Aquaculture Research

Aquaculture Research, 2017, **48**, 1183–1193 doi: 10.1111/are.12960

Molecular cloning and genetic ontogeny of some key lipolytic enzymes in large yellow croaker larvae (*Larimichthys crocea* R.)

Zuonan Cai[1,2], Fengjun Xie[1,2,3], Kangsen Mai[1,2] & Qinghui Ai[1,2]

[1]Key Laboratory of Aquaculture Nutrition and Feed (Ministry of Agriculture), Ocean University of China, Qingdao, Shandong, China

[2]Key Laboratory of Mariculture (Ministry of Education), Ocean University of China, Qingdao, Shandong, China

[3]Laboratory of Fish Nutrition, School of Marine Sciences, Ningbo University, Ningbo, China

Correspondence: Q Ai, The key laboratory of Mariculture (Ministry Education of China), Ocean University of China, 5 Yushan Road, Qingdao, Shandong 266003, China. E-mail: qhai@ouc.edu.cn

Abstract

This study was conducted to investigate the genetic ontogeny of some key lipolytic enzymes in large yellow croaker *Larimichthys crocea* larvae. Partial cDNA sequences of bile activated lipase (BAL) and pancreatic enzyme secretory phospholipase A_2 group IB ($sPLA_2$ IB) were cloned and characterized. Real-time quantitative PCR (RT q-PCR) was conducted from 1 day after hatching (DAH) to 40 DAH in larvae fed the following sequence: rotifers, *Artemia nauplii*, copepods and then frozen copepods. In this study, 1181 bp and 205 bp partial cDNA sequences were cloned for BAL and $sPLA_2$ IB respectively. The mRNA of all lipolytic enzymes were already present before mouth opening. The mRNA expression of BAL and $sPLA_2$ IB during larval development showed a similar pattern: both of them increased gradually from 1 DAH to 25 DAH, followed by a slight decrease up to 40 DAH. The mRNA expression of lipoprotein lipase (LPL) also increased first and then decreased, peaked at 20 DAH. However, the mRNA expression of hepatic lipase (HL) increased constantly up to 40 DAH. These results suggest that the transcript levels of these enzymes were mainly regulated by age in early stage and by the exogenous diet in late stage of large yellow croaker larvae. Energy available for fish larvae, originating from lipid oxidation, may have been reduced as indicated by decreasing expression of LPL due to lower lipid levels in frozen copepods compared to fresh copepods. Besides, lipid hydrolysis for utilization in the liver of large yellow croaker larvae from chylomicron-remnant, very low density lipoprotein (VLDL)-remnant and high density lipoprotein increased along larval development of large yellow croaker which was reflected by the increasing concentration of HL mRNA.

Keywords: development, gene expression, large yellow croaker larvae, lipolytic enzymes

Introduction

The successful rearing of fish during their larval stage relies on many aspects, of which dietary lipids are regarded as one important nutritional factor that can greatly affects the growth and development of fish larvae (Watanabe, Kitajima & Fujita 1983; Tocher 2003). Lipids, and specifically fatty acids, are the preferred source of metabolic energy for growth, reproduction and swimming in fish, especially marine fish (Rainuzzo, Reitan & Olsen 1997; Tocher 2010). Besides, they are important components of biological membranes and can function as carriers for non-fat nutrients, especially the fat-soluble vitamins (Watanabe 1982; Tocher 2010).

Triacylglycerol (TAG) and phospholipid are known to be the two main kinds of lipids in marine zooplankton (Lee 1974). Pancreatic lipase (PL) plays a critical role in TAG digestion in mammals, however it has been indicated that PL may be an unsuited lipase for fish when comparing to bile activated lipase (BAL) (Sæle, Nordgreen, Olsvik &

© 2015 John Wiley & Sons Ltd

Hamre 2010; Rønnestad, Yúfera, Ueberschär, Ribeiro, Saele & Boglione 2013). BAL has been recognized as the most important enzyme implicated in neutral lipid digestion in a majority of teleosts, including anchovy *Engraulis engrausicolus*, striped bass *Morone saxatilis*, pink salmon *Oncorhynchus gorbuscha*, leopard shark *Triakis semifasciata*, rainbow trout *Oncorhynchus mykiss*, Atlantic cod *Gadus morhua* and red seabream *Pagrus major* (Izquierdo, Socorro, Arantzamendi & Hernández-Cruz 2000; Sæle *et al.* 2010). BAL can completely hydrolyse TAG into free fatty acids (FFAs) and glycerol (Perez-Casanova, Murray, Gallant, Ross, Douglas & Johnson 2004). Phospholipid has been reported to be the major component of cell membranes and can enhance neutral lipid absorption and transport. In addition, they can also improve diet quality and supply essential components, such as choline and inositol (Cahu, Gisbert, Villeneuve, Morais, Hamza, Wold & Zambonino-Infante 2009). However, since fish larvae have limited capacity to synthesize phospholipid *de novo* to meet their requirement for phospholipid, dietary inclusion of phospholipid is essential (Tocher, Bendiksen, Campbell & Bell 2008). Among all 19 enzymes with PLA_2 activity in mammals, digestion of dietary phospholipid is mainly catalysed by pancreatic enzyme secretory phospholipase A_2 group IB ($sPLA_2$ IB), which hydrolyses the fatty acid ester bond at the sn-2 position of phospholipid and produces a non-esterified fatty acid and a lysophospholipid (Murakami & Kudo 2002). In fish, the existence of $sPLA_2$ IB has also been demonstrated in several species (Sæle, Nordgreen, Olsvik & Hamre 2011; Fujikawa, Shimokawa, Satoh, Satoh, Yoshioka, Aida, Uematsu & Iijima 2012).

In mammals, lipoprotein lipase (LPL) and hepatic lipase (HL) are key members in the lipase gene family (Wong 2002). The absorbed FFAs from the hydrolysis of dietary lipids are re-esterified to TGs and incorporated into plasma lipoproteins. Various kinds of endogenous lipases, including LPL and HL, then act on TAG present in plasma lipoproteins to release FFAs for either energy storage in adipose tissue or for oxidation in other tissues (Nilsson-Ehle, Garfinkel & Schotz 1980; Oku, Koizumi, Okumura, Kobayashi & Umino 2006). LPL participates in plasma chylomicron and very low-density lipoprotein metabolism, while HL is involved in chylomicron-remnant and high density lipoprotein metabolism (Santamarina-Fojo, Haudenschild & Amar 1998; Mead, Irvine & Ramji 2002).

Marine fish undergo major physiological and morphological change during their larval stage (Hansen, Folkvord, Grøtan & Sæle 2013). Although studies on genetic ontogeny of lipid digestive enzymes (BAL and $sPLA_2$ IB) in fish larvae have been the subject of attention by many authors throughout the years (Kurokawa, Suzuki, Ohta, Kagawa, Tanaka & Unuma 2002; Srivastava, Kurokawa & Suzuki 2002; Sæle *et al.* 2010, 2011; Kortner, Overrein, Øie, Kjørsvik, Bardal, Wold & Arukwe 2011; Fujikawa *et al.* 2012; Lee, Sivaloganathan, Walford & Reddy 2012), there is still only fragmentary information available on the molecular ontogeny of lipolytic enzymes, especially LPL and HL in larval stage of fish. Large yellow croaker *Larimichthys crocea* is an economically important marine fish species in southern China. Activities of enzymes involved in protein and carbohydrate digestion during the development of large yellow croaker larvae have been conducted in our laboratory (Ma, Cahu, Zambonino-Infante, Yu, Duan, Le-Gall & Mai 2005). However, there is a general lack of knowledge concerning the molecular basis underlying the ontogeny of lipolytic capability in this important fish species during their larval stage. The main purpose of this study was therefore to develop quantitative molecular markers for the ontogeny of lipid utilization in large yellow croaker larvae and contribute to comprehensive understanding of the molecular basis on lipid utilization in larval stage of marine fish by determining the genetic ontogeny of some key lipolytic enzymes, including BAL, $sPLA_2$ IB, LPL and HL.

Materials and methods

Experimental procedure

Large yellow croaker larvae used in this study were obtained and reared at the hatchery of the Aquatic Technology Extension Station of Ningde (Fujian, China). The parental fish of large yellow croaker were 2 years old males and females with body weights of 0.45–0.85 kg and 0.85–1.50 kg, respectively. Artificial induced spawning was conducted at 20–21.5°C. Eggs hatched within 31 h after spawning. Three thousand hatched embryos were equally transferred to three start feeding tanks (water volume 500 L). All tanks were supplied with seawater that had been filtered through two-grade sand filter. During the rearing period,

 © 2015 John Wiley & Sons Ltd,

water temperature was kept constant at 23 ± 1°C; pH ranged from 7.8 to 8.2 and salinity from 22‰ to 26‰. About 200–300% of the water volume was renewed daily and all rearing tanks were provided with continuous aeration. Fish were reared under a 14:10 h, light/dark regime. The maximum light intensity was 8.5 W m^{-2} at the water surface during daytime. Undissolved surface materials were skimmed with a polyvinylchloride pipe in time and accumulations of feed and faeces at the tank bottoms were syphoned twice daily.

Larvae were fed rotifers *Brachionus plicatilis* (0.5–1.5 × 10^4 individual L^{-1}) from 3 to 8 day after hatching (DAH), *Artemia nauplii* (1.0–1.5 × 10^4 individual L^{-1}) from 6 to 11 DAH, live copepods from 10 to 16 DAH and frozen copepods until 40 DAH (Fig. 1). Fish were manually fed to apparent satiation five times daily (06:00, 08:30, 12:30, 14:30 and 17:00 hours).

Sampling

Fifty individuals were randomly collected from each tank at day 1, 3, 7, 11, 15, 19, 25, 30, 35 and 40, and immediately frozen in liquid nitrogen and then stored at −80°C for mRNA expression analysis of target genes. Live prey including rotifers, *A. nauplii*, live and frozen copepods were collected and stored at −20°C for biochemical analysis.

Live prey composition

Dry matter, crude protein and lipid were determined according to standard methods (AOAC 2003). Dry matter after drying in an oven at 105°C until constant weight; crude protein (N × 6.25) by the Kjeldahl method after acid digestion using a Kjeltec system (Kjeltec-2300; Tecator, Hoganas, Sweden); crude lipid by petroleum ether extraction in a Soxtec System HT apparatus (B-801; Flawil, Switzerland).

RNA extraction and cDNA synthesis

Total RNA was extracted from whole larvae using Trizol Reagent (Takara, Tokyo, Japan). The quantity and quality of isolated RNA was determined by spectrophotometry with a NanoDrop® ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA), and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. Five hundred nanograms (500 ng) of total RNA was subjected to reverse transcription by TransScript TM One-Step gDNA removal and cDNA Synthesis Super Mix (TransGen Biotech, Beijing, China) in 20 μL volume according to reagent's instructions.

Cloning and sequencing of partial cDNA fragments of BAL and $sPLA_2$ IB

The degenerate primers were designed based on highly conserved regions from the genes of other fish available in the GenBank database (Table 1). Whole larvae cDNA at 40 day after hatching (DAH) was used as the template for amplification. The polymerase chain reaction (PCR) was conducted on an Eppendorf Mastercycler gradient (Eppendorf, Hamburg, German) to amplify target gene cDNA fragment. The PCR conditions were as follows: initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 54°C for 30 s, primer extension 72°C for 30 s with a final 10 min extension at 72°C. All PCR products were run on a 1.5% agarose gel, and then purified by SanPrep PCR Purification Kit (Sangon Biotech, Shanghai, China). PCR products were cloned into pEASY-T1 simple cloning vector (TransGen Biotech) and sequenced in BioSune Biotech (Shanghai, China).

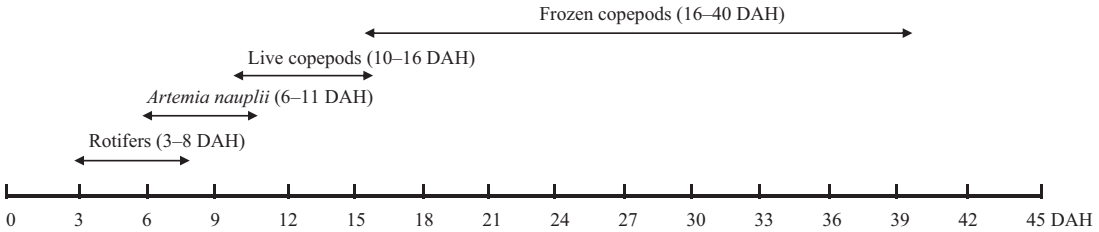

Figure 1 Feeding protocol during larval rearing.

Table 1 Degenerate primers for partial sequence cloning

Gene	Amplification size (bp)	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Annealing temperature (°C)
BAL	1000	TGYCTSTACCTSAACATYTGG	AAGTTDGTCCAGTADGCRAT	54
$sPLA_2$ IB	205	CACACCTGTGGATGATCTGG	CTTCTTGTCACACTCGCAGA	54

Table 2 Sequence of the primers used for q-PCR in this study

Target gene	Reference	Forward (5′–3′)	Reverse (5′–3′)	Annealing temperature (°C)
BAL		GAGGGTCAATGGGTGCTAACTTC	GAGTCTCCAGTGCTCAAGAAACC	55
$sPLA_2$ IB		TCTGGACAGGTGCTGTCAGG	AAGATGGGCCAGCACTCAGG	55
LPL	GenBank accession no. JQ327827	GAGAGGATTCATCTGCTGGGTTAC	ACATCAACAAACTGGGCGTCATC	56
HL	GenBank accession no. JX456350	TCCGTCCATCTATTCATTGACTCTC	GCCACTGTGAACCTTCTTGATATTG	56
β-actin	Yao, Kong, Wang, Ji, Liu, Cai & Han (2009)	TTATGAAGGCTATGCCCTGCC	TGAAGGAGTAGCCACGCTCTGT	60

Real-time quantitative PCR

After RNA extraction and reverse transcribed from 50 larvae each tank (as described above). Real-time quantitative PCR was carried out in a quantitative thermal cycler (Mastercyclerep *realplex*; Eppendorf). Specific primers were designed based on the partial cDNA sequences of BAL, $sPLA_2$ IB, LPL and HL (Table 2) and β-actin was evaluated for use as a reference gene. The amplification was performed in a total volume of 25 μL, containing 1 μL of each primer (10 μM), 1 μL of the diluted first strand cDNA product, 12.5 μL of 2× SYBR® Premix Ex Taq™II (Takara) and 9.5 μL of sterilized double-distilled water. The real-time PCR programme was as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, an annealing step for 10 s, and 72°C for 20 s. The annealing temperature was different for each primer pair (Table 2). At the end of each PCR reaction, melting curve analysis was performed to confirm that only one PCR product was present in these reactions. Standard curves were made with five different dilutions (in triplicate) of the cDNA samples and amplification efficiency was analysed according to the following equation $E = 10^{(-1/\text{Slope})} - 1$. The primer amplification efficiency of BAL, $sPLA_2$ IB, LPL, HL and β-actin was 1.045, 1.094, 1.024, 0.966 and 0.988 respectively. After the amplification efficiency of each gene was analysed, the ΔCT ($CT_{\text{target genes}} - CT_{\text{inner control gene}}$) was determined for each cDNA dilution. Then, a plot of the log cDNA dilution vs. ΔCT was made. The absolute values of all the slope were less than 0.1, which were close to zero, and it indicated that the ΔΔCT calculation for the relative quantification of target genes could be used. In addition, no significant differences in β-actin expression were observed among larval pools at different developmental stages, indicating that β-actin could be used as a reference gene in this study. Thus, to calculate the expression of BAL, sPLA2 IB, LPL and HL, the comparative CT method ($2^{-\Delta\Delta CT}$ method) was used as described by Livak and Schmittgen (2001).

Statistical methods

Similarity searches of the sequenced cDNA of BAL and $sPLA_2$ IB were done by BLAST programme at web servers of NCBI (www.ncbi.nlm.nih.gov/BLAST/). The multiple-sequence alignments were performed using CLUSTALW at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/). The deduced amino acid sequences were analysed with DNAman. The statistical analysis was performed by using SPSS 17.0 (SPSS Incorporation, Chicago, IL, USA) for Windows. All data were subjected to one-way ANOVA and differences between the means were tested by Tukey's multiple range test. The level of significance was chosen at 0.05 and the results were presented as means ± SEM.

 © 2015 John Wiley & Sons Ltd,

Table 3 Biochemical composition of the prey used in larval rearing (%, dry matter)

	Rotifers	*Artemia nauplii*	Live copepods	Frozen copepods
Crude protein	53.64 ± 0.26[a]	44.63 ± 0.30[b]	51.81 ± 0.83[a]	45.82 + 0.25[b]
Crude lipid	12.27 ± 0.16[a]	11.33 ± 0.28[a]	12.31 ± 0.22[a]	3.63 ± 0.20[b]

Means ± SEM (n = 3) with the same superscript letters are not significantly different by Tukey's test ($P > 0.05$).

Results

Biochemical composition of live prey

Biochemical composition of the live prey was different (Table 3). Protein content in rotifers and live copepods was significantly higher than that in *A. nauplii* and frozen copepods. The lowest lipid content was observed in frozen copepods, while there were no significant differences in lipid content among rotifers, *A. nauplii* and live copepods.

Cloning and sequencing of large yellow croaker BAL and sPLA$_2$ IB

The PCR products amplified by the degenerate primers were 1181 bp for BAL (GenBank accession no. KF954548.1) (Fig. 2) and 205 bp for sPLA$_2$ IB (GenBank accession no. KF954549.1) (Fig. 3) respectively. For BAL, the bile salt-binding site (GANFLDNYLY) was present at positions 36-45. The N-glycosylation site (NIT) and highly conserved active site serine motif (FGESAGG) (Wang & Hartsuck 1993) were also present, starting at positions 107 and 111 respectively. For sPLA$_2$ IB, the open reading frame contained the catalytic site (DXCCXXH) and the pancreatic loop (PYTXX), which started at positions 7 and 33 respectively.

Amino acid sequence alignment of BAL and sPLA$_2$ IB

The BLAST analysis revealed that BAL and sPLA$_2$ IB of large yellow croaker shared high identity to known BAL and sPLA$_2$ IB of teleost, respectively. For BAL, European seabass *Dicentrarchus labrax*, 91%; Pacific bluefin tuna *Thunnus orientalis*, 90%; winter flounder *Pseudopleuronectes americanus*, 90%; gilthead seabream *Sparus aurata*, 87%; haddock *Melanogrammus aeglefinus*, 78% (Fig. 4). For sPLA$_2$ IB, red seabream *P. major*, 81%; European seabass *Dicentrarchus labrax*, 79%; Zebrafish *Danio rerio*, 66% (Fig. 5).

Genetic ontogeny of lipolytic enzymes

The transcripts of all lipolytic enzymes were already present in large yellow croaker larvae at 1 DAH, before mouth opening. The mRNA expression of BAL was low at the beginning of larval development (1 DAH to 3 DAH) and increased steadily from 3 DAH to 7 DAH, followed by a significant increase up to 25 DAH ($P < 0.05$). Then, the mRNA concentration of BAL decreased gradually until 40 DAH (Fig. 6). The developmental profile of sPLA$_2$ IB mRNA showed a similar pattern with that of BAL mRNA: increased up to 25 DAH, and then decreased until 40 DAH, peaked at 25 DAH (Fig. 7). The mRNA expression of LPL increased about 4.46-fold from 1 to 19 DAH, at which the mRNA level of LPL reached a peak and then decreased about 2.29-fold between 19 and 40 DAH (Fig. 8), while the expression of HL showed a constant increase from 1 to 35 DAH, and then remained stable until 40 DAH (Fig. 9).

Discussion

Both BAL and sPLA$_2$ IB constitute the most important enzymes responsible for lipid digestion in teleost fish. In this study, the cDNA of BAL and sPLA$_2$ IB were cloned and characterized in large yellow croaker for the first time. The large yellow croaker BAL was the most nearly identical to that of European seabass and the bile salt-binding site (GANFLDNYLY) was 100% conserved among all the species analysed. Furthermore, the N-glycosylation site (NIT) and the active serine motif (FGESAGG) were also present in large yellow croaker and showed only minor difference when compared with that of other fish.

In mammals, the family of sPLA$_2$ includes four groups-I, III, V and X, which contain all ten enzyme forms (Dennis 1994). The sPLA$_2$ IB is one member of these ten sPLA$_2$ enzymes that share a highly conserved region for a catalytic site (DXCCXXH) (Murakami & Kudo 2002). In this

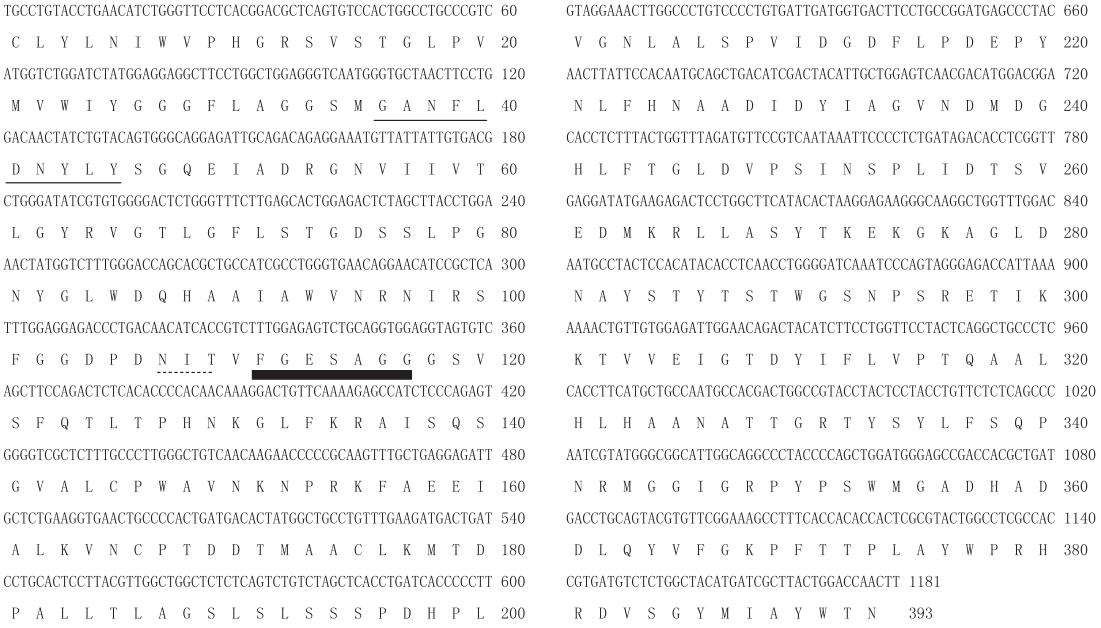

Figure 2 Nucleotide sequence of cloned bile salt-activated lipase (BAL) from large yellow croaker and its deduced amino acid sequence. Numbers refer to amino acid residues of the cloned fragment. The region involved in bile salt-binding is indicated by (——), the N-glycosylation site by (·····) and the active serine motif by (▬).

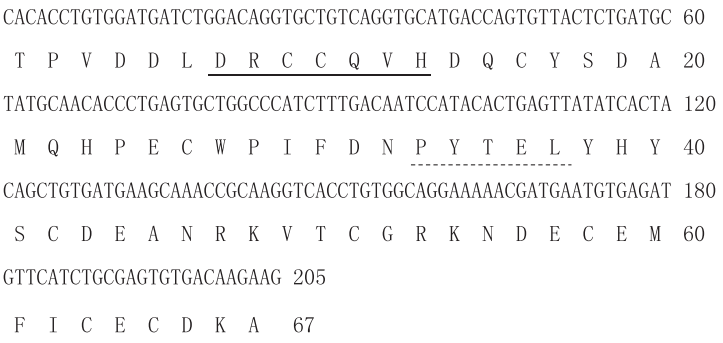

Figure 3 Nucleotide sequence of cloned pancreatic enzyme secretory phospholipase A_2 group IB ($sPLA_2$ IB) from large yellow croaker and its deduced amino acid sequence. Numbers refer to amino acid residues of the cloned fragment. The region involved in the catalytic site is indicated by (——) and the pancreatic loop by (·····).

study, the catalytic site was cloned and conserved among all the $sPLA_2$ IB amino acids sequences aligned. In addition, the pancreatic loop containing the domain PYTEL (PYTXX) was also present in large yellow croaker. In fish, together with mammals, the only PLA_2 digesting PL in the intestine is $sPLA_2$ IB, produced in the pancreas and recognized by the pancreatic loop domain (Murakami & Kudo 2002; Sæle *et al.* 2011). Thus, this confirmed that the PLA_2 sequence obtained in this study most likely encoded for $sPLA_2$ IB.

To fill the gaps in the understanding of molecular basis underlying the ontogeny of lipolytic capability in fish, gene expression patterns for those key lipolytic enzymes in large yellow croaker were monitored during their larval development. In this study, the transcript levels of BAL and $sPLA_2$ IB could be detected and remained stable at a low level until 3 DAH, before first feeding. These observations were similar to that found in Atlantic cod, Japanese eel, Japanese flounder, red sea bream and Asian sea bass (Kurokawa *et al.* 2002; Srivastava *et al.* 2002; Sæle *et al.* 2010, 2011; Kortner, Overrein, Øie, Kjørsvik, Bardal *et al.* 2011; Fujikawa *et al.* 2012; Lee *et al.* 2012), indicating that the expression of genes coding for digestive enzymes could be 'pre-programmed' before the mouth opening of fish larvae. Although the mRNA level of BAL and $sPLA_2$ IB remained at a constant low level, a significant increase was measured from 3

 © 2015 John Wiley & Sons Ltd,

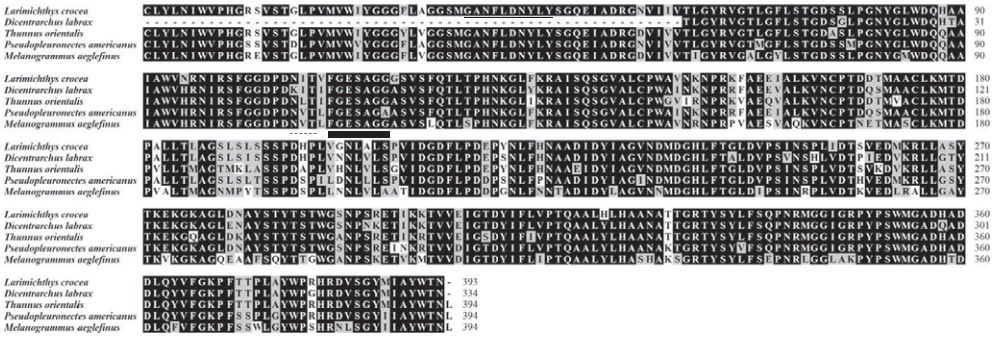

Figure 4 Amino acid alignment of BAL from large yellow croaker and other teleost. Sequences for the bile salt-binding site (——), the N-glycosylation site (·····) and the active serine motif site (▬).

Figure 5 Amino acid alignment of $sPLA_2$ IB from large yellow croaker and other teleost. Sequences for the catalytic site (——) and the pancreatic loop (·····).

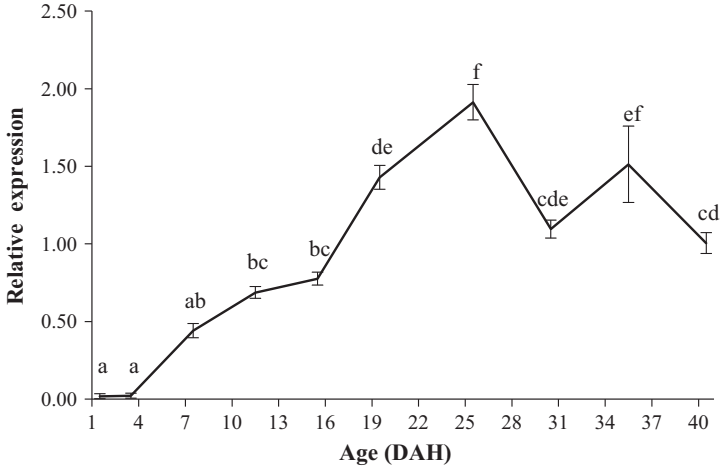

Figure 6 Genetic ontogeny of bile activated lipase (BAL) in large yellow croaker (*Larmichthys crocea*) larvae. Means ± SEM (n = 3) with the same superscript letters are not significantly different by Tukey's test ($P > 0.05$).

DAH to 25 DAH. Since pancreas differentiated at 3 DAH in large yellow croaker larvae (Mai, Yu, Ma, Duan, Gisbert, Zambonino-Infante & Cahu 2005), the increased mRNA concentration of BAL and $sPLA_2$ IB was probably due to the differentiation and development of the pancreas. Similarly, Sæle *et al.* (2010) also found an up-regulation in BAL and $sPLA_2$ IB expression levels with the development of the diffuse pancreas in cod larvae. In this study, it was noted that after 25 DAH, the genetic expression of BAL and $sPLA_2$ IB were significantly inhibited. In this study, biochemical composition of frozen copepods was quite different from other prey used in larval rearing, showing significant lower lipid content compared to live prey. Thus, the frozen copepod used after 25 DAH might account for the decreased expression of BAL and $sPLA_2$ IB. However, the mRNA levels of BAL and $sPLA_2$ IB increased constantly from 16 DAH to 25 DAH free from the negative influence of frozen copepod. That was probably because the expression of BAL and $sPLA_2$ IB were regulated through different mechanisms at different stages during the development of fish larvae and the mRNA of BAL and $sPLA_2$ IB were likely to be transcribed independent of the external diet before 25 DAH. This was in agreement with the findings of Kortner, Overrein, Øie, Kjørsvik and Arukwe (2011), who has demonstrated that no alterations in digestive gene expression in early stage of Atlantic cod larvae were observed in response to different diet, while the BAL/$sPLA_2$ expression pro-

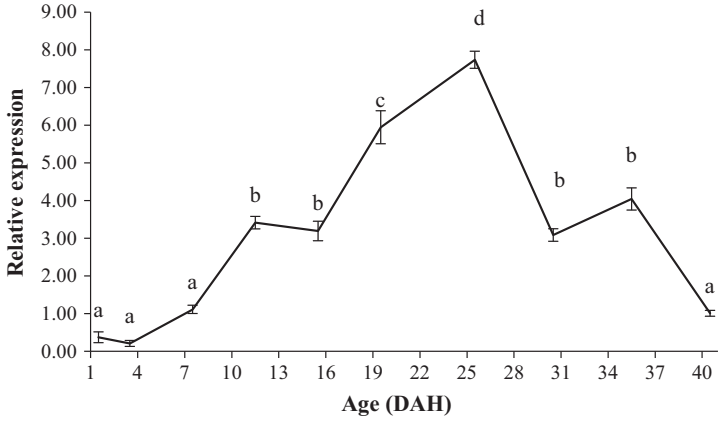

Figure 7 Genetic ontogeny of pancreatic enzyme secretory phospholipase A_2 group IB ($sPLA_2$ IB) in large yellow croaker (*Larmichthys crocea*) larvae. Means ± SEM ($n = 3$) with the same superscript letters are not significantly different by Tukey's test ($P > 0.05$).

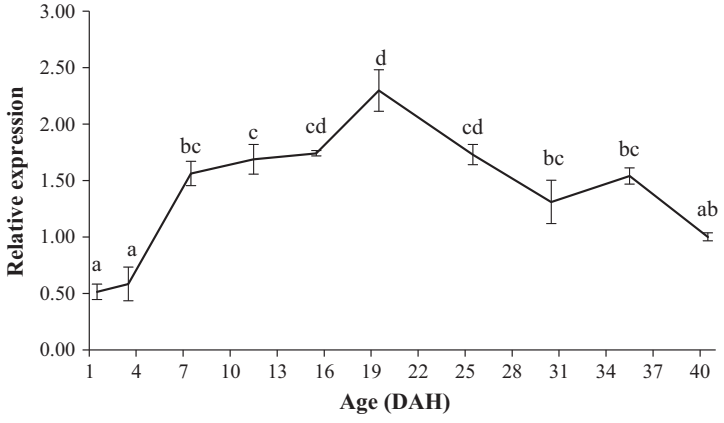

Figure 8 Genetic ontogeny of lipoprotein lipase (LPL) in large yellow croaker (*Larmichthys crocea*) larvae. Means ± SEM ($n = 3$) with the same superscript letters are not significantly different by Tukey's test ($P > 0.05$).

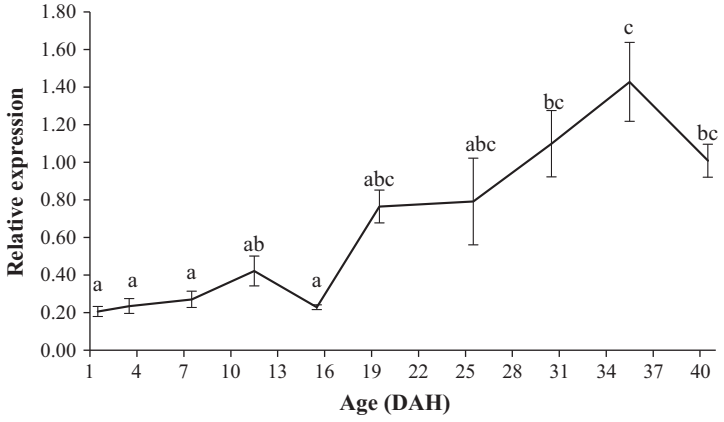

Figure 9 Genetic ontogeny of hepatic lipase (HL) in large yellow croaker (*Larmichthys crocea*) larvae. Means ± SEM ($n = 3$) with the same superscript letters are not significantly different by Tukey's test ($P > 0.05$).

files in late larval stage could be affected by different dietary constituents. In summary, gene expression of BAL and $sPLA_2$ were more age-dependently regulated in early stage of large yellow croaker larvae, while in late larval stage in which large yellow croaker has achieved maturation of its digestive functions, gene expression of these two lipid digestion enzymes were mainly controlled by the exogenous diet.

Lipoprotein lipase and HL are key members of lipase gene family involved in lipoprotein metabolism and remodelling (Nilsson-Ehle *et al.* 1980;

Tocher 2003). However, little information is available on molecular ontogeny of LPL and HL along larval development in marine fish. The LPL and HL might be critical for yolk lipid metabolism, as the transcript of HL and LPL could be detected before mouth opening, when fish larvae were totally dependent on internal nutrient-yolk. In this study, the mRNA expression of HL increased constantly until the end of the experiment. This was in agreement with the findings of Parma, Bonaldo, Massi, Yúfera, Martínez-Rodríguez and Gatta (2013). Since HL is found localized at the surface of liver sinusoidal capillaries and have hydrolytic activity on triglycerides, phospholipids of remnant lipoproteins (chylomicrons and VLDL), and high density lipoprotein (HDL) particles in mammals as well as in fish (Perret, Mabile, Martinez, Tercé, Barbaras & Collet 2002; Tocher 2003; Wang, Li, Sun, Fan & Liu 2013), the increased HL mRNA levels observed in this study indicated that lipid hydrolysis in these lipoproteins for utilization in the liver increased with the development of large yellow croaker larvae.

However, unlike the ontogenetic change in HL expression, the LPL mRNA level displayed an increase up to 20 DAH and down-regulation from 20 to 40 DAH. Ontogeny of the LPL transcript levels in large yellow croaker larvae confirmed the result observed in the study of Zheng, Zhu, Han, Yang, Lei and Xie (2010) who found that LPL showed an increase initially and then a decrease during the development of darkbarbel catfish larvae. The increase and decrease in LPL mRNA level indicated that LPL synthesis of large yellow croaker larvae during ontogeny was regulated at least at the transcriptional level. Unlike the exclusive production of HL in liver in fish, the mRNA of LPL is distributed in various tissues, including the liver, muscle and adipose tissue (Liang, Oku & Ogata 2002; Lindberg & Olivecrona 2002; Saera-Vila, Calduch-Giner, Gómez-Requeni, Médale, Kaushik & Pérez-Sánchez 2005; Cheng, Wang, Peng, Meng, Sun & Shi 2009). LPL is responsible for lipid utilization for these tissues through hydrolysing the triglycerides in chylomicrons and very low-density lipoproteins (Wang *et al.* 2013). Previous study has demonstrated that LPL gene expression is mediated by different fashion with tissue-specificities (Liang *et al.* 2002; Saera-Vila *et al.* 2005; Oku *et al.* 2006) and mRNA expression of LPL in the present study and darkbarbel catfish *Pelteobagrus vachelli* larvae were both analysed using the whole fish larvae instead of specific tissues. Thus, the ontogeny of LPL expression might not reflect the lipid utilization in the liver well and this could be partially responsible for the different changing pattern between LPL and HL mRNA expression. To better understand the mechanism regarding LPL gene expression change during larval stage of large yellow croaker, genetic ontogeny of LPL in the specific tissues needs to be further investigated. Furthermore, recent evidence suggests that LPL can act on circulating lipoproteins to release FFAs as peroxisome proliferator-activated receptor α (PPARα) ligands, eventually stimulates PPARα activity and the downstream responses involved in lipid oxidation (Ziouzenkova, Perrey, Asatryan, Hwang, MacNaul, Moller, Rader, Sevanian, Zechner, Hoefler & Plutzky 2003; Ruby, Goldenson, Orasanu, Johnston, Plutzky & Krauss 2010). Thus, in this study, the energy available for fish larvae, which was original from lipid oxidation, might be reduced due to low lipid level in frozen copepods as indicated by the decreasing expression of LPL. The nutritional value of live prey is usually unstable due to climate and/or environment variations as shown by comparison of biochemical analysis between live and frozen copepods in this study. These results emphasized the importance of sufficient dietary nutrients on fish larvae development and it was therefore necessary to develop a compound diet with stable nutrients level for fish larvae.

In conclusion, BAL and $sPLA_2$ cDNA were cloned and characterized for the first time in large yellow croaker. The molecular ontogeny of some important lipolytic enzymes was described for the first time in this species. This study demonstrated that developmental profiles of BAL and $sPLA_2$ transcripts were mainly regulated by age in early stage and by the exogenous diet in late stage of large yellow croaker larvae. Furthermore, lipid hydrolysis in remnant lipoproteins (chylomicrons and VLDL), and HDL particles for utilization in the liver of large yellow croaker larvae increased as the larvae grew, which was reflected by the increasing concentration of HL mRNA.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (grant no. 31172425 and 31372541) and the National Basic Research Program of China (973 Program,

grant no. 2014CB138600). We thank S.H. Feng, R.T. Zuo and X.J. Dong for their assistance in the study. There are no conflicts of interest to report.

References

AOAC (2003) *Official Methods of Analysis* (17th edn). Association of Official Analytical Chemists, Washington, DC, USA.

Cahu C.L., Gisbert E., Villeneuve L.A.N., Morais S., Hamza N., Wold P. & Zambonino-Infante J.L. (2009) Influence of dietary phospholipids on early ontogenesis of fish. *Aquaculture Research* **40**, 989–999.

Cheng H., Wang X., Peng Y., Meng X., Sun S. & Shi X. (2009) Molecular cloning and tissue distribution of lipoprotein lipase full-length cDNA from Pengze crucian carp (*Carassius auratus* var. Pengze). *Comparative Biochemistry and Physiology* **153A**, 109–115.

Dennis E.A. (1994) Diversity of group types, regulation, and function of phospholipase A_2. *Journal of Biological Chemistry* **269**, 13057–13057.

Fujikawa Y., Shimokawa M., Satoh F., Satoh O., Yoshioka D., Aida S., Uematsu K. & Iijima N. (2012) Ontogeny of gene expression of group IB phospholipase A2 isoforms in the red sea bream, *Pagrus major*. *Comparative Biochemistry and Physiology* **161A**, 185–192.

Hansen T.W., Folkvord A., Grøtan E. & Sæle Ø. (2013) Genetic ontogeny of pancreatic enzymes in *Labrus bergylta* larvae and the effect of feed type on enzyme activities and gene expression. *Comparative Biochemistry and Physiology* **164B**, 176–184.

Izquierdo M.S., Socorro J., Arantzamendi L. & Hernández-Cruz C.M. (2000) Recent advances in lipid nutrition in fish larvae. *Fish Physiology and Biochemistry* **22**, 97–107.

Kortner T.M., Overrein I., Øie G., Kjørsvik E. & Arukwe A. (2011) The influence of dietary constituents on the molecular ontogeny of digestive capability and effects on growth and appetite in Atlantic cod larvae (*Gadus morhua*). *Aquaculture* **315**, 114–120.

Kortner T.M., Overrein I., Øie G., Kjørsvik E., Bardal T., Wold P. & Arukwe A. (2011) Molecular ontogenesis of digestive capability and associated endocrine control in Atlantic cod (*Gadus morhua*) larvae. *Comparative Biochemistry and Physiology* **160A**, 190–199.

Kurokawa T., Suzuki T., Ohta H., Kagawa H., Tanaka H. & Unuma T. (2002) Expression of pancreatic enzyme genes during the early larval stage of Japanese eel *Anguilla japonica*. *Fisheries Science* **68**, 736–744.

Lee R.F. (1974) Lipids of zooplankton from Bute inlet, British Columbia. *Journal of the Fisheries Board of Canada* **31**, 1577–1582.

Lee C.S., Sivaloganathan B., Walford J. & Reddy P.K. (2012) Ontogeny of lipolytic enzyme gene expression in sea bass (*Lates calcarifer*) larvae. *Contributions to Marine Science* **13**, 113–119.

Liang X.F., Oku H. & Ogata H.Y. (2002) The effects of feeding condition and dietary lipid level on lipoprotein lipase gene expression in liver and visceral adipose tissue of red sea bream *Pagrus major*. *Comparative Biochemistry and Physiology* **131A**, 335–342.

Lindberg A. & Olivecrona G. (2002) Lipoprotein lipase from rainbow trout differs in several respects from the enzyme in mammals. *Gene* **292**, 213–223.

Livak K.J. & Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.

Ma H., Cahu C., Zambonino-Infante J., Yu H., Duan Q., Le-Gall M. & Mai K. (2005) Activities of selected digestive enzymes during larval development of large yellow croaker (*Pseudosciaena crocea*). *Aquaculture* **245**, 239–248.

Mai K., Yu H., Ma H., Duan Q., Gisbert E., Zambonino-Infante J.L. & Cahu C.L. (2005) A histological study on the development of the digestive system of *Pseudosciaena crocea* larvae and juveniles. *Journal of Fish Biology* **67**, 1094–1106.

Mead J., Irvine S. & Ramji D. (2002) Lipoprotein lipase: structure, function, regulation, and role in disease. *Journal of Molecular Medicine* **80**, 753–769.

Murakami M. & Kudo I. (2002) Phospholipase A_2. *Journal of Biochemistry* **131**, 285–292.

Nilsson-Ehle P., Garfinkel A.S. & Schotz M.C. (1980) Lipolytic enzymes and plasma lipoprotein metabolism. *Annual Review of Biochemistry* **49**, 667–693.

Oku H., Koizumi N., Okumura T., Kobayashi T. & Umino T. (2006) Molecular characterization of lipoprotein lipase, hepatic lipase and pancreatic lipase genes: effects of fasting and refeeding on their gene expression in red sea bream *Pagrus major*. *Comparative Biochemistry and Physiology* **145B**, 168–178.

Parma L., Bonaldo A., Massi P., Yúfera M., Martínez-Rodríguez G. & Gatta P.P. (2013) Different early weaning protocols in common sole (*Solea solea* L.) larvae: implications on the performances and molecular ontogeny of digestive enzyme precursors. *Aquaculture* **414–415**, 26–35.

Perez-Casanova J.C., Murray H.M., Gallant J.W., Ross N.W., Douglas S.E. & Johnson S.C. (2004) Bile salt-activated lipase expression during larval development in the haddock (*Melanogrammus aeglefinus*). *Aquaculture* **235**, 601–617.

Perret B., Mabile L., Martinez L., Tercé F., Barbaras R. & Collet X. (2002) Hepatic lipase structure/function relationship, synthesis, and regulation. *Journal of Lipid Research* **43**, 1163–1169.

Rainuzzo J.R., Reitan K.I. & Olsen Y. (1997) The significance of lipids at early stages of marine fish: a review. *Aquaculture* **155**, 103–115.

 © 2015 John Wiley & Sons Ltd,

Rønnestad I., Yúfera M., Ueberschär B., Ribeiro L., Saele Ø. & Boglione C. (2013) Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. *Reviews in Aquaculture* **5**, S59–S98.

Ruby M.A., Goldenson B., Orasanu G., Johnston T.P., Plutzky J. & Krauss R.M. (2010) VLDL hydrolysis by LPL activates PPAR-α through generation of unbound fatty acids. *Journal of Lipid Research* **51**, 2275–2281.

Sæle Ø., Nordgreen A., Olsvik P.A. & Hamre K. (2010) Characterization and expression of digestive neutral lipases during ontogeny of Atlantic cod (*Gadus morhua*). *Comparative Biochemistry and Physiology* **157A**, 252–259.

Sæle Ø., Nordgreen A., Olsvik P.A. & Hamre K. (2011) Characterisation and expression of secretory phospholipase A_2 group IB during ontogeny of Atlantic cod (*Gadus morhua*). *British Journal of Nutrition* **105**, 228–237.

Saera-Vila A., Calduch-Giner J.A., Gómez-Requeni P., Médale F., Kaushik S. & Pérez-Sánchez J. (2005) Molecular characterization of gilthead sea bream (*Sparus aurata*) lipoprotein lipase. Transcriptional regulation by season and nutritional condition in skeletal muscle and fat storage tissues. *Comparative Biochemistry and Physiology* **142B**, 224–232.

Santamarina-Fojo S., Haudenschild C. & Amar M. (1998) The role of hepatic lipase in lipoprotein metabolism and atherosclerosis. *Current Opinion in Lipidology* **9**, 211–219.

Srivastava A.S., Kurokawa T. & Suzuki T. (2002) mRNA expression of pancreatic enzyme precursors and estimation of protein digestibility in first feeding larvae of the Japanese flounder, *Paralichthys olivaceus*. *Comparative Biochemistry and Physiology* **132A**, 629–635.

Tocher D.R. (2003) Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in Fisheries Science* **11**, 107–184.

Tocher D.R. (2010) Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquaculture Research* **41**, 717–732.

Tocher D.R., Bendiksen E.Å., Campbell P.J. & Bell J.G. (2008) The role of phospholipids in nutrition and metabolism of teleost fish. *Aquaculture* **280**, 21–34.

Wang C. & Hartsuck J.A. (1993) Bile salt-activated lipase. A multiple function lipolytic enzyme. *Biochimica et Biophysica Acta* **1166**, 1–19.

Wang Z., Li S., Sun L., Fan J. & Liu Z. (2013) Comparative analyses of lipoprotein lipase, hepatic lipase, and endothelial lipase, and their binding properties with known inhibitors. *PLoS ONE* **8**, 1–15.

Watanabe T. (1982) Lipid nutrition in fish. *Comparative Biochemistry and Physiology* **73B**, 3–15.

Watanabe T., Kitajima C. & Fujita S. (1983) Nutritional values of live organisms used in Japan for mass propagation of fish: a review. *Aquaculture* **34**, 115–143.

Wong H. (2002) The lipase gene family. *Journal of Lipid Research* **43**, 993–999.

Yao C.L., Kong P., Wang Z.Y., Ji P.F., Liu X.D., Cai M.Y. & Han X.Z. (2009) Molecular cloning and expression of MyD88 in large yellow croaker, *Pseudosciaena crocea*. *Fish and Shellfish Immunology* **26**, 249–255.

Zheng K., Zhu X., Han D., Yang Y., Lei W. & Xie S. (2010) Effects of dietary lipid levels on growth, survival and lipid metabolism during early ontogeny of *Pelteobagrus vachelli* larvae. *Aquaculture* **299**, 121–127.

Ziouzenkova O., Perrey S., Asatryan L., Hwang J., MacNaul K.L., Moller D.E., Rader D.J., Sevanian A., Zechner R., Hoefler G. & Plutzky J. (2003) Lipolysis of triglyceride-rich lipoproteins generates PPAR ligands: evidence for an anti-inflammatory role for lipoprotein lipase. *Proceedings of the National Academy of Sciences* **100**, 2730–2735.