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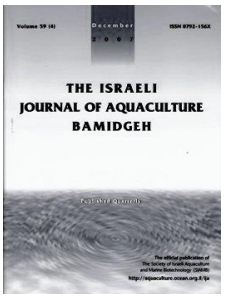

Intestinal Fatty Acid Binding Protein Gene (I-FABP) in Golden Pompano *Trachinotus ovatus* (Linnaeus 1758) Larvae: Ontogenetic Expression and Response to Water Temperature and Nutrition Manipulation

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Abstract

The gene for fatty acid binding proteins (I-FABP) in golden pompano *Trachinotus ovatus* larvae was cloned and analyzed from hatch to 18 days-post hatch (DPH). The I-FABP gene (GenBank accession: MF034871) of golden pompano is composed of 815 bp with an open reading frame of 399 bp, encoded in one amino acid with a molecular weight of 15.24 kDa. The predicted amino acid sequence of I-FABP genes from golden pompano showed high similarity and identity with Japanese sea bass *Lateolabrax japonicus* (97% and 87.9%, AOW69620.1). The highest tissue expression of I-FABP genes was found in the intestine, followed by the eye on 18 DPH. During the ontogenetic development, the expression of I-FABP genes remained at a low level during the first five days, and reached the highest level on 12 and 18 DPH. The expression of I-FABP genes was not significantly affected by environmental temperature on 12 DPH, but was significantly affected by the temperature on 18 DPH. Nutrition enhancement with algae containing high fatty acids significantly affected the expression of I-FABP genes. The highest expression was observed in the non-enriched treatment, but the lowest expression was in the *Nannochloropsis* feeding treatment. Results of the present study indicate that the expression of the I-FABP gene varies with environmental temperature and nutritional conditions during the ontogenetic development of golden pompano larvae. The expression of I-FABP genes may be potentially used as an indicator for assessing nutrient supply and functional development of the digestive system in fish larvae.

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Introduction

Fatty acid binding proteins (FABPs) belong to a multi-gene family of 14-16 kDa molecular mass and bind long chain fatty acids in both vertebrates and invertebrates (Alvite et al., 2008; Borchers et al., 1989; Kanda et al., 1989). The length of FABPs varies from 126 to 137 amino acids depending on species (Chen and Shi, 2009; Pelsers et al., 2005; Sharma et al., 2004). FABPs can mediate the transportation of free fatty acids for targeting specific metabolic pathways, protecting cells from cytotoxic effects of free fatty acids, modifying lipid metabolic enzymes, and participating in fatty acid signaling within the nucleus (Besnard et al., 2002; Lowe et al., 1987; Storch and McDermott, 2009). Different FABP types have been named after the mammalian tissue from which they were first isolated, such as intestine, heart, liver, myelin, and adipose tissues. Early studies have confirmed that the existence of these FABP types fulfilled specific roles associated with the histological structure and physiological functions of different tissues (Banaszak et al., 1994; Veerkamp et al., 1991; Veerkamp et al., 1993).

The intestinal fatty acid-binding protein (I-FABP) is a small cytosolic protein and has been considered to play a crucial role in intracellular fatty acid trafficking and metabolism in fish gut (Her et al., 2004). Evidence has indicated that the expression of I-FABP genes is an important marker for intestinal differentiation in humans (Sonnino et al., 2000), rats (Likic and Prendergast, 1999), frogs (Chalmers et al., 2000), and fish (Pierce et al., 2000). The expression levels of I-FABP genes may be related to the status of tissue damage and regeneration (Schroyen et al., 2012; Simula et al., 2010). In commercially cultured fish, the I-FABP has been selected as a marker to investigate physiological function and response to the nutrition change in fish diets (Overland et al., 2009; Venold et al., 2013; Yamamoto et al., 2007), but its role in fish larvae is largely unknown.

Golden pompano belongs to the family of Carangidae and is a good candidate species for aquaculture owing to fast growth and suitability for cage culture (Ma et al., 2014). Although artificial breeding and culture of this species have made substantial progress, the poor quality of juvenile fish is a major issue hindering further expansion of production of this species in hatcheries (Ma et al., 2016b; Zheng et al., 2016). The understanding of the ontogeny of the digestive system and nutritional requirement of fish larvae may improve management of fish feeding and fingerling quality in the hatchery production system. This study aims to quantify the expression of I-FABP genes at different water temperatures and feed types in golden pompano larvae from hatching to the formation of a functional stomach. The expression pattern of I-FABP genes could provide essential information to assess the functional change of the digestive system of golden pompano larvae during early ontogeny. In addition, the expression level of I-FABP genes may be used as a potential indicator to predict nutrient malformation of fish larvae in aquaculture.

Materials and methods

Larval rearing of golden pompano. The fish specimens in this study were obtained from a previous feeding trial in our laboratory (Ma et al., 2016a) in which fertilized eggs of golden pompano hatched in 500L fiberglass incubators at 26.5°C. On 2 DPH, larvae were stocked into three 1000L larval rearing tanks, supplied with upwelling filtered seawater (5-μm pore size) from the bottom of each tank and a daily exchange rate of 200%. Two air stones were used in each tank to maintain dissolved oxygen close to saturation. Light intensity was maintained at 2400 lux. The light regime was set at 14 h light and 10 h dark. Salinity was maintained at 33 ± 0.8‰, and water temperature was 26.5±1.0°C throughout the experiment. Rotifers (*Brachionus rotundiformis*) were fed to fish larvae from 2 DPH to 10 DPH at a density of 10-20 ind/mL. Artemia nauplii were added into the rearing tank from 10 DPH until completion of the experiment. Both rotifers and Artemia nauplii were enriched with DHA Protein Selco (INVE Aquaculture, Salt Lake City, USA) according to the manufacturer's instructions.

Response of I-FABP gene to rearing temperature. Upon arrival, all eggs were transferred into 500L incubators and hatched at 26°C. The experimental conditions included three constant temperatures, 23, 26, and 29°C with three replicates each. On 2 DPH, yolk sac larvae were acclimatized at each of these temperatures for 5 h, and

stocked in 500L fiberglass tanks at a density of 60 fish/L. Apart from the different rearing temperatures, all feeding protocols and rearing conditions were the same as described above.

Response of I-FABP gene to nutrition manipulation. The current experiment included three dietary treatments with three replicates each. Artemia nauplii were nutritionally used in three ways: (1) enriched with instant microalgal paste (*Nannochloropsis* sp., Qingdao Hong Bang Biological Technology Co., Ltd, Qingdao, China); (2) enriched with Algamac 3080® (Aquafauna, USA); and (3) with no enrichment as control. In the experimental diet, the Nannochloropsis enriched diet contained the highest content of polyunsaturated fatty acids (49.63 ± 3.78 % of total fatty acids), while the lowest one was observed in the non-enriched diet (40.64 ± 4.39 % of total fatty acids) (Yang et al., 2015).

Total RNA extraction and reverse transcription. On 0, 1, 2, 3, 4, 5, 12, and 18 DPH, approximately 300 mg (wet weight) fish larvae were sampled from rearing tanks in triplicate. Approximately 50 individuals were collected on 12 DPH and 18 DPH to assess the effects of temperature and nutrition manipulation. On 18 DPH, a total of 100 individuals were collected and examined under a dissecting microscope for the analysis of gene expression in tissues. Total RNA was extracted using TRIzol (Invitrogen, USA). RNA integrity was verified by electrophoresis on a formaldehyde-agarose gel (1.2%). The RNA concentration was measured by absorbance at 260 nm and the purity was determined at the ratio of absorbance between 260 nm and 280 nm (260/280). RNA was reverse-transcribed to cDNA with oligo (dT) primers using a PrimeScript first strand cDNA synthesis kit (TaKaRa Biotechnology, Dalian Co., Ltd). The cDNA was used as a template in subsequent PCR.

Cloning of the gene cDNA and real-time PCR. Based on a preliminary study on golden pompano transcriptome sequences in our laboratory (Illumina HiSeq2000, annotated by NR, KOG, kegg, and Swissprot), the primers for genes cloning were designed with Primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA) (Table 1). The PCR reaction systems included 1 μL of golden pompano larval cDNA, 1 μL of gene-specific forward primer (F), 1 μL of gene-specific reverse primer (R), 0.5 μL of ExTaq, 5 μL of PCR buffer, 4 μL of dNTP mixture (2.5 μM) and 37.5 μL of ddH_2O, adding up to a total volume of 50 μL. The PCR conditions were denaturation at 94°C for 1 min, 35-cycles of 94°C for 30 s, annealing temperature of each gene for 30 s, 72°C for 4 min, followed by a 10 min extension at 72°C. The PCR products were cloned into the PMD-19T vector (TAKARA, Japan), and then sequenced.

Table 1 Sequences of primers used in this study.

Primers	*Sequence (5'-3')*	*Amplicon sizes (bp)*
I-FABP -F	GGCATGGCACAGTTCTT	689
I-FABP -R	CACTTTTCACAGGTTATTAGGT	
I-FABP- qF	CGGCTCCTGGAAAATTGATC	111
I-FABP- qR	ATGGTTATCTTGAGGTTGTCGTG	
EF-1α-qF	CCCCTTGGTCGTTTTGCC	101
EF-1α-qR	GCCTTGGTTGTCTTTCCGCTA	

Quantitative real-time PCR was used to analyze the level of I-FABP gene expression in golden pompano larvae. Gene specific primer pairs for the I-FABP gene (Table 1) were amplified in the LightCycler480 II system (Roche, Switzerland). EF-1α was used as the internal reference and amplified. The cycling conditions for I-FABP genes and EF-1α were as follows: 1 min at 95°C, followed by 40-cycles 95°C for 15 s, and 60°C for 1min. Dissociation curves were used to guarantee that only one single PCR product was amplified in each gene reaction. For each test, three replicates were performed. The relative quantification (RQ) was calculated using the ΔΔCT (comparative threshold cycle) method:

ΔCT = CT of target gene - CT of EF-1α,

ΔΔCT = ΔCT of any sample - ΔCT of calibrator sample.

The efficiencies of the primers (E) were E I-FABP = 0.1001.

Sequences and phylogenic analysis. The I-FABP gene cDNA sequences were analyzed by BLAST at the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The complete ORF regions and amino acid sequences were deduced with ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The molecular weight (Mw) and isoelectronic point (pI) of deduced amino acids were computed by the pI/Mw tool of ExPASy (http://web.expasy.org/compute_pi/). Protein domains were predicted using SMART (http://smart.embl-heidelberg.de/). Multiple sequence alignments of amino acids were performed by ClustalX 2.1. The phylogenetic tree was constructed by the neighbor-joining (NJ) method in MEGA 6.0, and the bootstrap values were replicated 1000 times to derive the confidence value for the analysis (Tamura et al., 2013). Pairwise deduced amino acid sequence identity and similarity matrices of the I-FABP family sequences from various species were performed using Matgat 2.02 (Campanella et al., 2003). The three-dimensional structures of golden pompano I-FABP were constructed through homology modelling (http://swissmodel.expasy.org/workspace/index.php).

Statistical analysis. The data were expressed as mean ± SD, and compared with one-way ANOVA (PASW Statistics 18.0, Chicago, SPSS Inc.). Tukey's test was used for multiple range comparisons with the level of significant difference set at $P < 0.05$. All data were tested for normality, homogeneity and independence to satisfy the assumptions of ANOVA.

Results

Cloning and sequencing of golden pompano intestinal fatty acid binding protein (I-FABP) gene cDNA. The length of I-FABP gene cDNA sequence in the golden pompano (GenBank accession: MF034871) was 815 bp with an open reading frame (ORF) of 399 bp, which encoded one amino acid (aa) with a calculated molecular weight (Mw) of 15.24 kDa and theoretical isoelectric point (pI) of 6.13. The bioinformatics analysis of the deduced polypeptide sequence revealed the signature sequence of a cytosolic fatty-acid binding protein (Fig. 1). The molecular modelling of golden pompano I-FABP is shown in Fig. 1. The golden pompano I-FABP sequence shared 67.94% identity with the rat intestinal fatty acid binding protein (PDB ID: 1ifc.1. A). There was one beta sheet, two helixes in N-terminal amino acids and 10 anti-parallel beta sheets forming a hydrophobic pocket.

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   1 TGCAAACAGTTCTGCCATTCAAAAAGAATACAGAGTCATTTGGCATGGATTATTGGGCAT 60
  61 GGCACAGTTCTTTCTATTCAAAACCACTGTGTTGCCCTATCAGCTGCAGTGCTAGATGTT 120
 121 GCCATGAGAAAATTTGAGCTTTAAACGGCCACATCAGCATTCAGATAGATGACGAGATAA 180
 181 GAGAGTGTGTGGTTTAAAAGGAGCGGCAGACTTTGAGTAAGACACTCCTTGCTGCAGAGT 240
 241 TGTCCAGTTCAGCTCCCACCGCCACCatgaccttcaacggctcctggaaaattgatcgca 300
   1                          M  T  F  N  G  S  W  K  I  D  R  N  12
 301 atgaaaactatgagaaattcatggaacaaatgggaattaacatggtgaagaggaagctgg 360
  13  E  N  Y  E  K  F  M  E  Q  M  G  I  N  M  V  K  R  K  L  A  32
 361 ctgctcacgacaacctcaagataaccattgaacagactggagacaagtttcatgtcaagg 420
  33  A  H  D  N  L  K  I  T  I  E  Q  T  G  D  K  F  H  V  K  E  52
 421 agagcagtaatttccgcactctggaaatagacttcaccctgggggtcacctttgagtaca 480
  53  S  S  N  F  R  T  L  E  I  D  F  T  L  G  V  T  F  E  Y  S  72
 481 gccttgcagatggaacagaactaacaggctcatggaccattgagggagacatgatgaagg 540
  73  L  A  D  G  T  E  L  T  G  S  W  T  I  E  G  D  M  M  K  G  92
 541 gggttttcatcagaaaggacaatggaaagcagctgacaacaaccagaatcattcaaggag 600
  93  V  F  I  R  K  D  N  G  K  Q  L  T  T  T  R  I  I  Q  G  D 112
 601 atgaactcgtacagagctacaactatgatggtgtggacgcaaagaggattttcaagaggg 660
 113  E  L  V  Q  S  Y  N  Y  D  G  V  D  A  K  R  I  F  K  R  G 132
 661 gttagACCACAAATGTTTGATTACAGGATTACATACAGTATTGTGATAAATCATTGACTT 720
       *
 721 ATACCTAATAACCTGTGAAAAGTGCACTTCTTGTAATGCCATATATTTGAATTGCATTGG 780
 781 ATTTTGATACTTGCAGTAATAAAGTGATACTGTAA                          815
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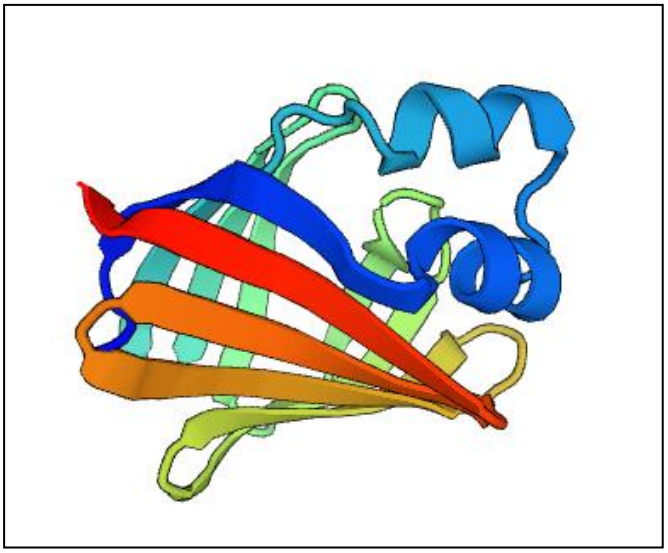

Fig. 1 Nucleotide sequence and deduced amino acids of the intestinal fatty acid binding protein (I-FABP) gene and predicted tertiary structure of I-FABP from golden pompano Trachinotus ovatus (Linnaeus 1758) Cytosolic fatty-acid binding proteins signature was underlined.

Multiple sequence alignments and phylogenetic analysis. Multiple sequence alignment of the deduced amino acid sequences of I-FABP genes with some known I-FABP family amino acid sequences from various species is shown in Table 2. The predicted amino acid sequence of I-FABP genes from golden pompano showed high similarity and identity with Japanese seabass *Lateolabrax japonicus* (97% and 87.9%, AOW69620.1) and large yellow croaker *Larimichthys crocea* (93.2% and 85%, ALP43793.1), but different similarity (82.6-93.9%) and identity (65.2-82.6%) with other species (Table 2). The phylogenetic tree of hedgehog genes (Ma et el., 2017) comprised two main clusters, i.e., the fish clusters, and the bird and mammal clusters (Fig. 2). The deduced I-FABP amino acid sequences of eight fishes and three other vertebrates contained the cytosolic fatty-acid binding proteins signature, and all showed high identity and similarity (Fig.3).

Table 2. Identity and similarity of I-FABP between golden pompano and other species homologue.

Species	*Accession NO.*	*AA*	*Similarity (%)*	*Identity (%)*
Trachinotus ovatus	Present study	132		
Lateolabrax japonicus	AOW69620.1	132	97	87.9
Larimichthys crocea	ALP43793.1	132	93.2	85
Oncorhynchus kisutch	XP_020352621.1	132	93.9	81.1
Danio rerio	AAF00925.1	132	90.2	82.6
Cyprinus carpio	ADF28554.1	132	90.2	80.3
Salmo salar	ACI66628.1	132	92.4	79.5
Ictalurus punctatus	NP_001187833.1	132	89.4	76.5
Columba livia	NP_001269737.1	132	86.4	75.8
Columba livia	NP_000125.2	132	82.6	65.2
Mus musculus	NP_032006.1	132	82.6	65.9
Rattus norvegicus	NP_032006.1	132	83.3	67.4

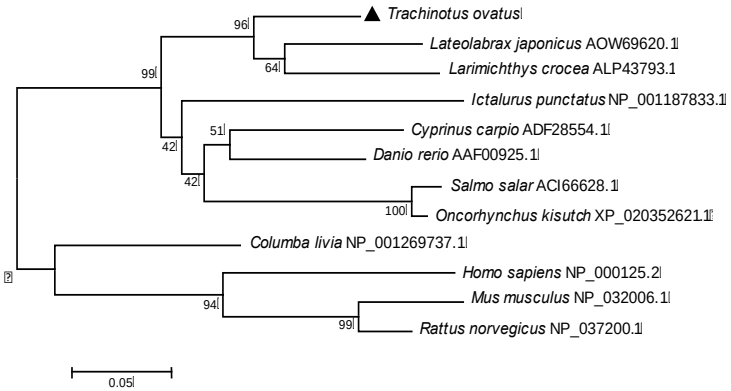

Fig 2. Phylogenetic tree of intestinal fatty acid binding protein. The numbers represent the frequencies with which the tree topology presented here were replicated after 1000 bootstrap iterations.

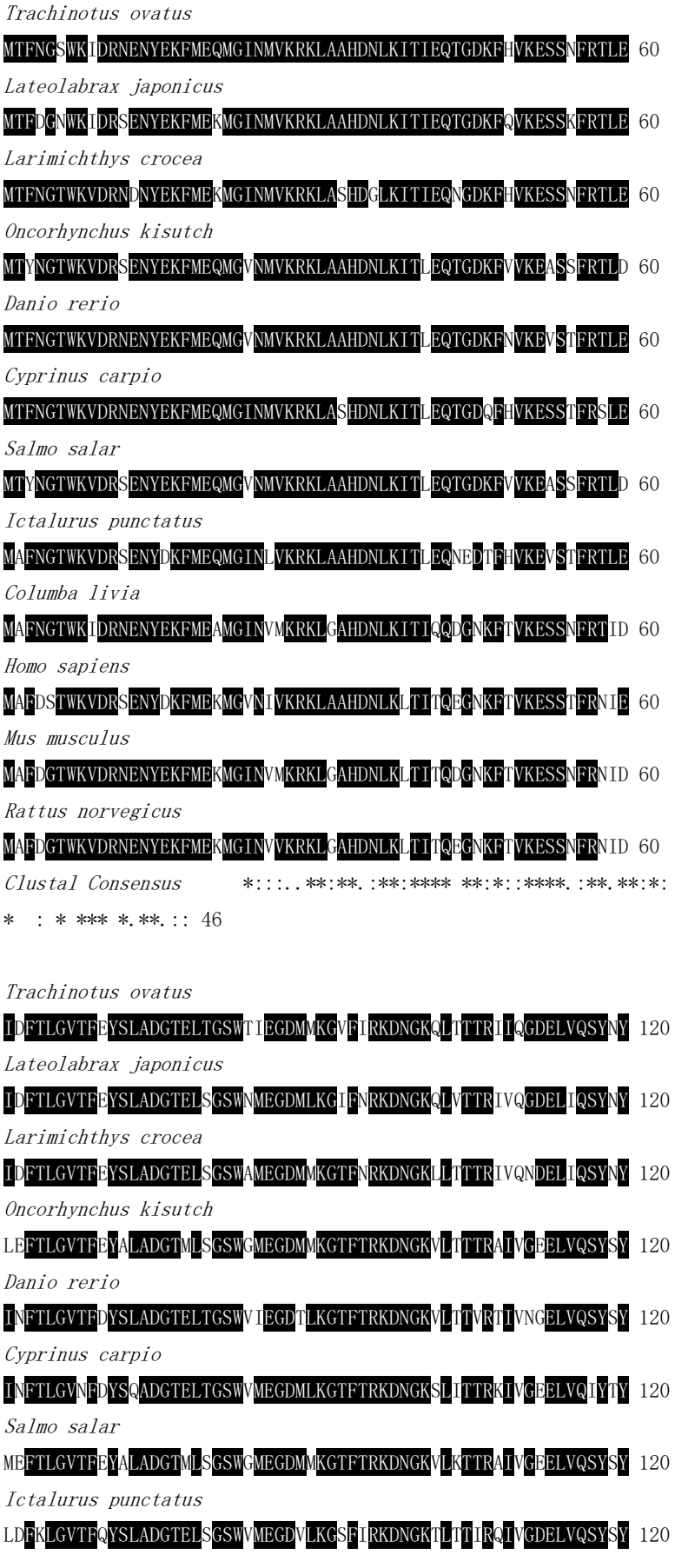

Fig 3. Multiple sequence alignment of the deduced amino acid sequence of I-FABP with other known homologous I-FABP amino acid sequence.

Ontogenetic expression of I-FABP gene. The expression level of I-FABP genes in golden pompano larvae was low at hatching, but slowly increased with the increase of fish age from 0 DPH to 5 DPH (Fig. 4). The expression of I-FABP genes reached the highest level on 12 DPH ($P < 0.05$), and remained at the similar level until the end of the experiment on 18 DPH.

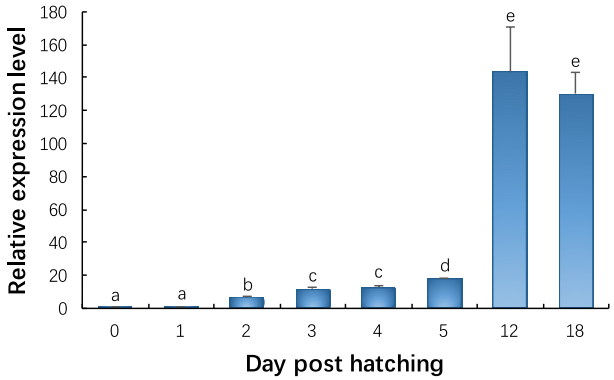

Fig. 4. Ontogenetic expression of the I-FABP gene in golden pompano larvae. Data with different letters were significantly different ($P < 0.05$).

Tissue expression of I-FABP gene in golden pompano. On 18 DPH, the highest expression of I-FABP gene in golden pompano was observed in the intestine ($P < 0.05$, Fig. 5), followed by in the eye. The expressions of I-FABP genes in the brain, gills, head-kidney, spleen, and stomach were significantly lower than the expression observed in the liver, muscle, and heart ($P < 0.05$). The expression of I-FABP gene in the muscle and heart was not significantly different ($P > 0.05$).

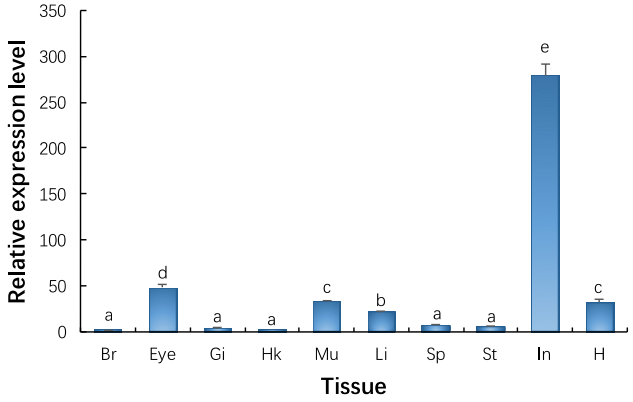

Fig. 5 Tissue expression of I-FABP gene in golden pompano larvae. Data with different letters were significantly different ($P < 0.05$). Abbreviations: Br, Brain; Gi, Gill; Hk, Head-kidney; Mu, Muscle; Li, Liver; Sp, Spleen; St, Stomach; In, Intestine; H, Heart; K, Kidney.

Response of I-FABP genes to water temperature and nutrition manipulation. On 12 DPH, the expression of I-FABP gene was not significantly affected by rearing temperature ($P > 0.05$, Fig. 6). On 18 DPH, the highest expression of I-FABP gene was found at 29°C ($P < 0.05$), but the expression of I-FABP gene was not significantly different between fish cultured at 23°C and 26°C ($P > 0.05$). The expression of I-FABP gene was significantly

affected by nutrition manipulation ($P < 0.05$, Fig. 7). The highest expression of I-FABP gene was observed in the non-enriched group, and lowest expression of I-FABP gene was found in the *Nannochloropsis* enriched group.

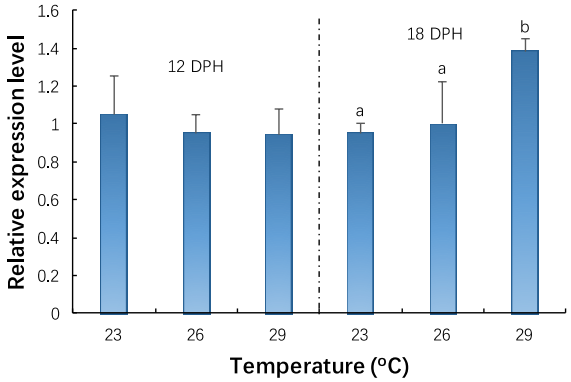

Fig. 6 Response of I-FABP gene to water temperature in golden pompano larvae. Data with different letters were significantly different ($P < 0.05$).

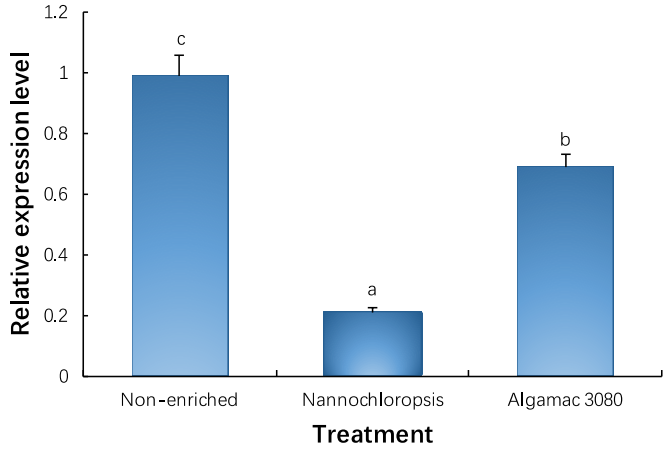

Fig. 7 Response of I-FABP gene to nutrition manipulation in golden pompano larvae. Data with different letters were significantly different ($P < 0.05$).

Discussion

In the present study, the I-FABP gene in golden pompano larvae was successfully isolated and identified. Similar to the FABP obtained from other species, such a unique structure in I-FABP allows it to actively participate in transporting fatty acids and other lipid soluble substances within cells (Andre et al., 2000; Hsu and Storch, 1996; Venold et al., 2012).

Expression of the I-FABP gene during ontogenetic development. In the present study, the expression level of I-FABP gene in fish remained at a low level at hatching, but slowly increased before 5 DPH. On 12 DPH, the expression of I-FABP sharply increased and reached the highest level. This expression pattern is consistent with the development of the digestive tract of golden pompano larvae, as the digestive system of golden pompano is primitive at hatching, and a functional digestive system appeared around 15 DPH (Ma et al., 2014). The expression of I-FABP gene during embryogenesis and early development has been reported in zebrafish through in situ hybridization (Andre et al., 2000; Sharma et al., 2004). To the best of our knowledge, there was no report on the expression level of I-FABP gene during early development of fish larvae. In terrestrial species such as pigeon, chickens, and turkeys, the expression of I-FABP gene significantly increases after hatching (Ding and Lilburn, 2002; Katongole and March, 1980; Xie et al., 2013), while the expression level of I-FABP gene in mice rises rapidly on day 17 during embryonic development (Green et al., 1992). Furthermore, the increase of I-FABP over time after hatching may be correlated with the uptake of dietary fatty acids

after the formation of a functional digestive tract at the late developmental stage of larval golden pompano.

Expression of I-FABP gene in different tissues. Although FABPs were originally named from the tissue where they were discovered, they are widely expressed proteins, and their expressions are species dependent in animal tissues. For instance, the expression of I-FABP gene is only observed in the intestinal tissue of humans (Sweetser et al., 1987), while the expression of I-FABP gene in zebrafish (*Danio rerio*) can be detected in intestine and brain (Sharma et al., 2004). In Atlantic salmon (*Salmo salar*), the expression of I-FABP gene can be observed in various tissues such as stomach, pyloric caeca, intestine, spleen, muscle, and brain (Venold et al., 2013). In the present study, the highest expression of I-FABP gene was observed in intestine, followed by the eye, and then in the muscle and heart. Furthermore, the expression of I-FABP gene was also observed in the stomach of larval golden pompano, but the expression level was lower than that reported in Atlantic salmon (Venold et al., 2013). In the present study, the expression of I-FABP gene in the eye of larval golden pompano was not previously reported in fish, and the functional expression of this gene in the eye may be related to vision development but this claim warrants further investigation.

Response of I-FABP gene to water temperature. Temperature is an important environmental factor in larval fish development, and can significantly affect fish feeding behavior and metabolism (Blaxter, 1992; Ma, 2014). Early studies have demonstrated that environmental temperature can regulate fatty acid metabolism and composition in fish (Farkas et al., 1980; Kemp and Smith, 1970; Skalli et al., 2006). As an important fatty acid binding protein in fish, I-FABP plays an essential role in fatty acid absorption (Storch and Thumser, 2010). However, it is unclear if temperature can affect the expression of I-FABP gene during the early development of fish larvae. In this study, the expression of I-FABP gene was not significantly affected by water temperature on 12 DPH, but was significantly affected by temperature on 18 DPH. Such difference may reflect the developmental stage of the digestive system in larval golden pompano as the functional stomach did not appear until 18 DPH.

Response of I-FABP gene to nutrition enhancement. Fatty acid binding proteins can affect gene regulation, leading to up-regulation of lipid related genes via activation of the peroxisome proliferating receptor (Lawrence et al., 2000; Tan et al., 2002). In Atlantic salmon, the expression level of I-FABP genes reduced when fish were fed with soybean meal (Venold et al., 2013). The reduction of the expression level of I-FABP gene is related to inflammation in the distal intestine of Atlantic salmon due to inclusion of soybean meal in the diet and is a sign of functional loss for the ability to bind dietary fatty acids (Venold et al., 2013). In the present study, the expression of I-FABP gene was significantly affected by nutrition enhancement. The highest expression of I-FABP gene was observed in the non-enriched feeding group, and the lowest expression was found in the *Nannochloropsis* enriched group. This expression pattern is inversely proportional to the total amount of polyunsaturated fatty acids in the diet. In the experimental diet, the *Nannochloropsis* enriched diet contained a higher content of polyunsaturated fatty acids than the non-enriched diet (Yang et al., 2015). This may suggest that the total poly unsaturated fatty acid in the diet has a negative effect on the expression of I-FABP gene in golden pompano larvae. However, there is no direct evidence to prove it, and this may need further investigation.

In summary, the I-FABP cDNA was cloned and analyzed in golden pompano larvae in this study. The expression of I-FABP gene in golden pompano larvae was significantly affected by water temperature and fatty acid content in the feed when the functional stomach formed on 18 DPH. The time dependent expression of I-FABP gene in fish larvae is important to understand the ontogenetic development and growth of fish larvae in early life. The monitoring of I-FABP gene expression in golden pompano larvae may serve as a useful indicator in the field and on fish farms, leading to a rapid assessment of environmental conditions and nutrition impact on fish development.

Acknowledgements

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