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ONTOGENY OF LIPOLYTIC ENZYME GENE EXPRESSION IN SEA BASS (*LATES CALCARIFER)* LARVAE

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ABSTRACT. — In this study, larvae of Asian seabass *Lates calcarifer* were fed, from mouth opening to 11 days post hatching (dph), with rotifers and then with newly hatched *Artemia* nauplii. Gene expression patterns of the lipolytic enzymes phospholipase A2 (PLA2) and triacylgycerol lipase (TGL) were determined by quantitative real time-PCR. The larvae were sampled at 0, 1, 2, 3, 5, 7, 10, 12, 15, 17, 20, 22, 24, 26, 28 and 30 dph. Larval dry weight was also measured. In order to quantify the mRNA levels of PLA2 and TGL, a 244-bp fragment of β-actin complementary DNA was cloned and sequenced. Based on this sequence, a real-time reverse transcriptase polymerase chain reaction technique to monitor the change in the mRNA levels in the larvae was developed. PLA2 and TGL mRNA were present at hatching and their levels generally increased with larval age and then reached a plateau. PLA2 mRNA expression was highest at 17 dph while the highest TGL mRNA expression was observed at 20 dph.

KEY WORDS. — Phospholipase A2, triacylglycerol lipase, sea bass larvae, real-time PCR, mRNA expression

INTRODUCTION

Lipids are well known to be a major energy source for fish and play a central role in the development of marine fish larvae (Bell & Tocher, 1989; Olsen et al., 1991; Sargent et al., 1999). Lipids stored in the eggs provide energy and polyunsaturated fatty acids for synthesis of membranes and bioactive compounds in the early larvae until exogenous feeding (Fraser et al., 1988; Sivaloganathan et al., 1998). Phospholipids, triacylglycerols and wax esters together make up over 90% of the total lipids in marine zooplankton (Lee, 1974). Dietary phospholipids have been demonstrated to improve survival, increase growth and reduce deformities and malformations in fish (Geurden et al., 1995; Coutteau et al., 1997; Cahu et al., 2003). Phospholipids are more efficient than neutral lipids as a highly unsaturated fatty acid (HUFA) source (Koven et al., 1993; Salhi et al., 1999), and are also a major component in cell membranes (Kanazawa, 1985; Tocher et al., 2008).

Recent studies have shown that marine fish larvae produce specific digestive enzymes during the first few weeks post-hatching (Ma et al., 2001; Zambonino Infante & Cahu, 2001). Phospholipase A2 is one of the most important pancreatic enzymes that have been found in early yolk sac larvae and premetamorphosed larvae (Ozkizilcik et al., 1996; Hoehne-Reitan & Kjørsvik, 2004). Triacylglycerol is the predominant lipid storage form in many species of fish, particularly in the liver (Henderson & Tocher, 1987; Harmon et al., 1991). Dietary triacylglycerol is partially hydrolysed to free fatty acids and monoacylglycerol or completely hydrolysed to fatty acids and glycerol by pancreatic triacylglycerol lipase (Tocher & Sargent, 1984; Koven et al., 1994). A study

has shown that rainbow trout *Oncorhynchus mykiss* fed a krill-rich diet containing predominantly triacylglycerols, exhibited a higher activity of triacylglycerol lipase (Tocher & Sargent, 1984).

The ontogenetic changes of phospholipase A2 and triacylglycerol lipase activities have been studied in the eggs and larvae of striped bass (*Morone saxatilis*) using radioimmunoassays (Ozkizilcik et al., 1996). More recently, there has been increased use of molecular techniques to determine levels of RNA expression in marine fish larvae. Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) and real-time PCR have been developed for quantification of DNA in fish (Ma et al., 2001; Panserat et al., 2009; Zheng et al., 2010). Real-time PCR techniques are based on analysis of fluorescence in 96-well plates. The use of double-stranded DNA dye SYBR allows the detection of as low as 10 to 120 copies in real-time quantification of the mRNA (Simpon et al., 2000). Real-time PCR was employed to develop quantitative molecular markers for the ontogeny of digestive capability and to study gene expression patterns of phospholipase A2 in Atlantic cod larvae (*Gadus morhua*)(Kortner et al., 2011). However, there is a very little information on the mRNA levels of lipolytic enzymes in tropical fish larvae.

The present study used molecular approaches to characterize the ontogeny of lipolytic digestive functions in Asian sea bass (*Lates calcarifer*) larvae. Asian sea bass is a fast-growing marine fish cultured extensively in Southeast Asian countries and the Indo-Pacific region (Thirunavukkarasu et al., 2004). This species has contributed approximately 20,066 metric tonnes annually to the total world aquaculture production of 3.6 million tonnes (Tucker et al., 2002; Thirunavukkarasu et al., 2004). However, there is a general lack of knowledge concerning the molecular basis underlying larval digestive capability in Asian sea bass. The aim of the present study was to quantify the gene expression patterns of phospholipase A2 (PLA2) and triacylglycerol lipase (TGL) during early larval development using real-time PCR.

MATERIAL AND METHODS

Larval rearing. — Eggs of Asian sea bass (*Lates calcarifer*) were collected from floating net-cages (San Lay Marine Culture Pte. Ltd., Ubin Island, Singapore). Experiments were conducted between December 2003 and June 2005 at the Tropical Marine Science Institute, Singapore. The eggs were washed with UV-treated seawater several times. Buoyant eggs were transferred and stored in a plastic container with 20 L of UV-treated mildly aerated seawater. Three thousand newly hatched larvae were transferred to 100 L fiberglass tanks containing UV-treated seawater. Green algae (*Nannochloropsis*) were introduced to the larval rearing tanks at 0 days post hatching (dph). From 2 dph, larvae were fed once a day with rotifers (10-20 individuals ml^{-1}) until 11 dph and from then on larvae were fed with newly hatched *Artemia* nauplii (1-8 individual ml^{-1}). Throughout the larval rearing period, the water temperature and salinity were monitored (29-31°C and 30-32‰, respectively). Mild aeration was provided to each tank and the oxygen level was maintained above 6 mg l^{-1} by water exchange.

Larval sampling. — Sampling was carried out in the morning before feeding at 0, 1, 2, 3, 5, 7, 10, 12, 15, 17, 20, 22, 24, 26, 28 and 30 dph. Three replicate samples were collected for mRNA quantification on each of the sampling days. Samples were immediately frozen on dry-ice to stop the enzyme activities. All samples were then immediately stored at -80°C until assayed. To monitor growth, 30 larvae were sampled at 0, 8, 14, 18, 24 and 30 dph and were freeze dried using ALPHA 2-4 freeze dryer (Christ). Dry weight was measured using an electronic balance (Sartorius).

Reverse transcriptase-PCR analysis. — Total RNA from 3-dph larval samples were extracted using TRIzol reagent (Life Technologies) according to the manufacturer's procedure. Total purity and integrity of RNA was verified spectrophotometrically by determination at 260 nm. The purified total RNA (1 µg) was reverse transcribed into complementary DNA (cDNA) using a ThermoScript™ RT-PCR system (Invitrogen). cDNA was then amplified using 10 µmol of each primer, 0.2 units of Taq DNA Polymerase (Promega), 10 mM dNTP mix, 10 × PCR buffer, 25 mM $MgCl_2$ and 0.1% DEPC-treated water, in a final volume of 25 µl. Degenerate Polymerase chain reaction (PCR) primers were designed based on highly conserved regions from phospholipase A2 (PLA2), triacylglycerol lipase (TGL) and β-actin sequences of other fish available in Genbank. Primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/). Primer sequences and optimal annealing temperatures are shown in Table 1. The size of primers was designed to be less than 300 bp. The specific primers designed did not amplify the DNA from rotifers and *Artemia* nauplii. The primers were also designed to amplify at differing positions of cDNA fragments derived from genomic DNA, and when products of different sizes were obtained.

Amplification was performed in a TPersonal Thermocycler (Biometra). PCR was carried out by an initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 30 sec, specific annealing temperature (Table 1) for 30 sec, and 72°C for 45 sec; and a final extension of 72°C for 7 min. PCR products were detected by addition of 4 µl of sample mixed with 3µl of loading dye to a 1.0% agarose gel in 1 × Tris-borate-EDTA and then stained with ethidium bromide. Electrophoresis was performed at 120mV in a submarine gel unit at room temperature. Gels were analysed using a MultiImage® Light Cabinet (Alpha Innotech Corporation) and photographed. DNA bands of expected size were excised from agarose gel and purified with QIAquick Gel Purification Kit (QIAGEN).

Cloning and sequence analysis. — The purified PCR product of β-actin (244-bp fragment) was ligated into pGEMT-Easy Vector (Promega), and then transfected into *Escherichia coli* XL10-Gold Ultracompetent cells (Stratagene), plated on LB agar plates containing ampicilin, IPTG and X-Gal (Sigma) and incubated overnight at 37°C. White colonies

Table 1. Primer pair sequences and annealing temperature conditions for genes used for reserve transcriptase-PCR amplification and real-time PCR

Gene	Forward primers (5'- 3')	Reverse primers (5'- 3')	Annealing temperature (°C)
PLA2	cacacctgtggatgatctgg	cttcttgtcacactcgcaga	60
TGL	gcctcgaccctaaaaacaaa	gccaatcaaatgagactttc	57
β-Act	ggaatccacgagaccaccta	gctggaaggtggacagagag	60

were selected and transferred to 5 ml LB broth containing ampicillin, cultured at 37°C for 4 h. The cloned plasmid DNA was purified from the bacteria cells using a QIAprep Spin Miniprep Kit (QIAGEN). Cloned plasmid DNA and the DNA of PLA2 and TGL were sequenced using BigDye® Terminator v3.1 Sequencing Kit and analysed on ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems Inc., USA). All sequences were confirmed using the Basic Local Alignment Search Tool software (BLAST, available at http://www.ncbi.nim.nih.gov).

Construction of the standard curve. — For determination of the absolute copy number of target genes, cloned plasmid DNA with β-actin gene was used to generate a standard curve. The copy number of the cloned plasmid was calculated according to the molecular masses of the plasmid (1 mol of molecular masses: 6×10^{23} copies gene). The cloned plasmid DNA was serially diluted at a range 0.55 fg ml^{-1} to 355 pg ml^{-1} (or 10^2 to 10^8 log copies). The standard curve was automatically generated by plotting the crossing point versus the known copy number of plasmid DNA in quantitative PCR analyses.

Quantitative (real-time) PCR analysis. — Total RNA was extracted from whole-larva samples using RNeasy® Mini Kit (QIAGEN). Larval samples were homogenized and divided into 3 aliquots. RNeasy-Free DNAse Set (QIAGEN) also was used to clean up DNA contamination in the samples and total RNA was purified. The purity and integrity of the RNA samples were verified by a spectrophotometer and agarose gel electrophoresis. The concentration of total RNA was determined by spectrophotometric analysis.

Lipolytic enzyme gene expression levels were quantified by real-time PCR. One-step reverse transcribed-PCR was performed from 1 µg of total RNA from all samples using a combination of random hexamer and poly-T primers from the iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio-Rad). Real-time PCR was performed in the MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad® Laboratories Inc.). Quantitative PCR analyses for each gene were performed in triplicate for each cDNA sample. The total volume of the PCR reaction was 25 µl containing 12.5 µl of iQ™SYBR® Green Supermix (Bio-Rad), 2 µl of diluted cDNA and 0.5 µl of each specific primers (10 µmol). The three-step real-time PCR program included an enzyme activation step at 95°C for 3 min, 50 cycles of 95°C for 30 sec, 57-60°C (depending on the primers used, Table 1) for 30 sec, 72°C for 20 sec. Following the final step of the quantitative PCR, melting curve was systematically monitored (with a heating rate of 0.1°C s^{-1} from 55 to 95°C) to ensure that only one fragment was amplified. Melting curve was used to determine the specificity of PCR product and also to discriminate primer dimmers from PCR product. A set of negative controls was prepared without addition of the cDNA template to assess the accuracy of the quantification in each real-time PCR analysis. The real-time PCR products were checked by gel electrophoresis to confirm the specificity of real-time PCR.

The calculations for quantification of DNA concentration were automatically generated when the fluorescence rose above background fluorescence (crossing point). Results were recorded as cycle threshold (Ct) values. Ct values were converted into mRNA copy number using standard plots of Ct versus log copy number. The standard plots were generated for each target sequence using known amounts of plasmid containing β-actin. Real-time PCR efficiency (E) was determined using Bio-Rad® software. β-actin was expressed as a housekeeping gene with an endogenous standard. Quantification of mRNA was based on the crossing point of each sample and data were converted into copy numbers.

RESULTS

Identification of lipolytic enzyme genes. — The partial nucleotide sequences and deduced amino acid sequences of PLA2 and TGL were amplified from PCR and identification was confirmed by sequencing. The sequence of PLA2 revealed a fragment of 205 bp highly homologous to *Pagrus major* (86% similarity), *Takifugu rubripes* (86% similarity) and *Dicentrarchus labrax* sequences (85% similarity)(Fig. 1). The TGL sequence of 186 bp was found to be highly similar to *Paralabrax maculatofasciatus* (88% similarity), *D. labrax* (85% similarity) and *Menidia estor* sequences (84% similarity)(Fig. 2). By agraose gel electrophoresis for reverse transcriptase- PCR reaction, a single amylase-specific product was generated using specific primers (Fig. 3).

Quantification of mRNA using real-time PCR. — Figure 4 shows the growth of sea bass larvae during the experimental period. Specific primers were designed to amplify sea bass PLA2 and TGL DNA for real-time PCR. The specific primers did not amplify the DNA from rotifers and *Artemia* nauplii. No amplification products were obtained in the negative control reactions in real-time PCR.

A series of diluted recombinant plasmid DNA with β-actin gene was used to generate a standard curve (Fig. 5A). Fig. 5B shows that the crossing point decreased linearly with increasing amounts of plasmid DNA copy numbers. All data were located within this linear amplification range.

The copy numbers of recombinant plasmid were used to quantify the mRNA levels for PLA2 and TGL using the standard curve.

Changes in mRNA levels of lipolytic enzymes in developing sea bass larvae over time are shown in Fig. 6. The mRNA could be detected as early as 0 dph for both PLA2 and TGL. mRNA expression of PLA2 was low at the beginning of larval development (0 dph and 1 dph) and the mRNA expression increased from 2 dph until 17 dph, followed by a slight decrease until 30 dph. A similar pattern in TGL mRNA expression was also observed (Fig. 6). A slight increase of TGL mRNA expression was observed after 1 dph and mRNA expression remained at that level until 7 dph; this was followed by an increase till 20 dph, and then a slight decline after 20 dph (Fig. 6).

These results suggest that PLA2 and TGL act as important lipolytic digestive enzymes during early development of sea bass larvae. The pattern of mRNA expression for both these digestive enzymes strongly suggests a defined regulatory system that controls lipolytic digestion.

DISCUSSION

In marine fish, phospholipase A2 cDNA has been isolated and the mRNA levels of lipolytic gene expression have

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Asian seabass       ------CACACCTGTGGATGATCTGGACAGGTGCTGCCAAGTGCATGACC  44
European seabass    CTCTGGCACACCTGTGGATGAACTGGACAGTTGCTGCTACGTCCATGACC  50
Pufferfish          CTCCGGCACGCCTGTGGATGATCTGGACAGGTGCTGCCAAGTGCACGACC  50
Red sea bream       CTCGGGCACACCCGTGGATGATCTGGACAGATGCTGCCAAGTGCACGACG  50

Asian seabass       AGTGTTACAGTGATGCCATGCAGCATCCTGACTGCTGGCCCATCCTTGAC  94
European seabass    AGTGCTATAATGATGCTATGCAACACCCTGAGTGCTGGCCCATCATCGAC  100
Pufferfish          AGTGTTACAGCGACGCCATGCAGCATCCCGAGTGCTGGCCCATCCTGGAC  100
Red sea bream       CGTGTTACACTGATGCCATGCAGCACCCCGAGTGCTGGGCCATCCTGGAC  100

Asian seabass       AACCCTTACACTGAGTTCTACGATTACAGCTGTGACAAACAAAACAGGAA  144
European seabass    AATCCTTACACCGAGTTCTACGACTACAACTGCGACGAGAACAACAAGAA  150
Pufferfish          AACCCCTACACCGAACTTTACGACTACAGCTGCGATGAGGCTAACAAGAA  150
Red sea bream       AATCCTTACACCGAGTTCTACGCCTACAACTGTGACGAGCAGAGCAAGAA  150

Asian seabass       GGTCACCTGTGGCAACAAAAACAACGAATGTGAGATGTTTATCTGCGAGT  194
European seabass    GGTCACATGTGGCAGCTCAAACAACGAATGTGAGATGTTCATCTGTGAGT  200
Pufferfish          GGTCACCTGTGGCAGCAAGAACAATGTATGCGAGATGTTCATCTGCGAGT  200
Red sea bream       GGTCACCTGTGGCAACAACAATGACGAATGCGAGATGTTCATCTGTGAGT  200

Asian seabass       GTGACAGGAAG --------                                205
European seabass    GTGACAGGAAGGCCGCCGAG                               214
Pufferfish          GCGACCGCAAGGCGGCAGAA                               214
Red sea bream       GCGACAGGAAGGCTGCCGAG                               214
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Fig. 1. Alignment of amino acid sequence of Asian seabass (*L. calcarifer*) PLA2 with PLA2 sequences of other fish species, red sea bream (*Pagrus major*, accession number AB009286), pufferfish (*Takifugu rubripes*, accession number CA844975) and European seabass (*Dicentrarchus labrax*, accession number AJ132762).

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Asian seabass       -------------------------GCCTCGACCCTAAAAACAAATCT  23
European seabass    ATGATGGAGAGTTGGGTGGTCAGGTTAGCTACAACCCTAAAGACAAATCT  50
Mexican silverside  ATGATGGAAAACTGGGTGACCAGACTGGCTTCAACTCTGAAGCTGAATCT  50
Spotted sand bass   ATGATGGAGGGCTGGGTGGTCAGGTTAGCTCGACCCCTGAAGACAACTCT  50

Asian seabass       GATACATGTTAACGTGGTGATCACTGACTGGCTGACCCTGGCACACCAAC  73
European seabass    AAAAGACGTCAACGTGGTGATTACTGATTGGCTGTCCCTGGCTCACCAGC  100
Mexican silverside  TGTTGATGTAAATGTGGTGATTACGGACTGGCTGTCCCTGGCTCAGCAGC  100
Spotted sand bass   AATAGACGTCAACGTGGTGATTACCGACTGGCTGTCACTGGCTCACCAGC  100

Asian seabass       ACTACCCTATAGCAGCACAGAGCACCCGCATTGTTGGAAAAGACATCGCT  123
European seabass    ACTACCCCACAGCAGCACAGAGCACCCGCACTGTGGGAAAAGACATAGCC  150
Mexican silverside  ACTACCCCATAGCTGTACAGAGCACCCGCACTGTCGGGAAGGACATAGCT  150
Spotted sand bass   ACTACCCCACAGCTGCACAGAGCACCCGCACTGTTGGAAAAGACATAGCT  150

Asian seabass       CACCTGCTGGAGACTTTTAAGGATCTGTACCAGTACCCAGTTAGAAAGTC  173
European seabass    CACCTGCTGCAGTCACTTCAGGTACACTACCAGTACCCAGTTAGAAAGGC  200
Mexican silverside  TACCTGCTGGGATCACTTAAGGAAAAGTACCAGTATTCCGCTAGGAAAGC  200
Spotted sand bass   CACCTGCTGCAGTCACTCCAGGTACACTACCAGTACCCGGTTAAAAAGGT  200

Asian seabass       TCATTTGATTGGC---------------                        186
European seabass    TCATTTGATTGGCTACAGCCTTGGAGCG                       228
Mexican silverside  TCATCTGATTGGCTACAGCCTTGGAGCG                       228
Spotted sand bass   TCATTTGATTGGCTACAGCCTTGGAGCG                       228
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Fig. 2. Alignment of amino acid sequence of Asian seabass (*L. calcarifer*) TGL with TGL nucleotide sequences of other fish species, European seabass (*Dicentrarchus labrax,* accession number AJ275976), Mexican silverside (*Menidia estor*, accession number FJ859999) and spotted sand bass (*Paralabrax maculatofasciatus*, accession number AJ418039).

been studied in young larvae (Zambonino Infante & Cahu, 1999; Cahu et al., 2003). The present study showed the levels of gene expression of PLA2 and TGL right from hatching, and the level of TGL was especially high. Both these lipolytic enzyme activities increased after the onset of feeding. TGL gene expression has been detected from hatching and throughout the larval period in winter flounder using reverse transcription-PCR (Murray et al., 2003). The enzyme activities of PLA2 and TGL have also been reported

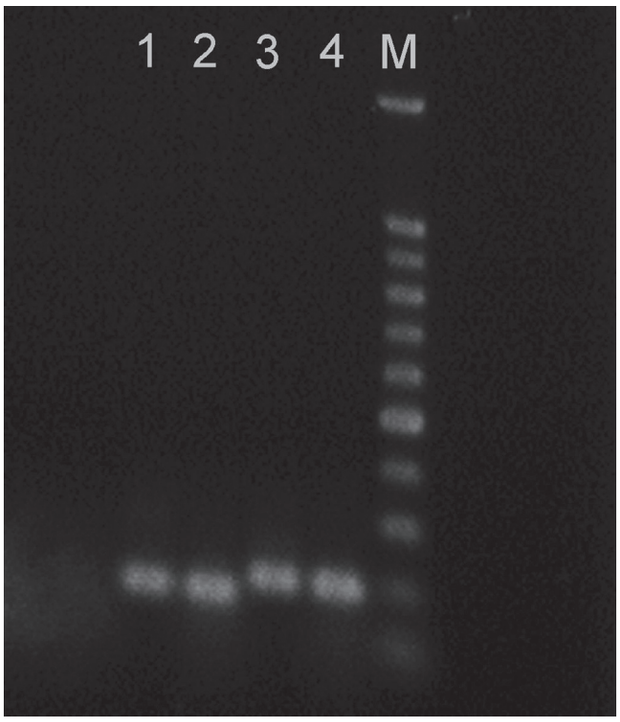


Fig. 3. Agarose gel electrophoresis reverse transcriptase-PCR product of PLA2 and TGL in sea bass larvae. Lane 1 and 2 were amplification products of TGL (186 bp); lane 3 and 4 were amplification products of PLA2 (205 bp). The PCR products were resolved in 1% agarose gel using a 100 bp DNA ladder (Promega) as marker (lane M).

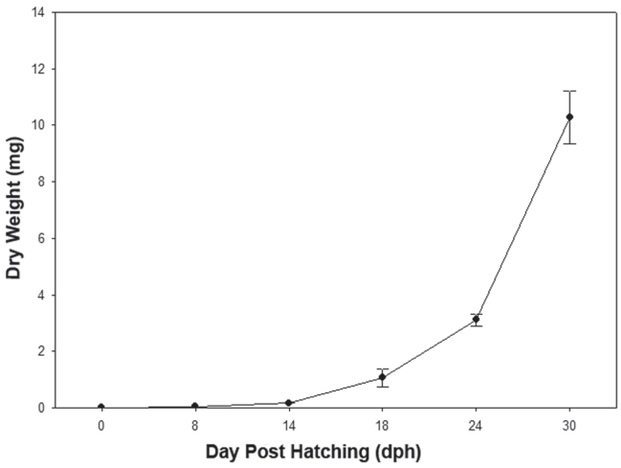
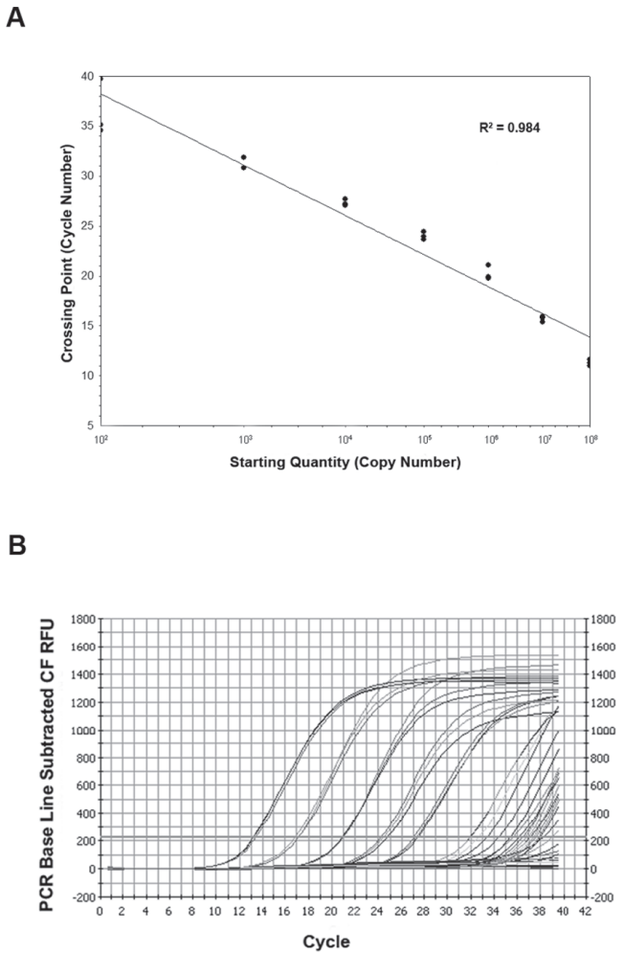


Fig. 4. Dry weight (mg) of whole-body Asian sea bass larvae fed rotifers and *Artemia* nauplii, and sampled at 0, 8, 14, 24 and 30 dph. Data are given as mean values ± standard error.



Figs. 5. Real-time PCR using a cloned plasmid DNA with β-actin gene as template: A. Calibration curve obtained by correlating crossing point and plasmid copy number from B; B. Real-time PCR amplification from 0 to 1.0 × 10^7 copies of plasmid DNA with β-actin genes.

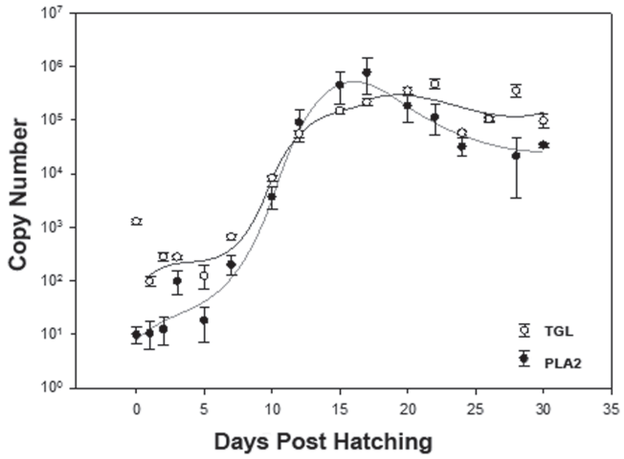


Fig. 6. Real-time PCR anlaysis of mRNA expression for PLA2 and TGL during early development of sea bass larvae. Data analysis was based on the crossing point of each sample and data were converted into copy numbers according to the standard curve obtained in Fig. 5A.

in premetamorphosed larvae of striped bass *Morone saxatilis* using radioimmunoassays (Ozkizilcik et al., 1996).

An increase in mRNA levels was observed up to day 17 after hatching in the present study, followed by a decrease after that. This suggested that the ontogenic changes in PLA2 and TGL gene expressions are transcriptionally regulated during larval development. The transcription regulation has been demonstrated for almost all major digestive enzymes at significant levels before mouth opening in marine fish (Zambonino Infante & Cahu, 2007). The present study also suggested that the gene expressions were primarily "pre-programmed" since mRNA was already detected before first feeding, and even at hatching. Gene expression continued to increase in the larvae fed with rotifers and *Artemia* nauplii. It has been suggested in previous studies that ingested diet can modulate enzyme mRNA levels in marine fish larvae (Cahu et al., 2003; Kortner et al., 2011). Furthermore, a change of feeding from rotifer to *Artemia* nauplii can influence TGL gene expression in *Chirostoma eastor* (Toledo-Cuevas et al., 2011). Another study also showed the mRNA levels of PLA2 positively responded to a high lipid diet in young fish larvae (Zambonino & Cahu, 1999). This suggests that formulated diets or live prey enriched with lipids may be beneficial to the larvae during a specific window of time.

Anatomical and functional development of the digestive tract and changes in the activity of lipolytic enzymes continue during the first month of life in marine fish. In newly hatched fish, the larvae do not have a distinct pancreas until metamorphosis, but rather diffuse pancreatic cells associated with the gut or in the liver (Yardley, 1988). The stomach and pyloric caeca do not form until 13 days after hatching and are not completely formed until day 17 in Asian sea bass (Walford & Lam, 1993). As the sea bass larvae get older, their stomach and pyloric caeca get larger and the pH levels in the stomach become more acidic on day 17 (Walford & Lam, 1993). Before the stomach has become fully functional, the larvae are dependent on lipolytic enzyme activities which hydrolyse lipids in the gut. However, when the development of the digestive system becomes more complete, larvae do not only rely on lipase activities. The functional change of enzyme activity is linked to the development of the digestive tract.

Real-time PCR is generally more specific than using enzymatic assays (Ma et al., 2003) and molecular techniques provide a better and more sensitive approach to quantifying gene expression in early fish development. This molecular approach offers opportunities to understand the regulation of lipolytic enzymes in marine fish larvae.

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