically observed in Fabp2 and Fabp4 (Figure 2). The salmonid-specific whole genome duplication even generated pairs of duplicated fabp genes [9, 26, 27]. However, the present study may not cover all the fabp isoforms in these two fish species due to the lack of available sequence information. Further efforts are needed to characterize the complete repertoire of fabps from turbot and tiger puffer.

3.3. Tissue Distribution of Turbot and Tiger Puffer Fabps. Generally, each turbot fabp had the highest expression level in a characteristic tissue, as observed in other fish species [9, 10, 15, 16], namely, fabp1 and fabp10 in the liver; fabp2 and fabp6 in the intestine; fabp3 in the heart; fabp4 in adipose tissue; and fabp7 in brain (Figure 3). These tissue distribution patterns were in accordance to the putative functions of each fabp. Fabp2 is reported to be able to facilitate the

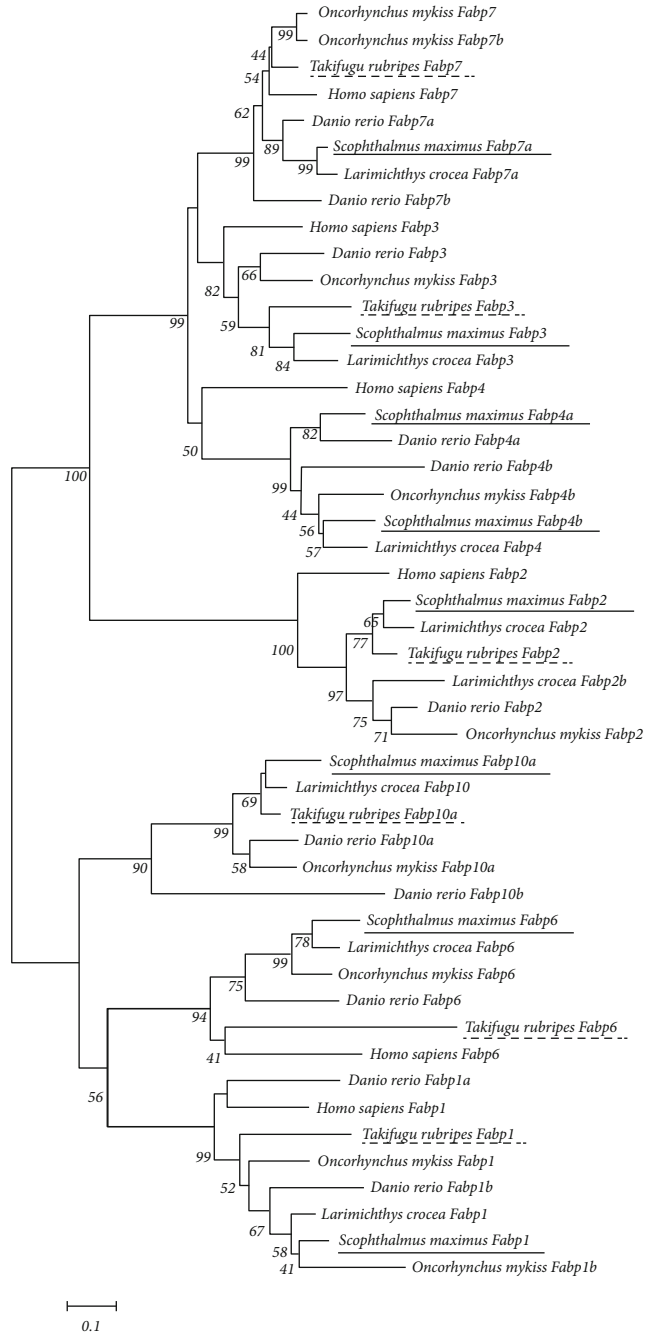

Figure 2: Phylogenetic tree of fabps. The amino acid sequences used in the phylogenetic tree included turbot and tiger puffer fabps, as well as those from other teleosts and human: human (Homo sapiens) FABP1 (NP_001434.1), FABP2 (NP_000125.2), FABP3 (NP_004093.1), FABP4 (NP_001433.1), FABP6 (NP_001035532.1), and FABP7 (NP_001437.1); zebrafish (Danio rerio) Fabp1a (NP_001038177.1), Fabp1b (NP_001019822.1), Fabp2 (NP_571506.1), Fabp3 (NP_694493.1), Fabp4a (NP_001004682.1), Fabp4b (NP_001018394.1), Fabp6 (NP_001002076.1), Fabp7a (NP_571680.1), Fabp7b (NP_999972.1), Fabp10a (NP_694492.1), and Fabp10b (NP_001373767.1); rainbow trout (Oncorhynchus mykiss) Fabp1 (XP_021446645.2), FABP1b (XP_036836002.1), Fabp2 (XP_021474749.1), Fabp3 (NP_001118185.1), Fabp4 (XP_021440549.1), Fabp6 (XP_021479883.1), Fabp7 (XP_020352070.1), Fabp7b (NP_001268271.1), and Fabp10a (XP_020353832.1); large yellow croaker (Larimichthys crocea) Fabp1 (XP_010731481.1), Fabp2 (XP_010735508.2), Fabp2b (XP_010743217.3), Fabp3 (XP_010733106.1), Fabp4 (XP_010755203.2), Fabp6 (XP_010742930.1), Fabp7 (XP_010744948.3), and Fabp10 (XP_010736463.2); turbot (Scophthalmus maximus) Fabp1 (XM_035640764.1), Fabp2 (XP_035476385.1), Fabp3 (XP_035477431.1), Fabp4a (XP_035477268.1), Fabp4b (XP_035491811.1), Fabp6 (XP_035491454.1), Fabp7a (XP_035466098.1), and Fabp10a (XP_035460943.1); tiger puffer (Takifugu rubripes) Fabp1 (XM_003974807.3), Fabp2 (XP_003976052.1), Fabp3 (XP_003968881.2), Fabp6 (XP_003970445.1), Fabp7 (XP_003971525.1), and Fabp10a (XP_003965684.1). The horizontal branch length is proportional amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented here was replicated after 1000 bootstrap iterations.

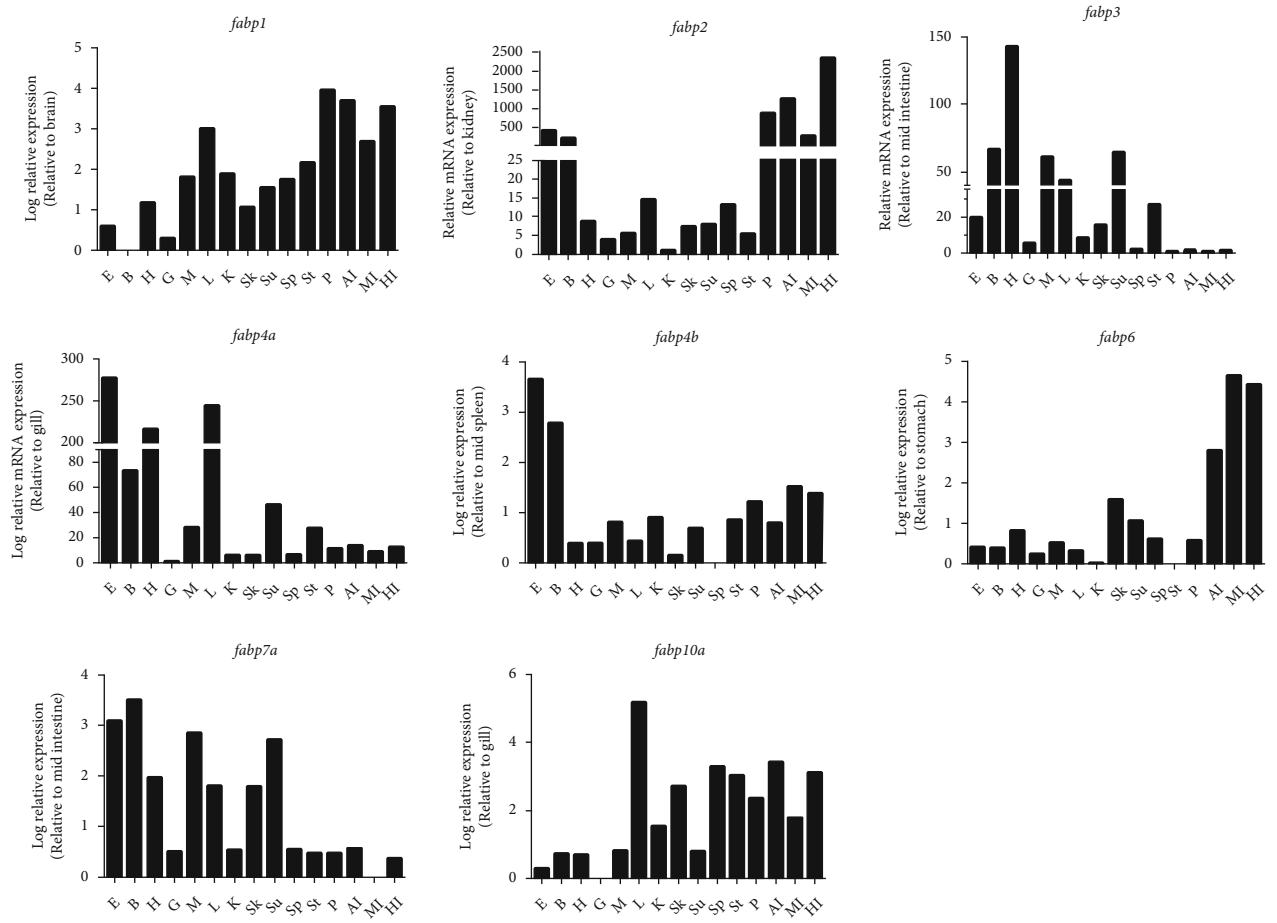

Figure 3: Tissue distribution of fabp expression in turbot. Results are expressed as mRNA expression relative to the lowest tissue expression (or log value). The following tissues were analyzed: eye (E), brain (B), heart (H), gill (G), muscle (M), liver (L), kidney (K), skin (Sk), and subcutaneous adipose tissue around the fin (Su), spleen (Sp), stomach (St), pyloric caecum (P), anterior intestine (AI), mid intestine (MI), and hind intestine (HI).

uptake and subsequent intracellular transport of diet-derived fatty acids [28, 29]. In zebrafish larvae, the expression of fabp2 in the intestine was correlated to the intracellular storage of lipid droplets and synthesis of very low-density lipoproteins [30]. High expression of fabp2 in the brain was also observed in zebrafish and common carp (Cyprinus carpio) [11, 31], indicating the diverse function of Fabp2. Fabp3 is reported to function as a transport protein mainly for mitochondrial β-oxidation in the muscle or heart [32–34]. The high expression of fabp7 in the brain was associated with its function in the transport of long chain-polyunsatureated fatty acids (LC-PUFA) [34, 35]. Our previous studies also revealed a high fabp7 expression in the liver of Japanese seabass, which may be related to the LC-PUFA biosynthesis there [10, 36].

Fabp4 mainly targets fatty acids to lipogenesis and lipid storage [33, 37] and thus has high gene expression level in tissues with developed adipocytes. In general, Fabp4 has the highest gene expression in the intraperitoneal adipose tissue. However, both turbot and tiger puffer do not have intraperitoneal adipose tissue. The Fabp4 sequence for tiger puffer was even not available in GenBank, which was why Fabp4 was not investigated in tiger puffer in the present study.

Although the tissue distribution of turbot fabps was generally in accordance with expectation, some phenomena should be noticed. Firstly, fabp1 even had higher expression level in the intestine than in the liver. In spotted gar (Lepisosteus oculatus), the highest expression level of fabp1 was also not observed in the liver, but rather in the heart [38]. Fabp1 binds cholesterol, free fatty acids, and their coenzyme A derivatives and thus has a wider scope of intracellular lipid transport functions [39]. In mammalian liver, fabp1 was even the only expressed fabp [23]. Mammalian fabp1 may accomplish several of the metabolic functions corresponding to the different fabps present in fish liver [40]. Secondly, a big difference existed between the tissue distributions of the two isoforms of fabp4, namely, fabp4a and fabp4b. The isoform fabp4b had a relative lower expression level in the liver compared to fabp4a. This indicates the possible function diversification between these two isoforms. A recent study with gold pompano also suggested the possible function diversification between fabp4a and fabp4b, as well as between fabp6a and fabp6b [15].

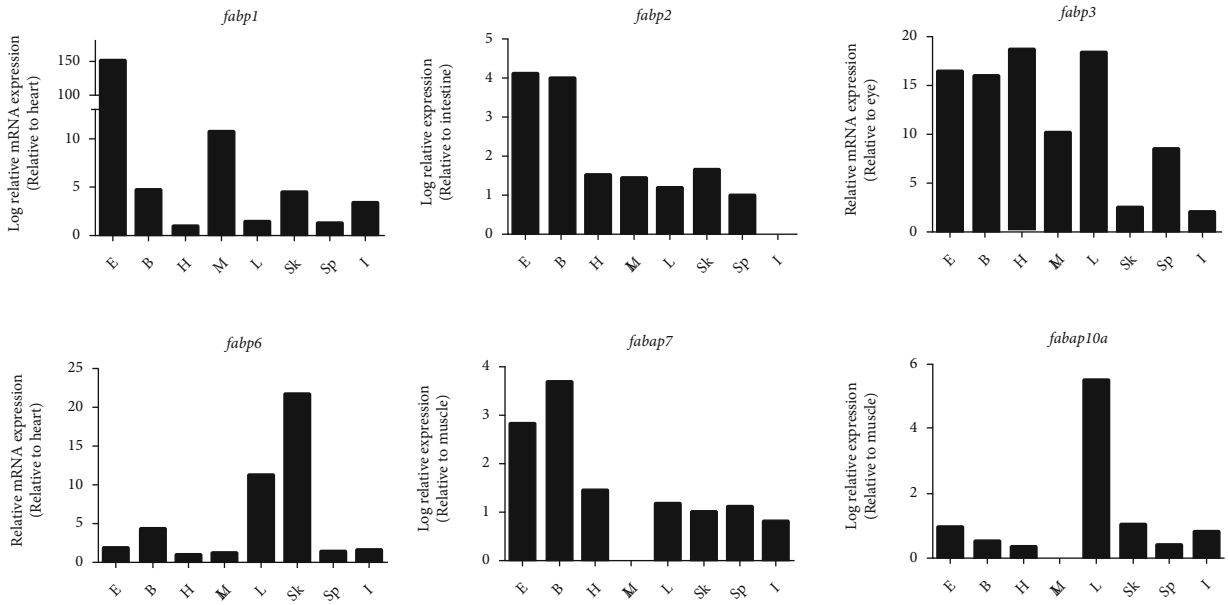

Figure 4: Tissue distribution of fabp expression in tiger puffer. Results are expressed as mRNA expression relative to the lowest tissue expression (or log value). The following tissues were analyzed: eye (E), brain (B), heart (H), muscle (M), liver (L), skin (Sk), spleen (Sp), and intestine (I).

However, when compared to turbot, the tissue distribution of tiger puffer fabps was not so regular (Figure 4). Both fabp1 and fabp2 of tiger puffer had the highest expression in the eye. The liver and intestine had very low expression level of fabp1 and fabp2, respectively. Similarly, tiger puffer fabp6 had the highest expression in the skin instead of intestine. All these features indicate the strange lipid metabolism in tiger puffer, considering fabp expression is closely related to lipid deposition [41]. Tiger puffer have a relatively special lipid storage pattern. They store lipid predominantly in the liver, have a very low lipid content in the muscle, and do not have intraperitoneal adipose tissue [17, 42]. The lipid metabolism in this fish species has evolved to adapt this special lipid storage pattern [43]. The lipid transport mechanisms, which were directly related to the fabp expression, could be special too in tiger puffer, but remain elusive to date and warrant further research. Moreover, the other subtypes of fabp1, fabp2, and fabp6 needed to be clarified by future studies.

3.4. The Liver fabp Expression in Response to Dietary Lipid Levels. In this study, the hepatic mRNA expression of fabps in both turbot and tiger puffer in response to dietary lipid levels was investigated, considering that the liver is the metabolic center and that all the analyzed fabps had considerable expression in the liver. In turbot, increasing dietary lipid levels decreased the mRNA expression of fabp2, fabp4b, and fabp6 (Figures 5 and 6). In fish fed the diet with the highest lipid level, the mRNA expression of fabp2, fabp4b, and fabp6 was only 1/4-1/3 of that in the control group. In tiger puffer, however, only the moderately high dietary lipid level decreased the mRNA expression of fabp2, fabp3, and fabp7.

Considering the important roles of fabps in intracellular lipid uptake and transport, the expression of fabps is assumed to be associated with the lipid levels in an animal body [41]. In recent fish studies, it has also been demonstrated that the reduced expression of fabps was associated with decreased lipid accumulation in fish tissues [44–47]. The decreased expression of fabps in the present study in response to high-lipid diets could be due to a self-protection system, which helps fish to prevent the overaccumulation of lipid in fish liver. As in terrestrial animals, fatty liver has been a serious health problem in aquaculture fish, especially when high-lipid diets are being more and more commonly used in aquaculture to spare the dietary protein. The roles of fabps in maintaining the lipid homeostasis in fish liver are beneficial to fish health when high-lipid diets are used.

Nevertheless, the response to dietary lipid level was different among different fabps. Unlike fabp2, fabp3, fabp6, and fabp7, the expression of fabp1 in both turbot and tiger puffer fed the extremely high-lipid diets was two-fold higher compared to the control group, whereas the expression of fabp4a in turbot and fabp10 in both fish species was not significantly affected by the experimental diets. It has been reported that Fabp1 can uniquely function in modulating the pattern of fatty acids esterified to phosphatidic acid, the de novo precursor of phospholipids and triacylglycerols [48]. In this consideration, when fish were fed high-lipid diets, the lipid-increasing roles of fabp1 and lipid-decreasing roles of fabp2 and fabp6 function concurrently to maintain the lipid homeostasis. Regarding the differences in the response of fabp expression to dietary lipid level between turbot and tiger puffer, it was difficult to explain these differences reasonably based on current data. Nevertheless, it is assumed that these differences were possibly

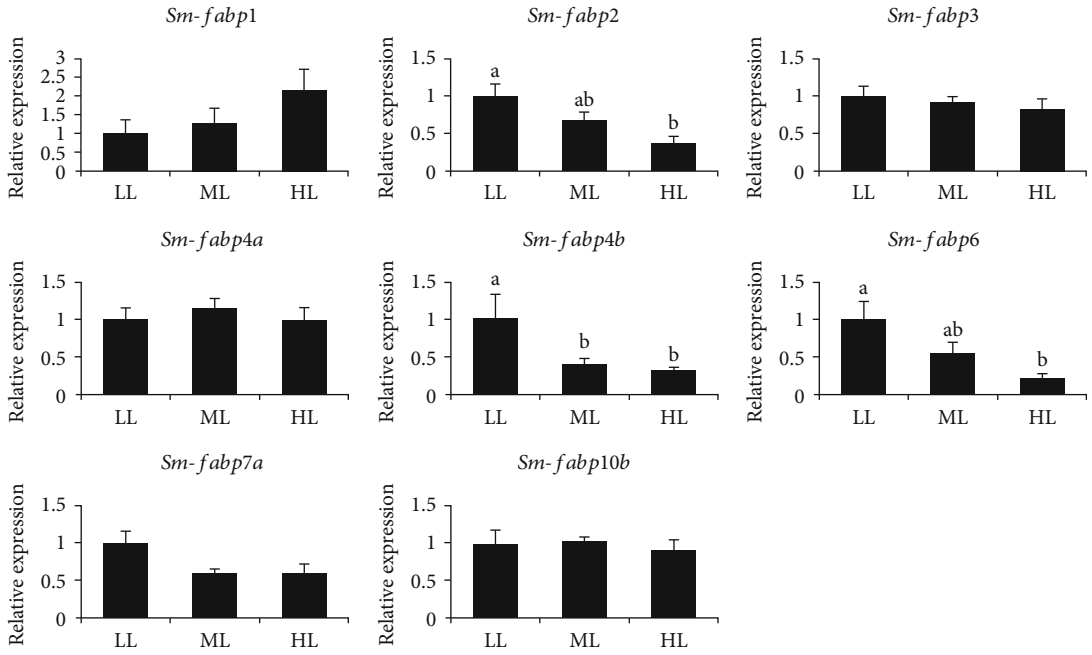

Figure 5: Effects of dietary lipid level on liver fabp expression of experimental turbot (mean ± standard error). Data bars not sharing the same letter were significantly different ($P < 0.05$).

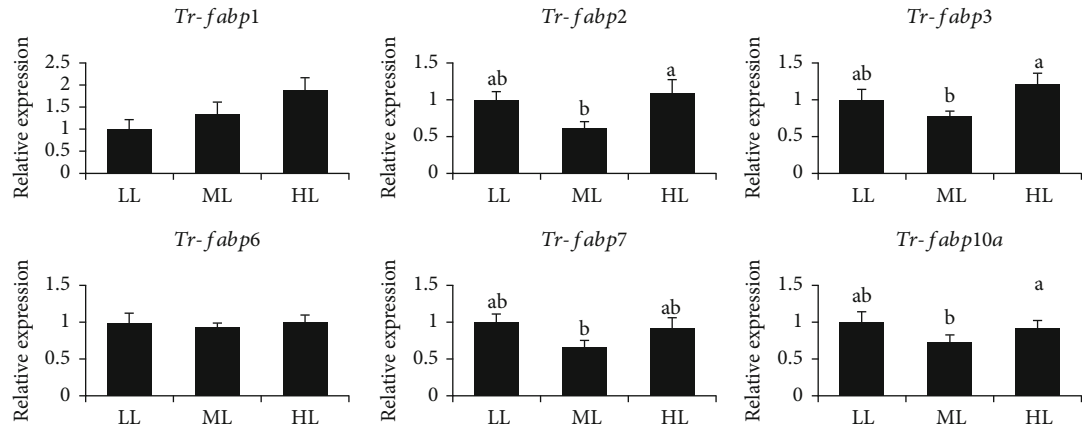

Figure 6: Effects of dietary lipid level on liver fabp expression of experimental tiger puffer (mean ± standard error). Data bars not sharing the same letter were significantly different ($P < 0.05$).

related to the difference in lipid storage pattern between these two fish species. Taking fabp3 as an example, since tiger puffer use the liver as a lipid storage site, an extremely high level in the diets, which usually led to high lipid accumulation in the liver, stimulated the motivation of lipid to β-oxidation mediated by fabp3. This prevented the further downregulation of fabp3 expression by extremely high dietary lipid levels as observed in turbot. The precise mechanisms involved in these differences between species warrant further research.

The response of fabp expression to dietary lipid level may also be related to the fatty acid profile of the diets. In general, different fabp isoforms reveal distinct binding preferences for specific fatty acids [35, 49]. In this study, the higher lipid level was obtained by adding fish oil into the control diet, resulting in high LC-PUFA levels in high-lipid diets. The stimulation of liver fabp1 expression by LC-PUFA has been reported in golden pompano, although the expression of fabp4 and fabp6 was also increased at the same time [50].

When the effects of dietary lipid sources on fabp expression were considered, the results in fish studies seem very complicated and diverse. In Atlantic salmon and hybrid grouper (♀Epinephelus fuscoguttatus × ♂E.lanceolatu), fish oil upregulated the fabp3 and fabp4 expression, compared to vegetable oil blend [51, 52]. However, in Senegalese sole larvae at 34 days posthatching, cod liver oil significantly downregulated the fabp3 expression compared to olive oil [53]. In Nile tilapia (Oreochromis niloticus), fish oil also

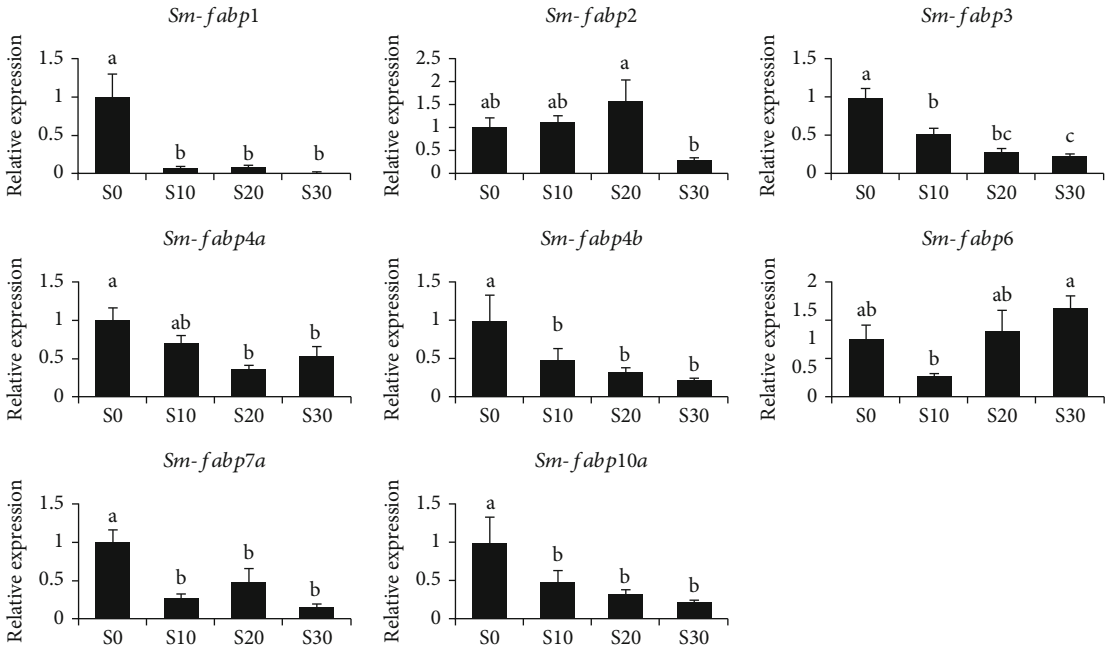

Figure 7: Effects of starvation period on liver fabp expression of experimental turbot (mean ± standard error). Data bars not sharing the same letter were significantly different ($P < 0.05$).

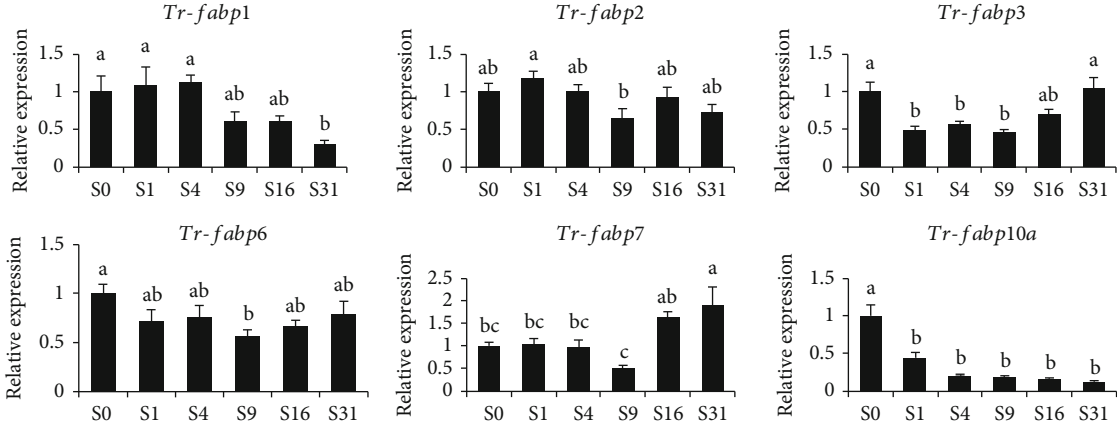

Figure 8: Effects of starvation period on liver fabp expression of experimental tiger puffer (mean ± standard error). Data bars not sharing the same letter were significantly different ($P < 0.05$).

downregulated the fabp3 expression when compared to palm oil [54]. In European sea bass (Dicentrarchus labrax) and grouper (Epinephelus coioides), the dietary lipid source even did not significantly affect the fabp expression [55, 56]. The discrepancies in different results could be due to the function diversification of fabps among fish species. In addition, the complex and dynamic fatty acid profiles in the lipid sources made it often difficult to compare the results directly [57].

A last notable result of the response of turbot and tiger puffer fabp expression to dietary lipid level was that differences were observed both between fabp4a and fabp4b of turbot and between turbot fabp6 and tiger puffer fabp6. These differences were in accordance to the sequence feature diversifications, indicating probable function diversifications.

3.5. The Liver fabp Expression in Response to Starvation. In the present study, starvation, in particular long-term starvation, generally resulted in downregulation of all analyzed fabps in both fish species (Figures 7 and 8). This was expected because starvation leads to substantially less active lipid metabolism. Relevant information was scarce in fish, but similar results have been observed in mammals [58]. Despite the expected results, other interesting results were observed when the two fish species or different fabps were compared. The downregulation of fabp expression in turbot was much more drastic and earlier compared to tiger puffer. This could be explained by the fact that turbot have much lower lipid deposition than tiger puffer, and consequently, turbot fabps were more susceptible to feed (lipid)

deprivation than their orthologs in tiger puffer. This difference was typical for fabp1, fabp3, and fabp7. Fabp3 plays an important role in transporting fatty acids to sites of β-oxidation inside cells [59]. The fluctuation in abundance of fatty acids, which are both suitable substrates for β-oxidation and high-affinity ligands of Fabp3, such as oleic acid (18:1n-9) and n-6 fatty acids, easily influences the fabp3 expression, as observed in Atlantic salmon [6, 60]. Fabp7 could be susceptible to the deprivation of dietary LC-PUFA.

Expression of both fabp4a and fabp4b in turbot was downregulated by starvation. This result indicates the possible similar roles of these two fabp4 subtypes in intracellular transportation of fatty acids for lipogenesis in adipocytes, despite the overall function diversification between them indicated by tissue distribution pattern.

Despite the ubiquitous downregulation of fabp expression by starvation, long-term (30-day) starvation increased the fabp7 expression (1.93 fold) in tiger puffer and tended to increase the fabp6 expression (1.56 fold) in turbot, indicating the starvation time-dependent characteristic. Long-term starvation may lead to compensating attempt in lipid uptake and utilization, as observed in javelin goby [16]. The compensating mechanism may also explain the upregulation of fabp3 expression by leptin injections in green sunfish (Lepomis cyanellus) [61], as well as the upregulation of fabp3 expression by stress in gilthead sea bream (Sparus aurata) [62].

4. Conclusions

In the present study, 7 fabp isoforms, i.e., fabp1, fabp2, fabp3, fabp4, fabp6, fabp7, and fabp10, in two marine teleosts, Scophthalmus maximus and Takifugu rubripes, were characterized in terms of sequence analysis, as well as expression in different tissues and under different nutritional status (different dietary lipid levels and different starvation periods). In general, turbot and tiger puffer fabp genes showed high identity to their orthologs in other fish species and mammals, but tiger puffer Fabp6 shared the lowest identity to its known orthologs in zebrafish and human. Tiger puffer fabps, especially Fabp1, Fabp2, Fabp6 and Fabp7, may have functions distinct from other teleosts, as indicated by the phylogenetic tree and the tissue distribution patterns. High dietary lipid levels downregulated the hepatic expression of fabp2, fabp3, fabp6, and fabp7a but tended to upregulate the hepatic expression of fabp1 in both fish species. Starvation ubiquitously downregulated the fabp expression in both fish species, but the downregulation of fabp expression in turbot was much more drastic and earlier compared to tiger puffer. Some specific differences in fabp expression were observed between fish species, among fabps, and between subtypes of a specific fabp. Future studies are needed to characterize the full repertoire of fabps in marine fish.

Data Availability

Raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

ML and HX are responsible for the conceptualization, funding acquisition, and supervision. DW, LZ, ZL, QB, and XM did the formal analysis and data curation. QM, MD, YW, and LC are assigned to the methodology and software. DW did the writing—original draft. HX did the writing—review and editing.

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