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Changes in digestive enzyme activities during larval development of Chinese loach *Paramisgurnus dabryanus* (Dabry de Thiersant, 1872)

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Abstract The digestive physiology of Chinese loach (*Paramisgurnus dabryanus*) was studied by assessing the specific and total activities of different pancreatic (trypsin, chymotrypsin, amylase and lipase), gastric (pepsin) and intestinal (alkaline phosphatase and leucine-aminopeptidase) enzymes from hatching to 40 days after hatching (DAH). Larvae were reared at 24.4 ± 0.4 °C and fed with rotifers from mouth opening (4 DAH) to 15 DAH, from 10 to 35 DAH with Cladocera and from 30 to 40 DAH with compound diet. Enzyme activities for trypsin, chymotrypsin, amylase and lipase were detected before the onset of exogenous feeding, indicating that these enzymes were genetically pre-programmed. Most of the pancreatic enzyme specific activities increased until 20 DAH and decreased thereafter. The pepsin activity of Chinese loach was firstly detected at 30 DAH, indicating the appearance of functional gastric gland. Alkaline phosphatase specific activity was detected from hatching onward, showed marked increase and reached the second peak at 20 DAH, while a gradual increase in specific leucine-aminopeptidase activity was observed until the end of the experiment. Accordingly, the larvae of Chinese loach possess a functional digestive system before the onset of exogenous feeding and the digestive capacity gradually increases as development progresses. The abrupt increase in intestinal enzyme activities between 10 and 20 DAH demonstrates onset of juvenile-like digestive mode in Chinese loach larvae. The increase in pepsin activity after 30 DAH indicates the shift from alkaline to acidic digestion in Chinese loach larvae, which may be considered as the onset of weaning.

Keywords *Paramisgurnus dabryanus* · Digestive enzyme · Ontogeny · Larval development

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Introduction

There is a great variety of species with potential for aquaculture in China, such as grass carp (*Ctenopharyngodon idellus*) and yellow catfish (*Pelteobagrus fulvidraco*). Recently, another species with potential for local aquaculture as well as Japan and Korea is the Chinese loach (*Paramisgurnus dabryanus*) (Hao et al. 2014), a freshwater omnivorous fish, which is distributed in East Asia. The culture of Chinese loach has become more and more popular as the market demand is gradually increasing in China, especially in recent years. However, most of the cultured Chinese loach larvae were obtained from wild, because of the intensive mortality in the artificial larvae production. It is well known that the wild larvae

are hardly to accept artificial compound diet and that the survival is not steady. Thus, it has become one of the main bottlenecks of the cultivation of Chinese loach. However, limited information is available in its early life history, especially in its digestive physiology, which is a limiting factor for the development of this species in aquaculture. In this case, there is great need to research in the area of digestive physiology, including studies about enzyme ontogeny, which allows keeping track of changes in the activities of digestive enzymes during larval development, determining the degree of maturation and functionality of the digestive system (Toledo-Solís et al. 2015).

Over the past decades, many new larval culture protocols have been developed with the aid of studies aiming to increase our knowledge with respect to larval digestive physiology (i.e., digestive enzyme activities and larval nutrition) of a species of interest, for example, for the Mayan cichlid (*Cichlasoma urophthalmus*) (López-Ramírez et al. 2011), the butter catfish (*Ompok bimaculatus*) (Pradhan et al. 2013), the common carp (*Cyprinus carpio*) (Farhoudi et al. 2013), the meager (*Argyrosomus regius*) (Suzer et al. 2013), the leopard grouper (*Mycteroperca rosacea*) (Martínez-Lagos et al. 2014) and the three-spot cichlid (*Cichlasoma trimaculatum*) (Toledo-Solís et al. 2015). These studies reflect the development of the digestive tract and digestive capability of the organism and can thus be used as an indicator of nutritional status at early life stage (Yúfera and Darías 2007) and can provide information for improving the feeding protocols of larviculture and offering a more suitable food item. Therefore, the aim of this was to assess the digestive capacity of Chinese loach based on the characterization of the digestive enzymes from hatching to 40 DAH, to provide information on feeding protocols.

Materials and methods

Larval rearing

Chinese loach larvae were obtained from Hubei Wuyuan Agricultural developmental Co., Ltd. (Jingzhou, P. R. China). Larvae were reared in three separated replicate tanks (9 m × 2 m × 1 m) with 0.5 m depth of water, and rearing density was 1000 larvae per m^2. The water was aerated by five air stones per tank. Water temperature, dissolved oxygen and pH of three tanks were monitored daily. Water temperature was 24.4 ± 0.4 °C and kept constant using steam-heated iron pipelines installed at the bottom of the tank and by mixing cold ground water (around 4 °C). During the experimental period, oxygen and pH were recorded as 7.1 ± 0.5 and 7.9 ± 0.4 mg L^{-1}, respectively. There is no significance difference among the water quality parameters of three replicated tanks.

Larvae were fed rotifers composed of *Brachiouns spp.*, *Asplanchnidae spp.* and *Filinia spp.* at a density of 10 individuals ml^{-1} from initial feeding (4 DAH) to 15 DAH and then Cladocera dominated by *Diaphanosoma spp.* and *Moina spp.* at a density of 5 individuals ml^{-1} from 10 to 35 DAH, and a commercial compound diet (crude protein 35 %, crude lipid 7 %, digestible energy 12 MJ kg^{-1}) at a feeding amount of 5 % per day from 30 DAH until the end of the experiment. The prey density was monitored three times each day using zooplankton quantitative method to assure the larval satiation.

Sample collection

Thirty individuals (ten individuals per tank) were collected everyday before the food distribution from hatching to 40 DAH to measure the body weight and total length. Digestive enzyme assays were performed using at least 1000 mg wet weight of larval tissue, representing 10–2000 larvae per sample, depending on their weight and age. The enzymatic analysis samples were randomly collected daily from 1 to 10 DAH, and subsequent samples were collected at 15, 20, 25, 30, 35 and 40 DAH. The samples were frozen at −80 °C following anaesthetization with MS-222 until enzyme assays.

Enzymatic assays

The samples were homogenized using a glass homogenizer placed in 4 °C ice-cold water with 4:1 v/w cold Tris–HCl buffer (0.05 M Tris, 0.1 M NaCl, 0.01 M EDTA, pH 8.0), centrifuged at 12,000*g* for 20 min at 4 °C to obtain the supernatant for subsequent enzymatic analyses. The soluble protein content was determined using the method reported by Bradford (1976) using bovine serum albumin as standard (0.563 g L^{-1}).

The trypsin (EC 3.4.21.4) activity was determined at 25 °C, 410 nm according to the method described by Erlanger et al. (1961) using *N*α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as the substrate. One unit of trypsin activity was defined as 1 μM BAPNA hydrolyzed per minute per ml of enzyme extract. Pepsin (EC 3.4.23.1) activity was determined at 37 °C, 680 nm according to Shan et al. (2008) using casein as the substrate. One unit of pepsin activity was defined as 1 μg tyrosine released per minute. Chymotrypsin (EC 3.4.21.1) activity was determined at 256 nm according to the method described by Ásgeirsson and Bjarnasson (1991) using benzoyl-tyrosine ethyl ester as the substrate. One unit of chymotrypsin was defined as the amount of enzyme required to liberate 1 μM of tyrosine per minute at 25 °C. Amylase (EC 3.2.1.1) activity was assayed at 540 nm using soluble starch as substrate according to the method reported by Métais and Bieth (1968). One unit of amylase activity was defined as the μM of maltose released in one minute. Lipase (EC 3.1.1.3) activity was measured using the method of McKellar and Cholette (1986) modified by Versaw et al. (1989) using β-naphtyl caprylate as substrate. One unit of lipase activity was defined as 1 μg naphthol released per minute at 540 nm. Determination of alkaline phosphatase (EC 3.1.3.1) activity was according to the method described by Bessey et al. (1946) using 4-nitrophenylphosphate as substrate. One unit of alkaline phosphatase activity was defined as the amount of enzyme, which hydrolyzes 1 nM of substrate in 1 min. The activity of leucine-aminopeptidase (EC 3.4.11.2) was determined according to Maroux et al. (1973) using L-leucine *p*-nitroanilide as substrate. One unit of leucine-aminopeptidase activity was defined as the amount of enzyme required to liberate 1 μM of p-nitroanilide per minute.

Statistic analysis

Values of the measured variables are expressed as mean ± standard deviation. The variance homogeneity of the data was performed using Levene's test. Data were compared by one-way ANOVA followed by Duncan's test when significant differences were found at 0.05 level. Statistics were performed using SPSS 18.0 software (SPSS Inc. Chicago, IL, USA).

Results

Larval growth

Growth of Chinese loach larvae in terms of total length (mm) and body weight (mg) and the feeding protocol during larval development are shown in Fig. 1.

Ontogeny of pancreatic enzymes

Activities of trypsin and chymotrypsin are shown in Fig. 2. Specific trypsin activity was detected as early as hatching and increased to the peak at 15 DAH. Then, a sharp decrease was observed in this activity until to the end of the experiment. Total trypsin activity was low at the first 20 days of development, rapidly increased until 35 DAH followed by significantly declining until the end of this experiment (Fig. 2a, b). Chymotrypsin specific activity was detected before the onset of exogenous feeding, reaching maximum specific activity levels by 20 DAH. Thereafter, a sharp decrease was observed until 40 DAH, while total chymotrypsin activity was low at first 10 days of development and increased at a significant rate until the end of the experiment (Fig. 2c, d).

A high level of specific amylase activity was detected at hatching and sharply declined from 2 to 5 DAH. Subsequently, the activity significantly increased to the maximum value at 20 DAH. Then, amylase specific activity decreased again until the end of this experiment. The total amylase activity was low

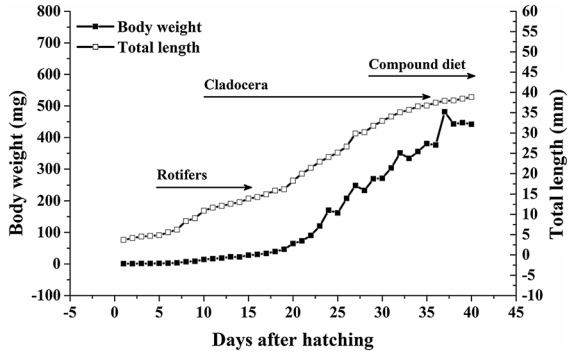

Fig. 1 Total length and body weight during larval development of Chinese loach up to 40 DAH. Data are expressed as mean ± SD ($n = 30$). Feeding protocol is summarized by *arrow*

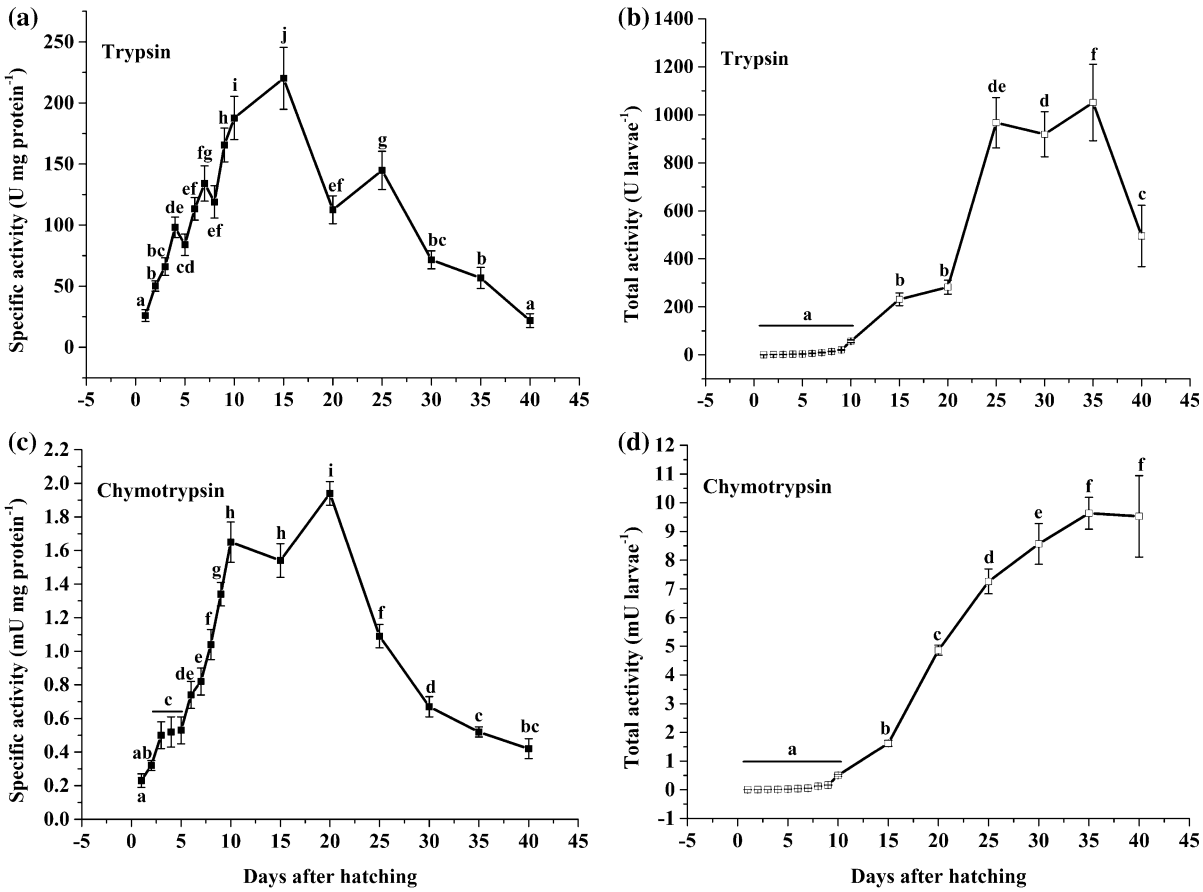

Fig. 2 Activity of digestive enzymes of Chinese loach larvae. Specific and total activities were determined for trypsin (**a**, **b**) and chymotrypsin (**c**, **d**). Values are mean ± SD ($n = 3$). The different superscripts are statistical difference ($P < 0.05$)

at the first 2 weeks of development and reached peak at 35 DAH followed by a sharp decrease until 40 DAH (Fig. 3a, b). Lipase specific activity was detected at 1 DAH and showed an obvious increasing pattern but with a slight variation at the first 15 days of development and then increased till 20 DAH. Thereafter, the activity significantly decreased till 30 DAH followed by increase to the maximum at 40 DAH, while the total lipase activity was low at first 15 days of development and then constantly increased until the end of this experiment (Fig. 3c, d).

Gastric enzyme

Pepsin activity is shown in Fig. 4. Both pepsin specific and total activities were detected at 35 DAH and increased until the end of the experiment.

Intestinal enzymes

Activity of intestinal enzymes (alkaline phosphatase and leucine-aminopeptidase) is shown in Fig. 5. Alkaline phosphatase specific activity was detected from hatching onward, reached the first peak at 7 DAH and dropped until 11 DAH. Then, this activity showed marked increase and reached the maximum peak at 20 DAH. Subsequently, the values of this activity sharply decreased until 30 DAH and remained a constant level until the end of this experiment, while the total alkaline phosphatase activity was low before 15 DAH; then, gradual increase was observed until 40 DAH (Fig. 5a, b). A gradual increase in specific leucine-aminopeptidase activity was observed until the end of the experiment. Total activity of leucine-aminopeptidase remained low until 25 DAH and then

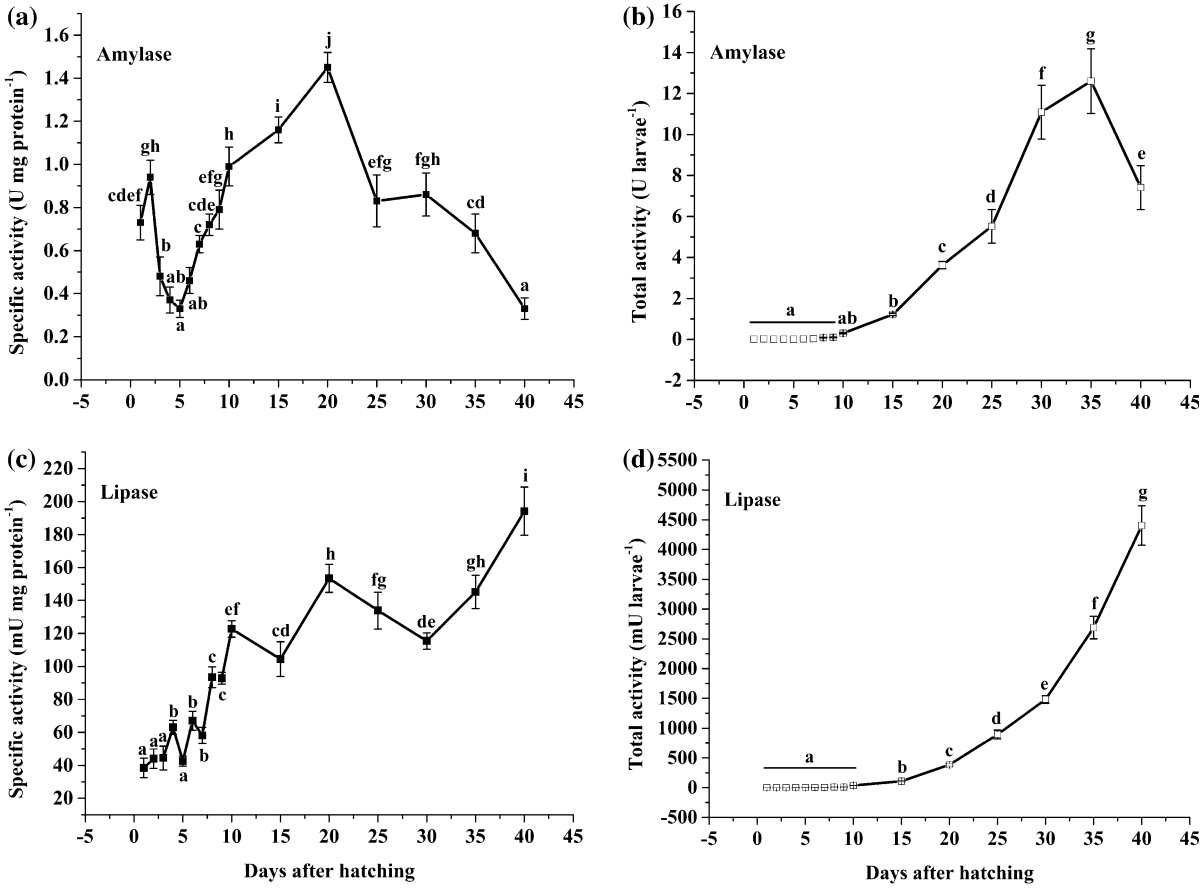

Fig. 3 Activity of digestive enzymes of Chinese loach larvae. Specific and total activities were determined for amylase (**a**, **b**) and lipase (**c**, **d**). Values are mean ± SD ($n = 3$). The different superscripts are statistical difference ($P < 0.05$)

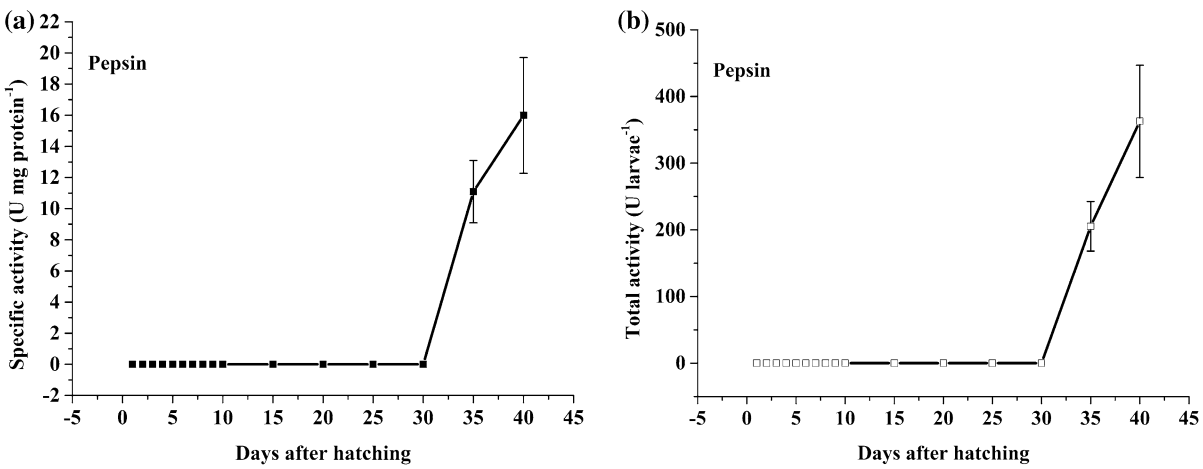

Fig. 4 Activity of digestive enzymes of Chinese loach larvae. Specific (**a**) and total (**b**) activities were determined for pepsin. Values are mean ± SD ($n = 3$)

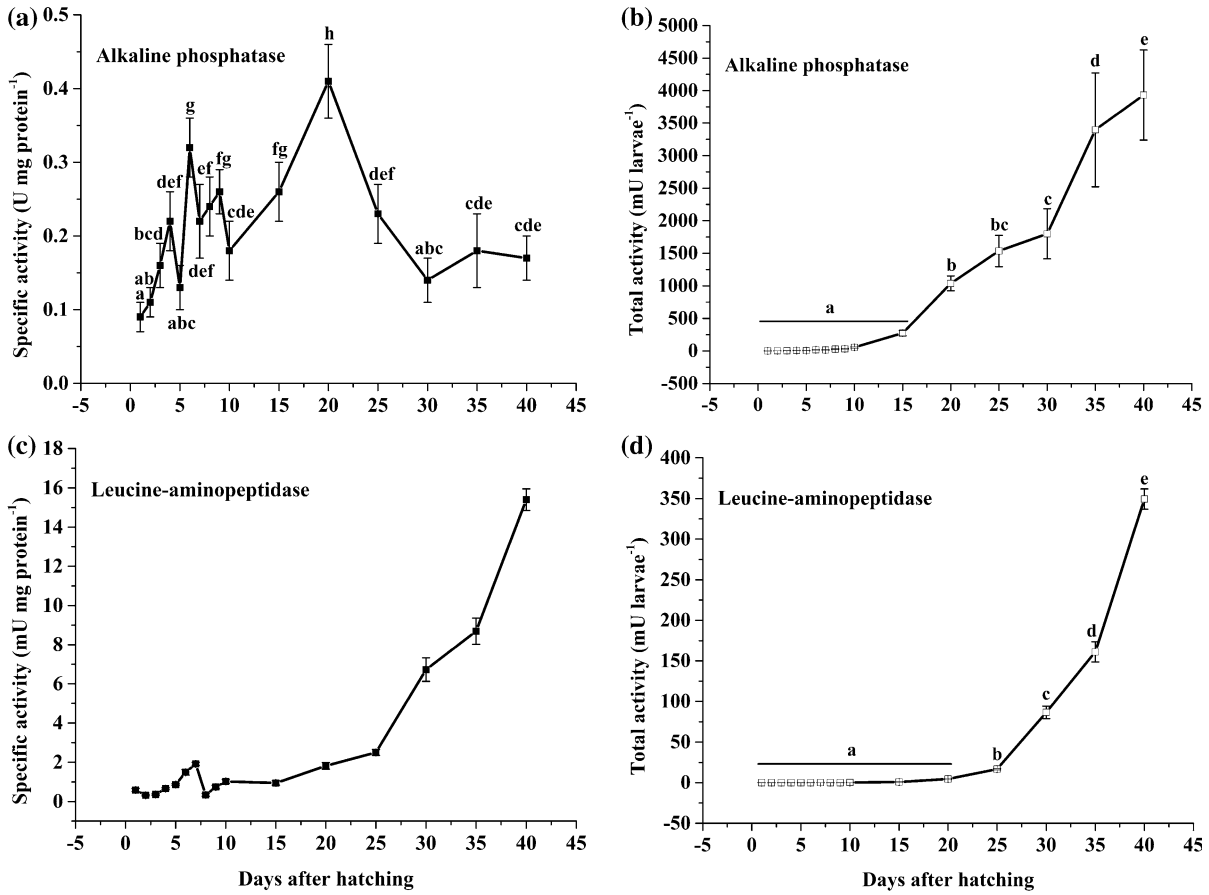

Fig. 5 Activity of digestive enzymes of Chinese loach larvae. Specific and total activities were determined for alkaline phosphatase (**a**, **b**) and leucine-aminopeptidase (**c**, **d**). Values are mean ± SD ($n = 3$). The different superscripts are statistical difference ($P < 0.05$)

significantly increased to the maximum value by the end of the experiment (Fig. 5c, d).

Discussion

In the present study, the activities of trypsin and chymotrypsin were detected as early as hatching (1 DAH) and increased to maximum values followed by sharp decrease, which has also been reported for fish species such as sharpsnout seabream (*Diplodus puntazzo*) (Suzer et al. 2007), common dentex (*Dentex dentex*) (Gisbert et al. 2009), Japanese eel (*Anguilla japonica*) (Murashita et al. 2013) and three-spot cichlid (Toledo-Solís et al. 2015). These results indicated that these enzymes were pre-programmed by gene rather than food induced (Ribeiro et al. 1999; Alvarez-González et al. 2006; Babaei et al. 2011). The sharp increase in pancreatic enzymes total activity of observed 10 DAH coincides with the initial supply of Cladocera. Several studies have found changes in enzyme activities in response to the quantity and quality of food, because this has been shown to stimulate digestive enzyme secretion in organisms (Cara et al. 2007; Martínez-Lagos et al. 2014). In addition, the increase in trypsin activity may be related to the formation of exocrine pancreas (Gisbert et al. 2004; Tong et al. 2012) from a morphohistological point of view. Our results suggested that the synthesis of trypsin and chymotrypsin is considered to be genetically pre-programmed and coincides with the changes in food supplementation.

Both amylase and lipase specific activities were detected as early as 1 DAH, suggesting that these enzymes were also genetically pre-programmed as reported in some other fish species (Cuvier-Péres and

Kestemont 2002; Lazo et al. 2007; Martínez-Lagos et al. 2014; Ma et al. 2014). The amylase specific activity around 5 DAH was low coincided with the initial feeding, suggesting the poor ability of Chinese loach to utilize carbohydrates at mouth opening (4 DAH). The amylase mRNA levels decreased independently of the dietary glucide concentration during the early stage of sea bass larvae (Zambonino-Infante et al. 2008). Therefore, the decrease in amylase activity of Chinese loach after hatching seems to be under genetic control, because of the low carbohydrate content of live prey (Cara et al. 2003). However, the marked increase in amylase specific activity from 7 to 20 DAH (live prey as main food) is probably attributed to the enhanced digestive ability result from the rapid digestive system development rather than the influence from live prey. The decrease in amylase activity after 20 DAH suggested that the secretion mechanisms of pancreas were operational before 20 DAH in Chinese loach. It was also observed around 25 DAH in sea bass (*Dicentrarchus labrax*) larvae (Zambonino-Infante and Cahu 1994), while it occurred around 21 DAH in Senegal sole (*Solea senegalensis*) larvae (Ribeiro et al. 1999).

The slight increase in lipase specific activity after hatching is typically associated with yolk lipid catabolism to provide energy for larval development before initial feeding. A decrease activity was observed after initial feeding as described in common dentex (Gisbert et al. 2009) and Japanese flounder (*Paralichthys olivaceus*) (Bolasina et al. 2006). It may be associated with the transition from endogenous to exogenous feeding (Oozeki and Bailey 1995). After 30 DAH, the significant increase in lipase specific activity coincides with weaning to a compound diet (30 DAH), and similar pattern is also observed in spotted rose snapper (*Lutjanus guttatus*) (Moguel-Hernández et al. 2014) and golden pompano (*Trachinotus ovatus*) (Ma et al. 2014), suggesting that the lipase activity may be strongly influenced by the type of diet and dietary lipid level.

The pepsin activity of Chinese loach was firstly detected at 35 DAH, indicating the appearance of functional gastric gland, which is species-specific in teleost (see Table 1). Compared with other species, the appearance of functional gastric in Chinese loach is considerable later (Table 1). The increase in pepsin activity after 30 DAH indicates the maturation of functional stomach. And this may be a proper opportunity of weaning to compound diet in Chinese loach because the larvae are well adapted to protein digestion of compound diet after 30 DAH. Moreover, the decrease in trypsin activity in this stage was correlated with the increase in pepsin activity, indicating a change in the digestive physiology and complete achievement of an adult-type protein digestion (Gisbert et al. 2009).

Alkaline phosphatase and leucine-aminopeptidase are considered to be a general maker of nutrient absorption and enterocyte differentiation (Zambonino-Infante and Cahu 2001) and are active at the intestinal brush border (Ribeiro et al. 1999; Tengjaroenkul et al. 2002; Suzer et al. 2007). In the present study, the alkaline phosphatase specific activity of Chinese loach showed abrupt increase between 10 and 20 DAH, indicating the rapid development and/or formation of brush border enterocyte (Moyano et al. 1996; Ribeiro et al. 1999; Kvåle et al. 2007) and an important development of the intestinal mucosa in terms of villi size and number (Pradhan et al. 2013). Our results are in agreement with many other studies on the digestive physiology of fish larvae that reported an increase in intestinal brush border enzymes around the first weeks of their life stage (Babaei et al. 2011; López-Ramírez et al. 2011; Uscanga-Martínez et al. 2011), indicating the onset of juvenile-like digestive mode (Ribeiro et al. 1999; Martínez-Lagos et al. 2014). The decrease in alkaline phosphatase specific activity was observed after 20 DAH as observed in yellowtail kingfish (*Seriola lalandi*) (Chen et al. 2006), Mayan cichlid (López-Ramírez et al. 2011) and butter catfish (Pradhan et al. 2013). This decline tendency is probably resulted from the marked increase in the soluble protein as reported by Kvåle et al. (2007) rather than a lowering in the enzyme activities.

In conclusion, the Chinese loach larvae have a diverse and characteristic digestive enzyme activities. The main digestive enzymes with the exception of pepsin were detected before the onset of the exogenous feeding stage, indicating an early functional development of the digestive system in Chinese loach. The larvae of Chinese loach possess a functional digestive system before the onset of exogenous feeding, and the digestive capacity gradually increases as development progresses. The abrupt increase in intestinal enzyme activities between 10 and 20 DAH demonstrates onset of juvenile-like digestive mode in

Table 1 Age of the appearance of pepsin in different fish species

Species	Age (DAH)	Rearing temperature (°C)	Reference
Mystus nemurus	1	Not mentioned	Srichanun et al. (2012)
Scophthalmus maximus	9	18–19	Tong et al. (2012)
Argyrosomus regius	15	20.0–22.0	Suzer et al. (2013)
Ompok bimaculatus	15	27	Pradhan et al. (2013)
Trachinotus ovatus	15	27-29	Ma et al. (2014)
Dentex dentex	19	19.2 ± 0.5	Gisbert et al. (2009)
Lutjanus guttatus	20	26.7 ± 0.21	Moguel-Hernández et al. (2014)
Oplegnathus fasciatus	22	24.0 ± 1.0	He et al. (2012)
Rachycentron canadum	22	Not mentioned	Salze et al. (2012)
Dicentrachus labrax	24	18–19	Zambonino-Infante and Cahu (1994)
Diplodus puntazzo	32	19.0–23.0	Suzer et al. (2007)
P. dabryanus	35	24.4 ± 0.4	Present study
Paralichthys lethostigma	37	18.5 ± 0.5	Faulk and Holt (2009)
Sparus aurata	40	19	Moyano et al. (1996)

Chinese loach larvae. The increase in pepsin activity after 30 DAH indicates the shift from alkaline to acidic digestion in Chinese loach larvae, which may be considered as the onset of weaning. Our results would provide valuable information to formulate diets and weaning protocols in Chinese loach larviculture.

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