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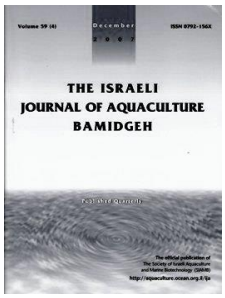

Ontogenetic Development of Digestive Enzymes in Orange-Spotted Grouper (*Epinephelus coioides*, Hamilton, 1822) Larvae

Huayang Guo[1, 2], Jianzhu Li[1], Zhenhua Ma[1*], Jiasong Zhang[1], Panlong Zhen[1], Dachuan Cheng[1]

[1]South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, 510300, China
[2]Key Laboratory of South China Sea Fishery Resources Exploitation and Utilization, Ministry of Agriculture, Guangzhou, 510300, China

Keywords: ontogenetic development; digestive enzyme; enzymatic assay; larvae; *Epinephelus coioides*

Abstract

The digestive physiology of orange-spotted grouper (*Epinephelus coioides*) was studied by assessing the specific and total activities of alkaline phosphatase, amylase, lipase, chymotrypsin, and trypsin, from hatching to 34 days post-hatching (DPH). From the onset of exogenous feeding, total alkaline phosphatase, amylase, lipase, and chymotrypsin activity fluctuated then sharply increased from 18 to 34 DPH. In contrast to other enzymes, the specific and total activity of trypsin remained at a low level from 1-26 DPH, and reached the maximum on 30 DPH. Digestive enzymes activity indicated that orange-spotted grouper were able to digest proteins, lipids, and carbohydrates at an early developmental stage. Results from the present study will improve understanding of the ontogeny of orange-spotted grouper during the larval stage, and provide a guide to hatchery feeding of this economically important fish.

Huayang Guo and Jianzhu Li contributed equally to this work.*Corresponding Author: Email: zhenhua.ma@hotmail.com

Introduction

Groupers are economically important marine fish, highly cultured in Asia for domestic consumption and overseas export. Orange-spotted grouper *Epinephelus coioides*, one of the most popular groupers, is commercially cultured in tropical and subtropical regions, particularly in Southeast Asia and China. The early developmental stage of marine fish presents a challenge in marine fish aquaculture (Ma et al., 2014). During this stage, fish larvae are extremely sensitive to environmental factors, and experience a transitional period from endogenous nutrition to exogenous feeding in the ontogenesis of the digestive system (Yang et al., 2010). Adequate nutrition is one of the principal factors influencing larval survival which depends on effective ingestion, digestion, and assimilation of feeds (Lazo et al., 2007). High incidence of mortality, skeletal deformities, and nutritional disorders normally occur in the early developmental stage due to inadequate feed uptake (Tong et al., 2012; Ma et al., 2012; Ma et al., 2014). A better understanding of the digestive functions and ontogenetic patterns of digestive enzyme activity could provide useful information on the nutritional requirements of fish larvae and feeding protocols for optimizing larval mass-rearing production (Tong et al., 2012).

Digestion is an important physiological process in animal metabolism since it determines the availability of nutrients needed for all biological functions (Gisbert et al., 2009). The digestive physiology of fish is critical at the larval stage, and the type and function of digestive enzymes largely determines the efficiency of the entire digestive process (Kolkovski, 2001). The ontogenetic development of digestive enzymes reflects the functional development of the digestive tract and digestive capacities of an organism, and consequently, has been used as a physiological biomarker to assess the nutritional status of fish at early life developmental stages (Yúfera and Darias, 2007). Understanding this process could improve growth and survival of fish larvae. The ontogenesis of digestive enzymes has been documented in many fish species, such as *Seriola lalandi* (Chen et al., 2006)*, Dentex dentex* (Gisbert et al., 2009), *Petenia splendida* (Uscanga-Martínez et al.,2011), *Acipenser persicus* (Babaei et al., 2011), *Centropomus undecimalis* (Jimenez-Martinez et al., 2012), *Ompok bimaculatus* (Pradhan et al.,2013), and *Trachinotus ovatus* (Ma et al., 2014).

A variety of small benthic shellfish and cephalopods are often caught and fed to *E. coioides* in culture. In commercial culture, artificial feeds are beginning to be administered to *E.coioides*. Low and inconsistent survival rates in grouper larvae are a significant obstacle in this initial feeding stage (Toledo et al., 1999). Knowledge of nutritional physiology is important for improving the seedling survival rates of larvae. The digestive capacity including development of the gastrointestinal tract of *E. coioides* larvae has been studied (Quinitio, et al., 2004a, b) as has the ontogeny of digestive enzymes (Eusebio et al., 2004; McBride, 2004). Our study was undertaken to examine the ontogenetic development of some other important digestive enzymes in orange-spotted grouper larvae, with the aim of qualifying and evaluating digestive enzyme activity during the initial ontogeny of *E. coioides,* and linking the data with the most critical phases of hatchery management.

Material and methods

Egg and larval fish rearing. Orange-spotted grouper larvae were cultured at the Tropical Fisheries Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Xincun Town, China. Fertilized orange-spotted grouper eggs were obtained from a spawning tank through natural fertilization in a commercial fish hatchery in Hainan Province, China. Upon arrival, eggs were hatched in 500L fiberglass incubators. During incubation, water temperature, oxygen content, and pH

were maintained at 26.5°C, >6.0mg/L, and 8.0, respectively. Eggs hatched after 96 h of incubation and were transferred to three indoor 1000L fiberglass rearing tanks at 60 fish/L. Fish were reared in a constant flow-through system with a daily exchange rate of 100% tank volume. Air stones were used in each tank to maintain dissolved oxygen at saturation, and to promote a homogeneous distribution of microalgae and live foods. Photoperiod was 14L:10D, with light intensity of 2400 Lux.

During the experimental period, salinity was maintained at 33‰, and water temperature was controlled at 27.6±0.7°C. pH and oxygen were recorded as 6.6±0.5 and 7.9±0.3 mg/L, respectively. Debris and dead animals were siphoned out of all tanks daily.

Rotifers (*Brachionus rotundiformis*) screened with a 120-μm mesh were fed to the larvae from initial feeding (3 DPH) until 10 DPH at a density of 10 individuals/mL. Instant microalgae (*Nannochloropsis* sp., Qingdao Hongbang Biotech. Pty Ltd., China) were fed to the rotifers. Before being placed into the larval rearing tanks, the rotifers were enriched with S. presso (INVE Aquaculture, Thailand) for 12 h. In addition, instant microalgae paste (*Nannochloropsis* sp.) was added to the larval fish tanks to feed rotifers and create a green ambient environment. Co-feeding based on rotifers and *Artemia nauplii* enriched with S. presso (INVE Aquaculture, Thailand) was conducted from 10-15 DPH, after which the study larvae were fed *Artemia nauplii* independently at 5 *A.nauplii*/mL until the end of the experimental period.

Fish sampling and growth measurements. Fish larvae were randomly collected in triplicate from three different rearing tanks daily from hatching to 4 DPH. They were then collected every 2–4 days up to 34 DPH to measure fish growth. On each sampling day, 20 larvae were collected and lightly anesthetized with tricaine methanesulfonate (MS-222, Sigma, USA) for growth measurements. Using a stereo microscope, standard fish length was measured from the upper jaw to the end of the vertebral column to the nearest 0.05 mm. Growth was determined by the specific growth rate (SGR) in % per day, and absolute growth rate (AGR) in mm/day, using the following equations (Hopkins, 1992):

$SGR = 100(\ln (SL_f) - \ln (SL_i)) / D_t$ and

$AGR = ((SL_f) – (SL_i)) / D_t$,

where SL_f and SL_i are the final and initial larval fish standard lengths (mm), respectively, and

Dt is the time between sampling intervals

Triplicate groups of orange-spotted grouper larvae were randomly collected in from each tank on 1, 3, 6, 10, 14, 18, 22, 26, 30, and 34 DPH to examine digestive enzyme development. To minimize the potential effects of exogenous enzymes, sampling was conducted in the morning prior to the first feeding. The sampled specimens were then thoroughly rinsed in distilled water to remove external salt, and immediately stored in liquid nitrogen until assayed.

Enzymatic assay preparation and procedure. For each assay, a pooled sample (10-200 individuals before 34 DPH) of frozen fish was partly thawed, weighed, and homogenized using a glass homogenizer on ice in five volumes of 0.2 M NaCl (W/V) (Ma et al., 2014). The suspensions were centrifuged at 13,300 *g* for 15 min at 2°C to obtain the supernatant for subsequent enzymatic analyses. The supernatant was incubated in the enzyme substrate under 25°C or 37°C, and read on a spectrophotometer (SpectraMax® M5, USA) at the target wavelength. All measurements were carried out in triplicate.

Chymotrypsin (EC 3.4.21.1) activity was determined at 256 nm and 25°C according to Ásgeirsson and Bjarnasson (1991). One unit of chymotrypsin was

defined as the amount of enzyme required to liberate 1 μmol/L of tyrosine per minute.

Alkaline phosphatase (E.C. 3.1.3.1) activity was assayed using an alkaline phosphatase test kit (Catalog No. TR11015, Thermo Electron, Waltham, MA, USA) according to the International Federation of Clinical Chemistry (IFCC) methods (Tietz et al., 1983). Increase in absorbance was measured at a wavelength of 405 nm for 3 min.

α-amylase (E.C. 3.2.1.1) activity was measured using an amylase activity colorimetric assay kit (Catalog No. K711-100, BioVision, Milpitas, CA, USA). In the assay, the ethylidene-pNP-G7 substrate was cleaved by α-amylase. The smaller fragments produced are acted upon by α-glucosidase, which causes the ultimate release of the chromophore, measured at 405 nm.

Trypsin (E.C. 3.4.21.4) activity was measured using a trypsin activity assay kit (Catalog No. K771-100; BioVision, Milpitas, CA, USA). In the assay, trypsin was cleaved in the substrate to generate p-nitroaniline (p-NA), which was detected at k = 405 nm.

Lipase (E.C. 3.2.1.1) activity was assayed using a lipase assay kit (Catalog No. K722-100; BioVision, Milpitas, CA, USA). In this assay, lipase hydrolyzed a triglyceride substrate to form glycerol, which was quantified enzymatically via monitoring a linked change in OxiRed probe absorbance at k=570 nm.

Enzymatic activity was expressed as total activity, defined as milli-units (mU) per larval fish or units per larval fish (mU/larva or U/larva) based on the whole-fish homogenate. Specific activity was expressed as milli-units per milligram of protein or units per milligram of protein (mU/mg protein or U/mg protein). Soluble protein of crude enzyme extracts was quantified using a bicinchoninic acid protein assay kit (Catalog No. BCA1 and B9643, Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis. Larval growth was evaluated with the model of exponential growth $y = ae^{bx}$;

where y = individual fresh length, e = exponential base, a = initial length, b = growth rate, and x = larval age in days.

The parameters of the model were evaluated through Mardquat interactions. Values of the measured variables were expressed as means±standard deviations. The measurement of each variable was presented as the mean of pooled fish larvae ranging from 10 to 200 individuals on a sampling day. Mean values of both total and specific activity of each digestive enzyme between each sampling date were compared with one-way ANOVA (PASW Statistics 18.0; Chicago, IL, USA), followed by Duncan's test when significant differences were below 0.05. All data were tested for normality, homogeneity, and independence before ANOVA. When the homogeneity of variances was violated, a nonparametric test (Welch test) was performed.

Results

Growth of *E. coioides* larvae in terms of standard length (mm) and feeding protocol during larval development are shown in Fig. 1. The average standard length of fish larvae was 1.67±0.04 mm at hatching and 25.24±3.18 mm on 34 DPH. The AGR and SGR were 0.74 mm/d and 7.99%/d, respectively. The growth of orange-spotted grouper larvae followed an exponential curve, which was fitted by: $y = 1.198e^{0.180x}$ (R^2 = 0.919).

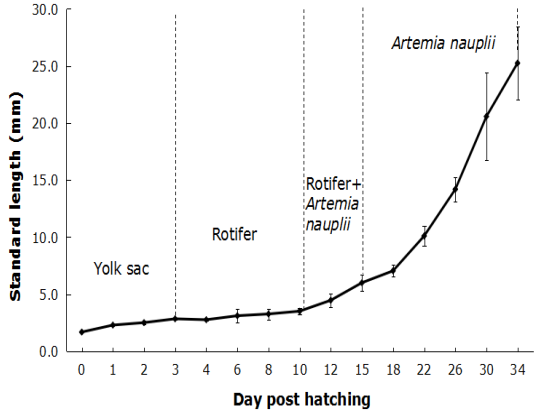

Fig. 1. Standard length of *Epinephelus coioides* larvae from 0 to 34 days post-hatching (DPH). Feeding protocol is presented above the growth curve. Data are expressed as mean±SD (n=10).

The activity of five digestive enzymes, chymotrypsin, alkaline phosphatase, amylase, trypsin, and lipase was evaluated from 1-34 DPH. Significant differences were detected in both total and specific activities of the digestive enzymes between sampling dates during ontogeny (P<0.05).

Alkaline phosphatase. Total alkaline phosphatase activity was 0.486±0.183 mU/larva on 1 DPH (Fig. 2). After first feeding, total activity increased progressively with fish age. On 14 DPH, total activity of alkaline phosphatase reached 14.028±5.418 mU/larva, then declined to 12.152±0.833 mU/larva on 18 DPH. Total activity then gradually increased to 45.491±10.535 mU/larva on 34 DPH, which was 90-fold higher than on 1 DPH (Fig. 2).

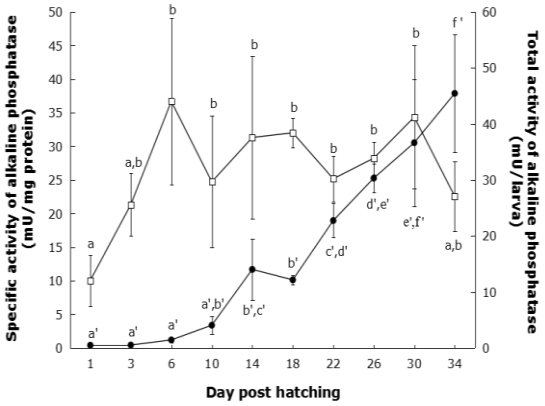

Fig. 2. Total activity of alkaline phosphatase in mU/larva (black circle) and specific activity of alkaline phosphatase in mU/mg protein (clear square) from 1 to 34 days post-hatching. Mean±SD (n = 3) with the same superscript letter are not significantly different in multiple comparisons (P > 0.05).

The specific activity of alkaline phosphatase was 10.055±3.797 mU/mg protein on 1 DPH, before reaching 36.691±12.399 mU/mg protein on 6 DPH (P<0.05, Fig. 2). After peaking (6 DPH), specific activity continuously remained at a high level. By 34 DPH, the specific activity was 22.560±5.228 mU/mg protein, which was 2.2-fold higher than that in newly hatched larvae. No significant differences were found between these two sampling points (P> 0.05, Fig. 2).

Amylase. The total activity pattern of amylase was similar to that of alkaline phosphatase (Fig. 3). Total amylase activity remained at a low level in the first 6 days, and increased sharply from 6 DPH to 34 DPH (Fig. 3). On 34 DPH, total amylase activity was 25.474±3.369 mU/larva, which was 26.1 fold higher than on 1 DPH (P<0.05).

Specific amylase activity on 1 DPH was 20.206±4.726 mU/mg protein, which increased to a maximum of 61.630±8.302 mU/mg protein on 6 DPH. A gradual decrease was then observed until the end of the experiment. Specific

activity dropped to 12.641±1.672 mU/mg protein on 34 DPH, which was significantly lower than on 1 DPH (P<0.05, Fig. 3).

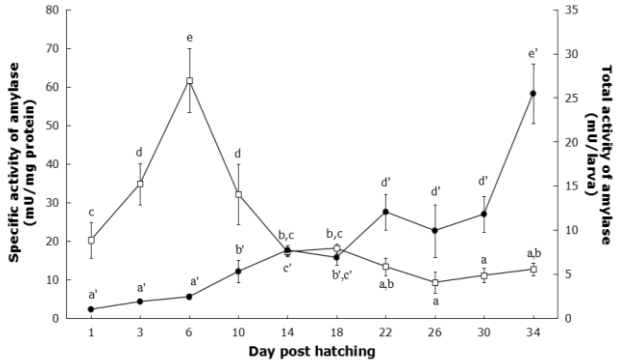

Fig. 3. Total amylase activity in mU/larva (black circle) and specific activity of amylase in mU/mg protein (clear square) from 1 to 34 days post-hatching. Means±SD (n=3) with the same superscript letter are not significantly different in multiple comparisons (P>0.05).

Lipase. Total lipase activity was 0.023±0.028 mU/larva on 1 DPH, and steadily increased to 0.440±0.141 mU/larva on 14 DPH (Fig. 4). Total activity then decreased to 0.301±0.061 mU/larva on 18 DPH, coinciding with the food shift from rotifers to *Artemia nauplii*. Total activity steadily reached a high level until on 30 DPH. Total activity then increased sharply to 1.914±0.267 mU/mg larva on 34 DPH (P < 0.05, Fig. 4).

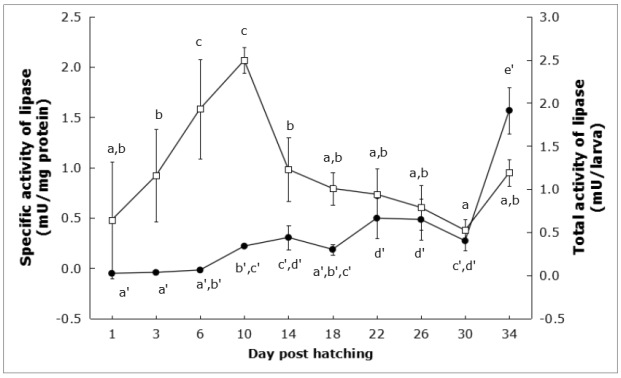

Fig. 4. Total activity of lipase in mU/larva (black circle) and specific activity of lipase in mU/mg protein (clear square) from 1 to 34 days post-hatching. Means±SD (n=3) with the same superscript letter are not significantly different in multiple comparisons (P > 0.05).

Specific lipase activity increased significantly from 0.479±0.583 mU/mg protein on 1 DPH to a maximum of 2.067±0.130 mU/mg protein on 10 DPH (P < 0.05, Fig. 4). After 10 DPH, specific activity dropped rapidly to 0.376±0.109 mU/mg protein on 30 DPH. On 34 DPH, specific activity increased and reached 0.950±0.267 mU/mg protein, which was similar to the level in newly hatched larvae (P > 0.05, Fig.4).

Chymotrypsin. The pattern of chymotrypsin activity is shown in Fig. 5. Total chymotrypsin activity was 2.698±0.236 mU/larva on 1 DPH. After first feeding, the activity increased sharply to 13.692±2.469 mU/larva on 6 DPH. Total activity peaked at 22.565±7.218 mU/larva on 14 DPH, coinciding with feeding on rotifers and *Artemia nauplii*. After 14 DPH, total activity progressively decreased to 4.991±2.696 mU/larva on 18 DPH, coinciding with feeding on *Artemia nauplii* alone. Total chymotrypsin activity reached a second peak on 22 DPH (31.129±2.671 mU/larva), and then dropped to 24.524±6.466 mU/larva on 30 DPH (Fig. 5). On 34 DPH, total activity reached a maximum of 35.698±4.461 mU/larva, which was 13.23 times greater than that in newly hatched larvae (1 DPH).

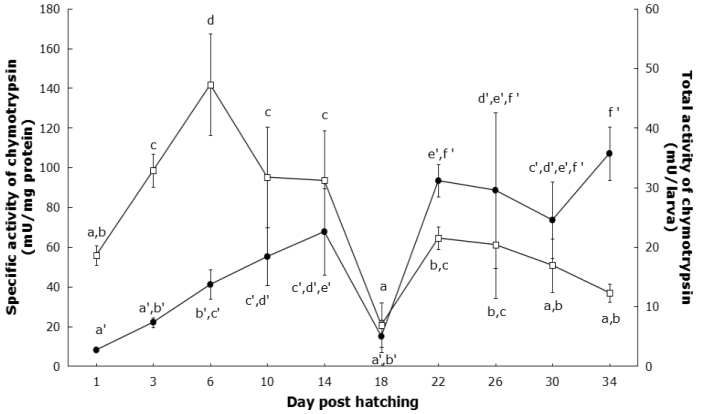

Fig. 5 Total chymotrypsin activity in mU/larva (black circle) and specific chymotrypsin activity in mU/mg protein (clear square) from 1 to 34 days post-hatching. Means±SD (n = 3) with the same superscript letter are not significantly different in multiple comparisons (P > 0.05).

Specific chymotrypsin activity increased significantly from 55.861±4.884 mU/mg protein on 1 DPH to 98.561±8.245 mU/mg protein on 3 DPH (after the first feeding), and peaked at 141.761±25.559 mU/mg protein on 6 DPH (P<0.05, Fig. 5). Specific activity then gradually decreased to 20.668±11.165 mU/mg protein on 18 DPH when food was switched from rotifers to *Artemia nauplii.* It was followed by a sharp increase to 64.459±5.530 mU/mg protein on 22 DPH and a progressive decrease to 36.959±4.619 mU/mg protein on 34 DPH, similar to the level on 1 DPH (P>0.05).

Trypsin. Total trypsin activity was 0.225±0.114 U/larva on 1 DPH, and remained at a low level until 26 DPH (Fig. 6). Total activity increased sharply from 0.583±0.410 U/larva on 26 DPH to 7.936±4.895 U/larva on 30 DPH (P<0.05), before decreasing to 2.574±0.154 U/larva on 34 DPH.

Trypsin specific activity was 4.655±2.366 U/mg protein on 1 DPH (Fig. 6). Activity then gradually decreased and maintained a low level until 26 DPH. It reached its highest level of 16.434±10.136 U/mg protein on 30 DPH (P<0.05), and then decreased to 2.665±0.159 U/mg protein on 34 DPH.

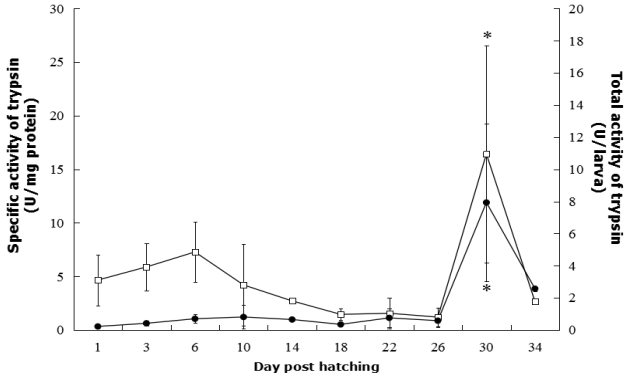

Fig. 6. Total trypsin activity in U/larva (black circle) and specific activity of trypsin in U/mg protein (clear square) from 1 to 34 days post-hatching. Means±SD (n = 3) with * denoting significant difference in multiple comparisons (P < 0.05).

Discussion

In commercial production of marine fish, larval culture is a crucial stage. Studies have showed that quality and quantity of live food (mainly rotifers and *Artemia nauplii*), feeding frequency, weaning period, and morphophysiological changes related to digestion (enzymatic activity), and absorption of nutrients by enterocytes, are the main factors affecting fish growth (Moyano et al., 1996). The standard length of orange-spotted groupers, which was slow during the initial period of life, increased rapidly after 10 DPH. This change may be caused by the type of food provided (rotifers and *Artemia nauplii* fed on 10 DPH) and the development of the digestive system. Previous studies have shown that the digestive tract of orange-spotted grouper larvae on 0 DPH is a simple primordial stomach,

which broadens into a voluminous pouch on 10 DPH (Quinitio et al., 2004a), and that grouper larvae are small and fragile with limited reserves of endogenous nutrition and low initial feeding rates (Ordonio-Aguilar et al.,1995). The same growth trends have been reported in several species, such as the common snook *Centropomus undecimalis,* (Jimenez-Martinez et al., 2012), Atlantic cod *Gadus morhua L.*, Atlantic halibut *Hippoglossus hippoglossus L.,* (Kvåle et al., 2007), turbot *Scophthalmus maximus* L., (Tong et al., 2012), and golden pompano *Trachinotus ovatus,* (Ma et al., 2014).

Previous studies have also indicated that the digestive physiology of marine fish larvae has a profound effect on many morphological and functional changes during ontogeny, and can substantially influence larval development (Tong et al., 2012). The presence and level of activity of digestive enzymes, as valuable tools for understanding the nutritional capabilities of fish larvae, have been widely reported in many marine and freshwater fish species (Rønnestad & Morais, 2007; Zambonino-Infante et al., 2008). In the present study, the specific activity of chymotrypsin, alkaline phosphatase, amylase, trypsin, and lipase, which are involved in the digestion of proteins, lipids, and carbohydrates, were present in the larvae before the onset of exogenous feeding and sharply increased from 6 DPH onward. Similarly, in golden pompano larvae, specific trypsin, amylase, and lipase activities were detected before the onset of exogenous feeding and sharply increased between 3 and 7 DPH after exogenous feeding (Ma et al., 2014) when yolk reserves had been completely absorbed (Micale et al.,2008). Our study also indicated that enzyme synthesis of chymotrypsin, alkaline phosphatase, amylase, trypsin, and lipase in early stages of orange-spotted grouper development was not triggered by dietary stimulation, but rather by an internal mechanism.

The developing patterns of chymotrypsin, trypsin, and lipase are closely related to the structural development of the digestive system (Quinitio et al., 2004a). Earlier research showed that proteins and free amino acids are the principal sources of energy, whereas during the endogenous feeding phase, lipids rather than proteins or carbohydrates are preferentially used during embryogenesis (Mourente et al., 1999). In this study, the specific chymotrypsin and trypsin activities were higher than that of lipase at hatching, indicating the importance of those enzymes during organogenesis and early larval development as both types of proteases might be involved in the cleavage of proteins contained in the yolk and in live feed (Gisbert et al., 2009). High chymotrypsin activity coincided with the transition from endogenous to exogenous feeding on 6 DPH and then dramatically decreased. This decrease during the endogenous feeding phase has also been observed in other marine fish species (Chen et al., 2006; Ma et al., 2005). The developmental pattern of trypsin activity in orange-spotted grouper larvae differed from that of the other digestive enzymes. Total and trypsin specific activity remained at low levels from 1 to 26 DPH, then increased rapidly and peaked on 30 DPH, followed by a decreasing trend. This pattern was different from that reported in other marine fish species. In yellow kingfish (*Seriola lalandi*), for example, trypsin specific activity gradually increased to 15 DPH, followed by a sharp decrease (Chen et al., 2006). In golden pompano, trypsin specific activity gradually increased until 7 DPH, followed by a sharp decrease, then an increase on 15 DPH, before finally exhibiting a decreasing trend (Ma et al., 2014). Low trypsin activity during the initial developmental period suggests that trypsin is not the main protein digestive enzyme at the beginning of larval development, and other protein enzymes, for example, pepsin not measured here might play an important role in protein digestion.

Previous research has indicated that high dietary glycogen levels in live prey might stimulate synthesis and secretion of amylase (Ma et al., 2005). In the present study, amylase was detected in orange-spotted grouper larvae on

1 DPH. Similar results have been reported in other fish species, such as yellow kingfish (Chen et al., 2006), red drum (Lazo et al., 2007), and golden pompano (Ma et al., 2014). However, other results (Zambonino-Infante & Cahu, 2001) differ somewhat from the results reported in the present study wherein amylase activity was not detected until mouth opening phase and the onset of exogenous feeding. After hatching, amylase specific activity progressively increased, reaching a maximum on 6 DPH and decreasing thereafter. This developmental pattern for amylase secretion has also been described in golden pompano (Ma et al., 2014) and common snook (Jimenez-Martinez et al., 2012). High amylase activity in the Persian sturgeon larval might indicate that dietary carbohydrates fill the energy gap between endogenous and exogenous protein requirements in larvae (Tanaka, 1973). Examining characteristics of amylase in orange-spotted grouper larvae can explain their ability to digest carbohydrates during early developmental stages, thereby assisting in the development of carbohydrate-rich (rather than protein rich) compound diets for fish larvae.

Alkaline phosphatase brush border activity is an indicator of intestinal maturation (Zambonino Infante & Cahu 2001). In the present study, alkaline phosphatase in *E. coioides* larvae was present on 1 DPH, after which it increased in activity from 6 DPH and remained at a high level until 34 DPH. The first peaks of activity occurred on 6 DPH and steadily reached a high level until 30 DPH suggesting that alkaline phosphatase was produced by a functional digestive tract at the beginning of hatching, and was at its optimal on 30 DPH, when the animal was morphologically formed. This developmental pattern has been seen in previous studies, and indicates enterocyte maturity and a well-developed digestive system, which are related to a simultaneous decrease in intestinal cytosolic enzymes (Uscanga-Martínez et al., 2011).

In conclusion the digestive enzymes, alkaline phosphatase, amylase, lipase, chymotrypsin, and trypsin in our study were present before the onset of exogenous feeding in orange-spotted grouper larvae. The relatively high specific activity of chymotrypsin and trypsin facilitate digestion of protein at an early stage. The pattern of primary digestive enzyme activity indicates early functional development of the digestive system. Knowledge of the specific activity of amylase in orange-spotted grouper larvae helps our understanding of the way in which their carbohydrates are digested during early developmental stages. This may assist in the development of carbohydrate-rich (rather than protein rich) compound diets for fish larvae. This study on digestive enzyme patterns will provide valuable information on the nutritional requirements of larvae and help establish feeding protocols for optimizing larval mass-rearing production.

Acknowledgements

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Ethics/Institutional Review Board Approval of Research

Compliance with ethical standards. This study was carried out in strict accordance with the animal welfare recommendations of the Chinese Academy of Fishery Sciences. The protocols, species, and numbers of animals used in this study were approved by the South China Sea Fisheries Research Institute

Animal Welfare Committee (Approval Number: 2014YJ01). Euthanasia procedures were performed using an overdose (40 mg/L) of AQYI-S® (New Zealand Ltd), and all efforts were made to alleviate fish suffering.

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