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Response of Liver-Type Fatty Acid-Binding Protein (*L-FABP*) Gene in Golden Pompano *Trachinotus ovatus* (Linnaeus 1758) to Temperature and Nutrient Manipulations

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SZ interpreted the results and wrote the manuscript. PW and CZ analyzed the data. MFB and JGQ modified the paper. LQ conceived and designed the experiments. ZM and ML initiated the project.

Key words
L-FABP, *Trachinotus ovatus*, Ontogeny, Temperature, Nutrition enhancement.

ABSTRACT

The liver-type fatty acid binding proteins (L-FABP) gene in golden pompano *Trachinotus ovatus* larvae was cloned in the present study. The full length of *L-FABP* cDNA from golden pompano was 604 bp, including a 5'-untranslated region (UTR) of 154 bp, a 3'-UTR of 69 bp and an open reading frame (ORF) of 281 bp. L-FABP encoding a polypeptide of 126 amino acids with a predicted molecular weight of 14.06 kDa and a theoretical isoelectric point of 8.73. The results of qRT-PCR showed that the highest tissue expression of *L-FABP* gene was observed in fish liver on 18 DPH. Along with the ontogeny of fish larvae, the expression of *L-FABP* gene was low at hatching, and quickly increased with the increase of fish age from 0 DPH to 4 DPH, and reached the highest level on 12 DPH. The highest expression of *L-FABP* gene was observed in fish cultured at 29°C on both 12 DPH and 18 DPH ($P< 0.05$). Nutrition enhancement significantly affected the expression of *L-FABP* gene, the highest expression was observed in the Algamac 3080 treatment, and lowest expression was observed in the *Nannochloropsis* treatment. This study provides useful information on the functional mechanism of *L-FABP* gene in the ontogenetic development of golden pompano.

INTRODUCTION

The liver fatty acid binding protein (L-FABP) is a 14-kDa cytoplasmic protein that binds long-chain fatty acids with high affinity (Veerkamp and Maatman, 1995). The mammalian liver fatty acid binding protein (L-FABP) is a small cytosolic protein in various tissues including liver, small intestine and kidney and is thought to play a crucial role in intracellular fatty acid trafficking and metabolism (Her *et al*., 2003; Saqlain *et al*., 2018). To date the complete primary structures of L-FABPs of non-mammalian vertebrates have been determined for chicks (Cecilian *et al*., 1994), frogs (Baba *et al*., 1999), catfish (Pietro *et al*., 1996) and shark (Medzihradszky *et al*., 1992). In birds, the changes of the expression level of *L-FABP* gene may relate to embryogenesis (Murai *et al*., 2009).

Golden pompano belongs to the family of Carangidae and is a good candidate species for aquaculture due to its rapid growth and suitability for culture (Ma *et al*., 2014). At the early life stage, the supplement of dietary fatty acids significantly affects the growth, survival, behavior and biological functions and processes of fish larvae (Izquierdo, 1996; Hamre *et al*., 2013). In several studies, fish larvae exhibit slow growth and low survival, when essential fatty acids are insufficient in feed (Cahu *et al*., 2003; Kattner *et al*., 2003; Hamre *et al*., 2013). Like most marine fish larvae, the pompano requires a high amount of fatty acids especially highly unsaturated fatty acids in larvae during early ontogeny, and the unbalanced supply of dietary unsaturated fatty acids can cause significant body malformations (Yang *et al*., 2015; Ma *et al*., 2017). Although many studies have explored the fatty acid

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requirement of fish larvae, the quantitative requirements for fish larvae in the same species reported by various authors are often contradictory (Izquierdo, 1996). In order to understand the requirement of fatty acids for target fish species, knowledge on the biochemical and molecular mechanisms underlying the requirement of essential fatty acids is essential. As L-FABP can mediate the transportation of free fatty acids for targeting to specific metabolic pathways (Storch and McDermott, 2009), the understanding of the functional expression of *L-FABP* gene will improve our knowledge on the digestive ontogeny and nutrition requirement of fish larvae, and ultimately improve fingerling quality (Ma *et al*., 2012).

Therefore, this study was designed to explore the expression of L-FABP during the ontogeny of golden pompano larvae in the first 18 DPH, and the effects of water temperature and nutrition manipulation on the *L-FABP* gene expression. Results from the present study would provide essential information on the digestive ontogeny of golden pompano larvae, and deliver a potential digestive functional indicator in the early development of fish larvae.

MATERIALS AND METHODS

Ethics statement

The *Trachinotus ovatus* is not endangered or protected species, and there is no requirement for permission to perform experiments involving this species in China.

Expression of L-FABP *gene in the first 18 days of golden pompano larvae*

The fish specimens in study were obtained from a previous feeding trial in our laboratory (Ma *et al.*, 2016). In brief, fertilized eggs of golden pompano hatched in 500-L fiberglass incubators at 26.5°C with a hatching rate of 97.5±1.5% (mean±SD). On 2 DPH, larvae were stocked into three 1000-L larval rearing tanks. Larval rearing tanks were supplied with filtered seawater (5-μm pore size) from the bottom of each tank through upwelling with a daily exchange rate of 200% tank volume. Water was discharged through an outlet screen (300 μm) on the upper side of each tank, and the screen was daily cleaned to reduce clogging. Light intensity was maintained at 2400 lux, and the light regime was controlled at 14 h light and 10 h dark. The salinity was maintained at 33 ± 0.8% and water temperature was 26.5 ± 1.0°C throughout the experiment. Rotifers *Brachionus rotundiformis* at a density of 10-20 ind mL^{-1} were used to feed the larvae from 2 DPH to 10 DPH. Rotifers fed with baker yeast were enriched with DHA protein Selco (INVE Aquaculture, Salt Lake City, UT, USA) for 12 h before they were added into the larval rearing tanks. Instant microalgal paste (*Nannochloropsis* sp.) was also added into larval fish tanks to create a green-water background. *Artemia nauplii* were first introduced at 0.1 nauplii mL^{-1} on 10 DPH, and then added with a daily increment of 90% by number. After five days of co-feeding, *Artemia nauplii* were gradually phased out at a daily reduction of 20% by number until the co-feeding period ended. *Artemia nauplii* were enriched with DHA Protein Selco (INVE Aquaculture, Salt Lake City, UT, USA) following the manufacturer's instruction.

Response of L-FABP *gene to rearing temperature*

Same batch of fertilized eggs as described above were transferred into 500-L incubators and hatched at 26°C. The experimental design included three constant temperatures 23, 26, and 29°C with three replicates each. On 2-days post hatch (DPH), yolk sac larvae were acclimatized at each desired temperature for 5 h, and then stocked in 500-L fiberglass tanks at a density of 60 fish L^{-1}. Except rearing temperature, all the feeding protocols and rearing conditions were the same as in experiment I.

Response of L-FABP *gene to nutrition manipulation*

This present study was derived from the same feeding trial in our previous study (Yang *et al*., 2015). The nutritional manipulation experiment included three dietary treatments with three replicates each. *Artemia nauplii* were treated in three methods (1) enriched with instant microalgal paste (*Nannochloropsis* sp., Qingdao Hong Bang Biological Technology Co., Ltd., Qingdao, China), and (2) enriched with Algamac 3080® (Aquafauna, USA), and (3) without any enrichment as control. For each treatment, three replicate tanks were used in this study.

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 the rearing tanks in triplicates for ontogenetic expression analysis. Approximately 50 individuals were collected in triplicate on 18 DPH for temperature and nutrient manipulation analysis. A total of 100 individuals were collected in triplicate, and examined under a dissecting microscope for tissue expression analysis. 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 at 260 nm and 280 nm (260/280) and agarose gel electrophoresis. RNA was reverse-transcribed to cDNA with oligo (dT) primers using a PrimeScript 1st 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 measured previously in our laboratory (Illumina HiSeq2000, annotated by NR, KOG, kegg, and Swissprot), the genes cloning primers were designed (L-FABP –F: 5'-ATTGCGATGGGACCCC-3'; L-FABP –R: 5'-TTAACTTCACTGCCAAGTT-3') with Primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA). The PCR reaction systems were as follows: 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 were mixed in 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 sequenced.

Quantitative real-time PCR (qPCR) was used to analyze the level of *L-FABP* gene expression in golden pompano larvae. Gene specific primer pairs for the *L-FABP* gene (L-FABP –qF: 5'-CAAGGACATCAAGCCAATTACTG-3'; L-FABP –qR: 5'-AATGGTAAAGGAATTGGTCACAG-3') were amplified in LightCycler480 II (Roche, Switzerland). *β-actin* gene (Accession number: KX987228) (β-actin -qF: 5'-TACGAGCTGCCTGACGGACA -3'; β-actin-qF: 5'-GGCTGTGATCTCCTTCTGC-3') was used as the internal reference and amplified. The cycling conditions for *L-FABP* genes and *β-actin* 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 employed to ensure 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 $^{\Delta\Delta}$CT (comparative threshold cycle) method ($^{\Delta}$CT = CT of target gene - CT of EF-1α, $^{\Delta\Delta}$CT = $^{\Delta}$CT of any sample - $^{\Delta}$CT of calibrator sample). The efficiencies of the primers (E) were $E_{L\text{-}FABP}$ = 0.9997.

Sequences and phylogenic analysis

The *L-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 the 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 acids sequence identity and similarity matrices of the Hh family sequences from various species were performed using Matgat 2.02 (Campanella *et al*., 2003). The three-dimensional structures of golden pompano L-FABP were obtained by homology modelling (http://swissmodel.expasy.org/workspace/index.php).

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  1  GTTACTCATTACACATTGCGATGGGACCCCTTTGCCTTCCAGTATAAGAAGGTTTGGTAG  60
 61  CACATTCACATTCTCCACATTGTGTTGAGCTTCACACAGCTGTCTCAGCCTCCACTCCAC 120
121  TTTGGTGAAGGAGATCCCAGACCTTCTAGAGAAGatggacttcaatggaacatggcaggt 180
  1                                     M  D  F  N  G  T  W  Q  V   9
181  ttactctcaggagaattacgagtcgttcctcagggccatggaactcccagaagatgtcat 240
 10  Y  S  Q  E  N  Y  E  S  F  L  R  A  M  E  L  P  E  D  V  I  29
241  caagatggccaaggacatcaagccaattactgagatcaaacagagtggcaatgactttgt 300
 30  K  M  A  K  D  I  K  P  I  T  E  I  K  Q  S  G  N  D  F  V  49
301  tgtcacctccaagacccctggaaagtctgtgaccaattcctttaccattggtaaggaggc 360
 50  V  T  S  K  T  P  G  K  S  V  T  N  S  F  T  I  G  K  E  A  69
361  tgaaatcaccaccatggacggcaagaagctcaagtgcatcgtcaatctggagggtggcaa 420
 70  E  I  T  T  M  D  G  K  K  L  K  C  I  V  N  L  E  G  G  K  89
421  aatggtgtgcaagactggcaagttctgccacatccaagagctcaagggaggagagatggt 480
 90  M  V  C  K  T  G  K  F  C  H  I  Q  E  L  K  G  G  E  M  V 109
481  tgagacattgaccatgggctcaacaactctcgtcaggaagagcaaaaagatgtaaACTTG 540
110  E  T  L  T  M  G  S  T  T  L  V  R  K  S  K  K  M  *       126
541  GCAGTGAAGTTAACCAATGTCTAATAAAAGTGTTGCTTGATAACAAAAAAAAAAAAAAA 600
601  AAAA                                                        604
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Fig. 1. Nucleotide sequence and deduced amino acids of liver-type fatty acid binding protein (L-FABP) gene from *T. ovatus* (Linnaeus, 1758). Cytosolic fatty-acid binding proteins signature was underlined.

Statistical analysis

The data were all 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

Characteristics of L-FABP *gene*

The full length of L-FABP cDNA from golden pompano (GenBank accession No. MF034872) was 604 bp, including a 5'-untranslated region (UTR) of 154 bp, a 3'-UTR of 69 bp and an open reading frame (ORF) of 281 bp encoding a polypeptide of 126 amino acids with predicted molecular weight of 14.06 kDa and theoretical isoelectric point of 8.73 (Fig. 1). The sequence structure analysis showed that the deduced protein sequences of had a cytosolic fatty-acid binding proteins signature, and this domain was also found in all detected sequences as showed in multiple sequences alignment (Fig. 2). The multi-sequence alignment revealed that the L-FABP of golden pompano share high identity to other known orthologs. Multiple sequence alignment of the deduced amino acid sequences of *L-FABP* genes with some known L-FABP family amino acid sequences from various species is shown in Table I. The predicted amino acid sequence of *L-FABP* genes from golden pompano showed the high similarity and identity with *Epinepheluscoioides* (97.6% and 95.2%, ADG29164.1) and *Oryziaslatipes* (98.4% and 84.1%, XP_004078356.1) (Fig. 2). The phylogenetic tree indicated that the L-FABPs from teleost and mammal were divided into two clusters, and the L-FABPs from golden pompano was closed to that from *Epinephelus coioides* (Fig. 3).

Table I.- Identity and similarity between *T. ovatus* L-FABP with other L-FABP homologue.

Species	Accession No.	AA	Similarity (%)	Identity (%)
Trachinotus ovatus	Present study	126	-	-
Epinephelus coioides	ADG29164.1	126	97.6	95.2
Oryzias latipes	XP_004078356.1	126	98.4	84.1
Cyprinus carpio	ACA64701.1	126	92.1	80.2
Danio rerio	NP_694492.1	126	92.9	76.2
Gallus gallus	NP_989965.1	126	87.3	70.6
Rattus norvegicus	NP_036688.1	127	62.2	40.9
Mus musculus	NP_059095.1	127	62.2	41.7
Homo sapiens	NP_001434.1	127	63.8	40.9

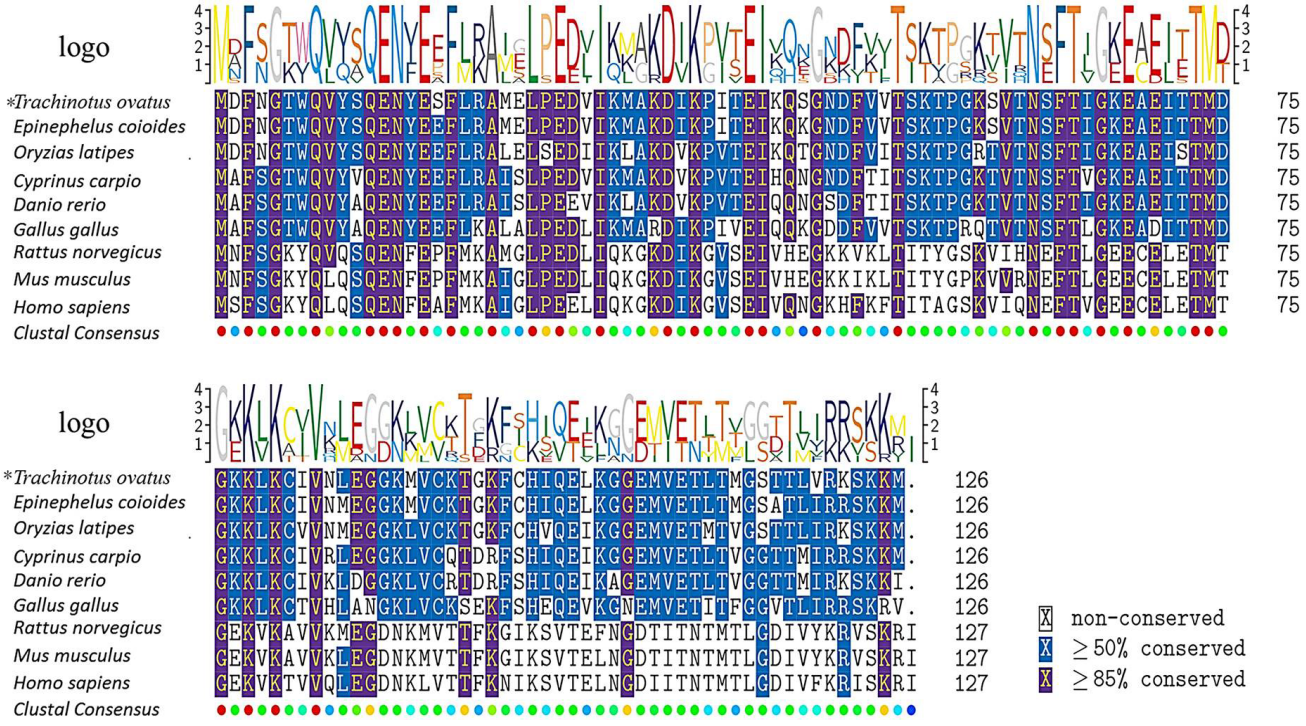

Fig. 2. Multiple sequence alignment of the deduced amino acid sequence of L-FABP with other known homologous H-FABP amino acid sequence.

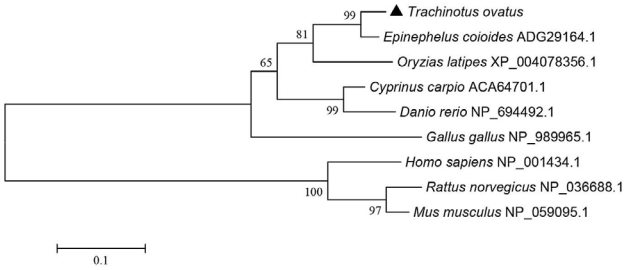

Fig. 3. Phylogenetic analyses of L-FABP. Alignment of amino acid sequences were produced by Clustal W, and the bootstrap neighbor-joining phylogeny tree was constructed by MEGA 5.03. GenBank accession numbers encoding L-FABP are showed in Table I.

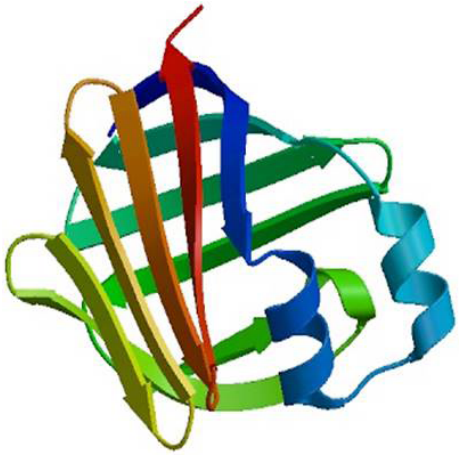

Fig. 4. Predicted tertiary structure of *T. ovatus* L-FABP.

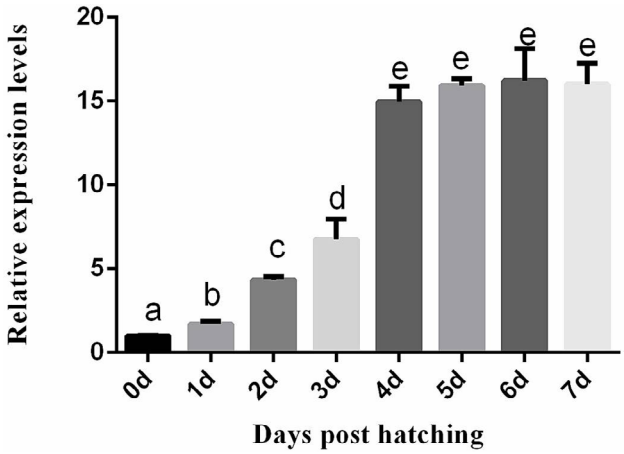

Fig. 5. Relative expressions of L-FABP was detected by quantitative RT-PCR analysis after 0d, 1d, 2d, 3d, 4d, 5d, 6d and 7 day post hatching. Each Bar represent the mean ± SD (n=3). Different lowercase letters indicate statistically significant differences (P<0.05).

The three-dimensional structure of L-FABP from golden pompano was predicted by SWISS-MODEL, and there were ten anti parallel beta sheets forming a hydrophobic pocket (Fig. 4).

Ontogenetic expression of L-FABP *gene and tissue expression of golden pompano larvae*

The expression level of *L-FABP* gene of golden pompano larvae was low at hatching, and was slowly increased with the increase of fish age from 0 DPH to 3 DPH (Fig. 5). The expression of *L-FABP* gene reached a relatively high level on 4 DPH ($P< 0.05$), the highest level was reached the on 12 DPH, and then remained at a similar level until the experiment was completed on 18 DPH. On 18 DPH, the highest expression of *L-FABP* gene in golden pompano was observed in liver ($P < 0.01$, Fig. 6). The expression of *L-FABP* gene in brain, gills, head-kidney, spleen, eye, intestine, stomach, muscle and heart, was significantly lower.

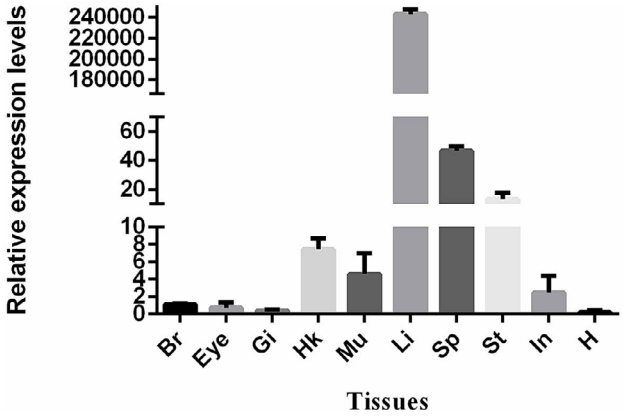

Fig. 6. Relative expressions of L-FABP in different brain (Br), gills (Gi), head-kidney (Hk), spleen (Sp), eye (Eye), intestine (In), stomach (St), liver (Li), muscle (Mu) and heart (H), by quantitative RT-PCR analysis. Each Bar represent the mean ± SD (n=3). Different lowercase letters indicate statistically significant differences (P<0.05).

Response of L-FABP *gene to water temperature and nutrition manipulation*

Both on 12 DPH and 18 DPH, the highest expression of *L-FABP* gene was found in 29°C ($P < 0.05$), and the expression of *L-FABP* gene was not significantly different between fish cultured at 23°C and 26°C ($P > 0.05$, Fig. 7). The expression of *L-FABP* gene was significantly affected by nutrition manipulation on 18 DPH ($P < 0.05$, Fig. 8). The highest expression of *L-FABP* gene was observed in Algamac 3080 feeding group, and lowest expression of *L-FABP* gene was found in the *Nannochloropsis* feeding group ($P < 0.05$).

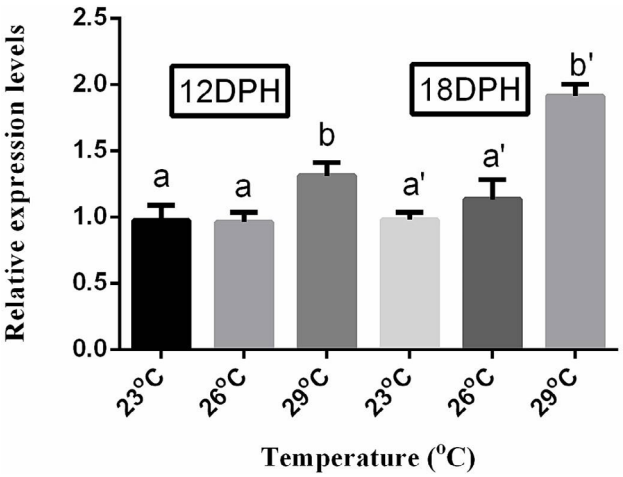

Fig. 7. *T. ovatus* larvae was treated in 23°C, 26°C and 29°C, respectively. The left side of 12DPH represents the experimental *T. ovatus* larvae was on 12 days post hatching, the right side of 18DPH represents the experimental *T. ovatus* larvae was on 18 days post hatching. Each Bar represent the mean ± SD (n=3). Different lowercase letters indicate statistically significant differences (P<0.05).

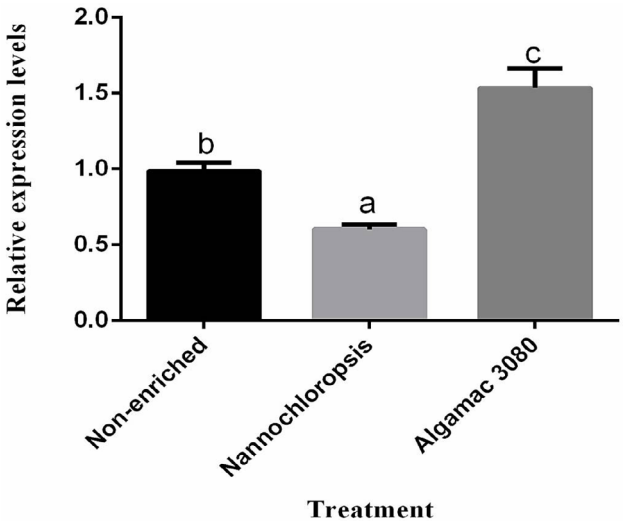

Fig. 8. Relative expression levels of *L-FABP* gene of *T. ovatus* under enriched instant microalgal paste (Nannochloropsis), Algamac3080 and non-enriched. Each Bar represent the mean ± SD (n=3). Different lowercase letters indicate statistically significant differences (P<0.05).

DISCUSSION

In this study, the *L-FABP* gene in golden pompano larvae was successfully isolated and identified. The predicted amino acid sequence of *L-FABP* genes from golden pompano showed high similarity and identity with *Epinephelus coioides* (97.6% and 95.2%, ADG29164.1) and *Oryzias latipes* (98.4% and 84.1%, XP_004078356.1). Similar to the FABP obtained from other species, such unique structure in L-FABP allows it to actively participate in transporting fatty acids and other lipid soluble substances within cells (Hsu and Storch, 1996; Andre *et al*., 2000; Venold *et al*., 2013), partitioning of fatty acids to different metabolic pathways (Storch and Corsico, 2008).

The expression of *L-FABP* gene was first observed in the embryos of zebrafish and then continuously to the adult stage (Her *et al*., 2003) through a green fluorescent protein (GFP) trans-genic strategy to generate liver-specific transgenic zebrafish. The study on the expression level of *L-FABP* gene during early development of commercial cultured fish larvae is rare. In birds such as chick and Japanese quail, only small amounts of the L-FABP mRNAs were detected in the liver during their embryogenesis (Murai *et al*., 2009), and the expression of *L-FABP* gene can be observed in both liver and intestinal tissues. In the present study, the expression of *L-FABP* gene in the liver of larval golden pompano was not previously reported in fish, and the functional expression of this gene in the liver may warrant further investigation. The results showed that the expression level of *L-FABP* gene in golden pompano larvae remained at a low level at hatch, and slowly increased before 3 DPH. On 4 DPH, the expression of L-FABP sharply increased and reached a high level and remained at a similar level until the experiment was completed on 18 DPH. This expression pattern suggests that the *L-FABP* gene in larval golden pompano expressed before the development of the digestive tract, as the digestive system of golden pompano was immature at hatch, and a functional digestive system appeared around 15 DPH (Ma *et al*., 2014). Furthermore, the increased expression in L-FABP may be correlated to the uptake of dietary fatty acids after a fully functional digestive tract developed in the larval golden pompano (Ma *et al*., 2014).

Although genetic factors are mainly involved to control fish growth (Ferguson and Danzmann, 1998; Ma and Qin, 2014), environmental parameters can also regulate fish development (Georgakopoulou *et al*., 2010). More and more evidence has showed that temperature is an important environmental factor in larval fish rearing, and can significantly affect fish feeding and metabolism (Blaxter, 1992; Ma *et al*., 2014). The digestive function of fish larvae can be significantly affected by water temperature (Hevrøy *et al*., 2012; Liu *et al*., 2017). Early studies have demonstrated that environmental temperature can regulate the metabolism and composition of fatty acids in fish (Kemp and Smith, 1970; Farkas *et al*., 1980; Skalli *et al*., 2006). However, it is unclear if temperature can affect the expression of *L-FABP* gene during early development

of fish larvae. In this study, the expression of *L-FABP* gene was significantly affected by water temperature on 12 DPH and 18 DPH, and obvious impact was observed with the increase of fish age. Such difference may reflect the development progress of the digestive tract in larval golden pompano, since the digestive system appeared to be more mature on 18 DPH (Ma *et al*., 2014).

Fatty acid binding proteins can affect gene regulation, leading to up-regulation of lipid related genes via the activation of peroxisome proliferating receptors (Lawrence *et al*., 2000; Tan *et al*., 2002). On the other hand, feeding stimulation only slightly stimulated expression of the *L-FABP* gene, and was not always its primary determinant (Atsushi *et al*., 2009). In addition, probably due to the initiation of feeding after hatch, whereas *L-FABP* gene expression did not change after hatch (Atsushi *et al*., 2009). In the present study, the expression of *L-FABP* gene was significantly affected by the nutrition enhancement. The highest expression of *L-FABP* gene was observed in the Acgamac3080 group, and the lowest expression was found in the *Nannochloropsis* enriched group. This expression pattern is parabola to the total saturated fatty acid content in the diet. In the experimental diet, the *Nannochloropsis* enriched group contained the lowest amount of total saturated fatty acid, and the Algamac3080 group contained the highest total saturated fatty acid (Yang *et al*., 2015). This may suggest that higher total saturated fatty acids in the diet promote the expression of *L-FABP* gene in golden pompano larvae.

CONCLUSION

We cloned and analyzed the L-FABP cDNA in golden pompano larvae in this study. The present study indicates that the expression of *L-FABP* gene in golden pompano larvae was significantly affected by the water temperature and nutrition manipulation on both 12 DPH and 18 DPH. The time dependent expression and tissue dependent of *L-FABP* gene in fish larvae is important to understand the ontogenetic development and growth of fish larvae in early life. The monitoring of *L-FABP* gene expressions in golden pompano larvae may serve as a useful indicator in the field or in a fish farming setting, leading to rapid assessment of environmental conditions and nutritional impact on fish development.

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Statement of conflict of interest

The authors declare no conflict of interest.

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