Changes of digestive enzymes activity in common carp (*Cyprinus carpio*) during larval ontogeny

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Abstract

This study was aimed to gain knowledge on the ontogeny of digestive enzymes in common carp larvae at the governmental Warm water Fish Aquaculture Center of Shahid Rajaee in Sari, Mazandaran, Iran. The ontogenetic development of pancreatic (trypsin, chymotrypsin, lipase and α-amylase) and intestinal (alkaline phosphatase and aminopeptidase-N) enzymes were assessed in common carp larvae from first feeding (3 days post hatching) to 33 days post hatching (dph). The larvae started to feed on rotifers at day 3 to day 7. Feeding on a commercial diet was started from day-8 onwards. Specific trypsin and chymotrypsin activities feed with rotifer were 0.011 U/mg protein and 0.003 U/mg protein at day 7, respectively; then the specific activities feed with dry food reached a maximum level at 33 dph (0.028 U/mg protein and 0.028 U/mg protein, respectively) (P<0.05) compared to live prey. The elevated alkaline proteases activity can be related to adaptation of larvae to digest protein content in the food. Specific lipase activity was 0.0006 U/mg protein at 7 dph; then the activity feed with dry food reached a maximum level at 15 dph (0.0011 U/mg protein) (P<0.05) compared to live prey. Specific α-amylase activity feed with rotifer was 85.86 U/mg protein on the 7 dph; consequently, its specific activity reached a maximum level at 15 dph (128.77 U/mg protein) (P<0.05). Specific alkaline phosphatase and aminopeptidase N activities feed with rotifer were 6 U/mg protein and 0.0106 U/mg protein at day 7, respectively; then the specific activities feed with dry food reached a maximum level at 33 dph (28.66 U/mg protein and 0.58 U/mg protein, respectively) (P<0.05) compared to live prey.

Keywords: Common carp, Pancreatic enzymes, Larval growth, Digestive enzymes, Ontogeny

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Introduction

Common carp (*Cyprinus carpio)*, habituates in southern Caspian Sea. It is one of the most economically important fish species in aquaculture and stock enhancement in the Caspian Sea. The eastern coasts of southern Caspian Sea, an estuarine environment, with salinity ranges from 12 to 15ppt, are the best habitats for common carp. During the last decade, production of common carp has been impeded due to low larval survival and nutritional disorders. For better survival of larvae, feeding must be initiated on digestible diets before or very soon after depletion of the endogenous energy sources, yolk and oil (Kim et al., 2001). The study of the digestive specificities of early stages will be of great help to adapt the nutrient nature and its form of supply in a micro diet (Ma et al., 2005). The assessment of the presence and level of activity of certain enzymes is used as a comparative indicator of the rate of development of the larvae, as well as their further survival rate (Ueberschar, 1993). However, there is no available information about the differentiation of the gastrointestinal tract of common carp during early its stages. A detailed knowledge of the changes correlate with digestive system development and food assimilation mechanism during larval development is essential for understanding of the nutritional physiology of larval fish (Gisbert et al., 1998), design of feeding techniques and formulation of dry diets (Chakrabarti and Rathore, 2009). Digestion is a key process in animal metabolism since it determines the availability of nutrients needed for all biological functions. Thus, the study of the digestive physiology is a very important issue, since the net efficiency of the whole digestive process mostly relies on the type and function of the digestive enzymes. In addition, the study of digestive enzymes is a key tool when studying the nutritional condition and adaptation of the organism to dietary change (Gisbert et al., 2009). In turn, knowing when to begin feeding is essential for survival and optimal growth. Therefore, initiate feeding must be based on understanding of the development of the digestive system (Gawlicka et al., 2000), as the larvae must be physiologically competent for digesting the food consumed (Vega-Orellana et al., 2006). Whereas, the production of live prey is costly, numerous efforts have been made to progress dry start diets such as microparticulate and microencapsulated (Murray et al., 2004).

The aim of the present study was to investigate the development of some digestive enzyme activities of common carp larvae fed on live prey and dry food during larval development, in order to obtain important information for the formulation of a compound diet adapted to larvae.

Materials and methods

Fish material and experimental design

This study was carried out at the governmental Warm water Fish Aquaculture Center of Shahid Rajaee in north of Iran. Common carp fertilized eggs were obtained from common broodstocks captured from freshwater environment. Broodstock spawned between April and May. Caught broodstock were transferred

to the center and were kept in spawning tanks (average water depth was 1m). Water temperature ranged 24.4 to 25.7°C. Males (weight 0.7 to 0.8 kg) and females (weight 1.0 to 1.2 kg) were stripped and eggs were transferred to vase incubator. Water temperature and pH in vase incubator was maintained 19.6 to 22.8°C and 7.0 to 8.5, respectively during the study period. Dead eggs were removed daily to prevent fungal contamination. Eggs hatched after 6-7 days and larvae 3 days post hatching were transferred to pools. Water temperature was 24.2°C to 25.5°C in the pools. After yolk sac absorption (day 3), the larvae started to feed on rotifers at day 3 to day 7. Rotifers were administered *ad libitum.* Co-feeding based on rotifers and commercial diet was comprised between 7 and 11 dph, whereas from then to the end of the experiment larvae were fed on a commercial diet at the rate of 4% body weight (SFC-1, 3-4 times per day, 40 % protein of dry weight, Chineh Company, Iran). Duration of the experiment was 30 days. Ingredients of diets (rotifer and dry food) are expressed as percentage in Table 1.

Table 1: Composition of the diets (Rotifer and Dry food)

Diets / Ingredients (%)	Rotifer	Dry food
Protein	36.26±0.06	40
Lipid	11.17±0.00	12
Carbohydrate	4.77±0.01	35
Ash	14.80±0.04	12
Moisture	94.35±0.04	10
Fiber	-	1.5
Vitamin Premix	-	3
Mineral premix	-	2

Ingredients of diets are represented as a percentage (%).
Dry food: SFC-1, Chineh Company, Iran

Sample preparation

Total length, from tip of the snout to posterior margin of the body of 20 larvae was measured in each period. Wet body weight of 20 larvae was measured after removing water with filter paper. The larvae were collected randomly at 9 A.M. before morning feeding at 3, 7, 11, 15, 19, 26 and 33 days post hatching (dph), respectively. Two hundred larvae were washed with distilled water and after removing water with a filter paper; samples were frozen in liquid nitrogen and stored at –80˚C for pancreatic and intestinal enzyme assays.

Treatment of the samples

The sample preparation and pancreatic enzymes (trypsin, chymotrypsin, amylase and lipase) assay methods described by Furne et al. (2005) methods. Whole larvae were homogenized at 0-4ºC in an electric

homogenizer (WIGGEN, D500, Germany). The samples were put into a clean tube and were homogenized in 9 volumes (v/w) of Tris-HCl (100 mM), EDTA (0.1 mM), Triton X-100 (0.1%), pH 7.8 for 30 s, then centrifuged at 30000 × g for 30 min at 4 °C (Hermle Z36HK, Germany) and supernatants were collected and stored in -80°C.

For determination of intestinal enzymes (alkaline phosphatase and aminopeptidase N) assay methods were in accordance to Cahu et al. (1999) methods. The samples were homogenized in 30 v/w fractions of Tris-HCl (2mM), Mannitol (50mM) buffer, pH 7. Then the homogenates were centrifuged (4°C) at 22000 rpm for 30 s. To prepare the brush border membrane (BBM) extracts as described by Crane et al. (1979). After addition of 0.1 M $CaCl_2$ to homogenate, this homogenate was centrifuged at 9000×g at 4°C (Hermle Z36HK, Germany) for 10 min. The supernatants were collected and stored in -80°C.

Enzyme assays

Trypsin (E.C.3.4.21.4) and chymotrypsin (E.C.3.4.21.1) activities were measured according to Erlanger et al. (1961), using BAPNA (N-α-benzoyl-Dl-arginine *p*-nitroanilide) and SAPNA (Succinyl-$(Ala)_2$-Pro-phe-*p*-nitroanilide) as substrate, respectively. BAPNA (1mM in 50 mM Tris-HCl, pH 7.5, 20 mM $CaCl_2$) was incubated with the enzyme extract at 37 °C. One unit of trypsin per ml (U) was defined as1 µmol BAPNA hydrolyzed per min per ml of enzyme extractat at 410 nm. SAPNA (50 mM Tris-HCl, pH 7.5, 20 mM $CaCl_2$) were incubated with the enzyme extract at 37 °C. One unit of chymotrypsin per ml (U) was defined as1 µmol SAPNA hydrolyzed per min per ml of enzyme extracted at 410 nm. The molar extinction coefficient of p-nitroanilide was 8,800 cm^2 mg^{-1}. Trypsin and chymotrypsin activity units were calculated by the following equation:

$$unit/mg\ protein = \frac{(Abs_{410}/\min)\times 1000\times ml\ of\ reaction mixture}{8800\times mg\ protein in\ reactin mixture}$$

Lipase (E.C.3.1.1) activity was assayed according to Iijima et al. (1998), using p-nitrophenyl myristate as substrate. Each assay (0.5 ml) contained 0.53 mM n-nitrophenyl myristate, 0.25 mM 2-methoxyethanol, 5 mM sodium cholate and 0.25 M Tris-HCl (pH 9.0). Incubation was carried out for 15 min at 30 °C, and the reaction was terminated by adding 0.7 ml of acetone / n-heptane (5:2, v/v). The reaction mixture was vigorously mixed and centrifuged at 6,080 g for 2 min. The absorbance at 405 nm in the resulting lower aqueous layer was measured. The extinction coefficient of n-nitrophenol was 16,500 M^{-1} cm^{-1} per liter. Lipase activity (U) was defined as the µmol of p-nitrophenol released per min per ml of enzyme extract at 405 nm.

Amylase (E.C.3.2.1.1) activity was assayed using 1% soluble starch, as substrate, with 3,5-dinitrosalicyclic acid (DNS) at 540 nm (Bernfeld, 1955). A unit (U) of activity was defined as the µmol maltose liberated per min per ml of enzyme extract at 540 nm.

Alkaline phosphatase (AP) (E.C.3.1.3.1) was quantified at 37 °C using 4-nitrophenyl phosphate (PNPP) as substrate

in 30 mM Na_2CO_3 buffer (pH 9.8). One unit (U) was defined as 1 μg BTEE released per min per ml of brush border homogenate at 407 nm (Bessey et al., 1946).

Aminopeptidase-N(AN) (E.C.3.4.11.2) activity was assayed spectrophotometrically with 0.4 mM Leu-NH-Np in 50 mM Tris/HCl at pH 8.0 and 25 °C, by following the continuous increase in absorbance at 405 nm due to the release of 4-nitroaniline; the extinction coefficient was 8800 M^{-1} cm^{-1} (Prescott and Wilkes, 1976; Spungin and Blumberg, 1989). One unit of enzyme activity (U) was defined as 1 μg *p*-nitroanilide released per min per ml of enzyme extract at 405 nm.

Total soluble protein content of whole larvae was determined by the method of Bradford (1976) using bovine serum albumin as standard. Enzyme activities were expressed as specific activity (U mg $protein^{-1}$). Three replicates were used for enzyme assays.

Statistical analysis

Results are given as mean ± S.D. (n=3 for enzymes; n=3 for body weight of 20 larvae; n=3 for total length of 20 larvae). Data of body weight and length were analyzed by using appropriate regression model analysis. Digestive enzyme activities were determined by a One-way analysis of variance (ANOVA) followed by Duncan test. Statistical analysis was performed using the SPSS for Windows software, version 15 (SPSS Inc., Chicago, IL, USA). Mean values were considered significantly different at $P<0.05$.

Results

The growth of common carp larvae is given as total length and wet body weight per 20 larvae in various larval stages (Figure 1). Total length increased until day 33 ($P<0.05$). Weight slowly increased until day 7 and then increased until day 33 ($P<0.05$). The results showed that the suddenly growth in weight and length between day 7 and day 11 may be related to larvae which fed rotifer and dry food (Mixed feeding phase) ($P<0.05$). Larval growth in weight and length followed a linear curve ($y = 32.063x - 46.55$, $R^2= 0.93$ and $y = 4.0987x - 0.3954$, $R^2= 0.93$, respectively). The weight of larvae in initial and at the end of experiment was 4.10 ± 0.01 mg and 199.5 ± 0.02 mg, respectively. The length of larvae in initial and at the end of experiment was 3.82 ± 0.03 mm and 29.09 ± 0.05 mm, respectively.

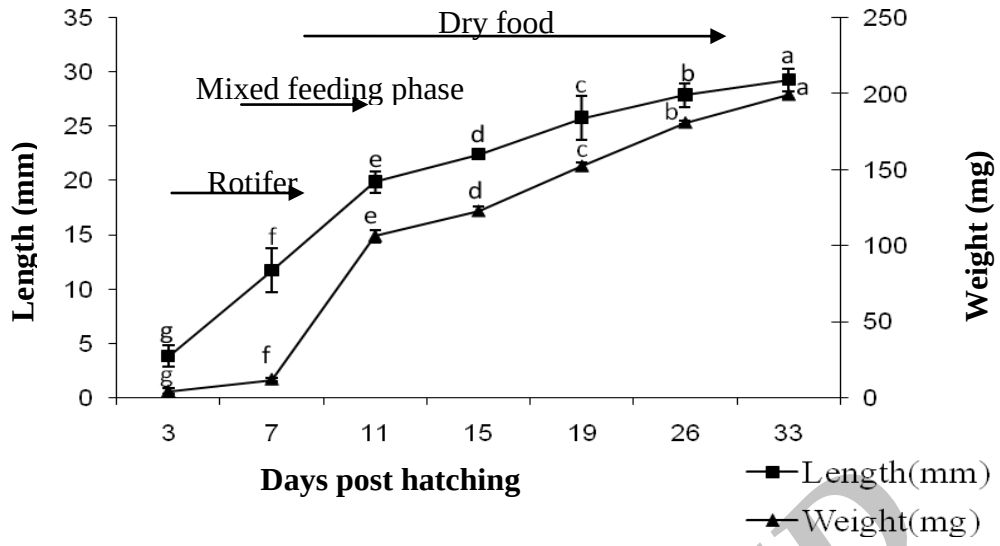

Figure 1: Growth in length (■) and weight (▲) of *Cyprinus carpio* during larval development. Each value represents mean ± SD (n=3). Data with different letters are significantly different (ANOVA, P<0.05).

Pancreatic enzymes

Specific trypsin activity was 0.01 U/mg protein at 3 dph. By the initiation of exogenous feeding at 3 dph, trypsin activity increased significantly (P<0.05) until day 15. Then, the enzyme activity decreased significantly (P<0.05) on day 19 than the enzyme activity on day 15. The enzyme activity increased significantly (P<0.05) from day 19 until day 33. Minimum trypsin activity was 0.010 U/mg protein at day 3 and maximum specific trypsin activity was 0.028 U/mg protein at day 33 (Figure 2a).

Specific chymotrypsin activity was 0.0024 U/mg proteins at 3 dph. By the initiation of exogenous feeding on rotifer from day 3 to day 7, the enzyme activity increased (P>0.05). From day 7 (0.003 U/mg protein) the chymotrypsin total activity tend to increase along larval development and peaking at 33 dph (0.028 U/mg protein) (P<0.05). Minimum and maximum chymotrypsin activity was observed at day 3 (0.001 U/mg protein) and day 33 (0.028 U/mg protein), respectively (Figure 2b).

Figure 2c shows Lipase activity in various larval stages. After the onset exogenous feeding on rotifer at day 3, the specific activity increased until day 7 (P>0.05). From day 7, with feeding of dry food, the specific lipase activity increased until day 15 (P<0.05) and 19 dph, the specific activity decreased and reached to 0.0005 U/mg protein (P<0.05). After that, total activity of lipase increased until day 26 and in day 33, again decreased and reached to 0.0006 U/mg protein (P<0.05). The specific activity of lipase showed two peaks: the first one was observed on day 15 and the second one was exhibited on day 26. The second peak on day 26 was less than the first peak on day 15. Minimum and maximum lipase activity recorded at day 1 (0.0003 U/mg protein) and day 15 (0.001 U/mg protein), respectively.

In Figure 2d amylase activity has been shown in different larval stages. After the first feeding of day 3, the activity showed an increase until day 7 (P<0.05). From day 7 to day 11, when dry food was offered, the amylase activity decreased (P<0.05). Afterwards, the specific activity of amylase increased and reached to a maximum level of 128.77 U/mg protein at day 15 (P<0.05). Decrease in activity of amylase was again observed between day 15 and day 26 (P<0.05). Minimum and maximum amylase activity recorded at day 26 (14.86 U/mg protein) and day 15 (128.77 U/mg protein), respectively.

Intestinal enzymes

The specific activity of alkaline phosphatase has been shown in Figure 2e. Alkaline phosphatase total activity was very low from day 3 (6.88 U/mg protein) until 15 dph (7 U/mg protein), but the specific activity of alkaline phosphatase abruptly increased at day 15 and reached a maximum value of 28.66 U/mg protein at day 33 dph (P<0.05).

The specific activity of aminopeptidase N has been shown in Figure 2f. Total activity of aminopeptidase N was almost similar to alkaline phosphatase activity. The specific activity of aminopeptidase N was very low at day 3 (0.057 U/mg protein) and increased slowly until day 15 but in the second half of the experimental stage of common carp, from day 15 to day 33, the enzyme activity increased sharply and reached the highest level at day 33 (0.058 U/mg protein) (P<0.05).

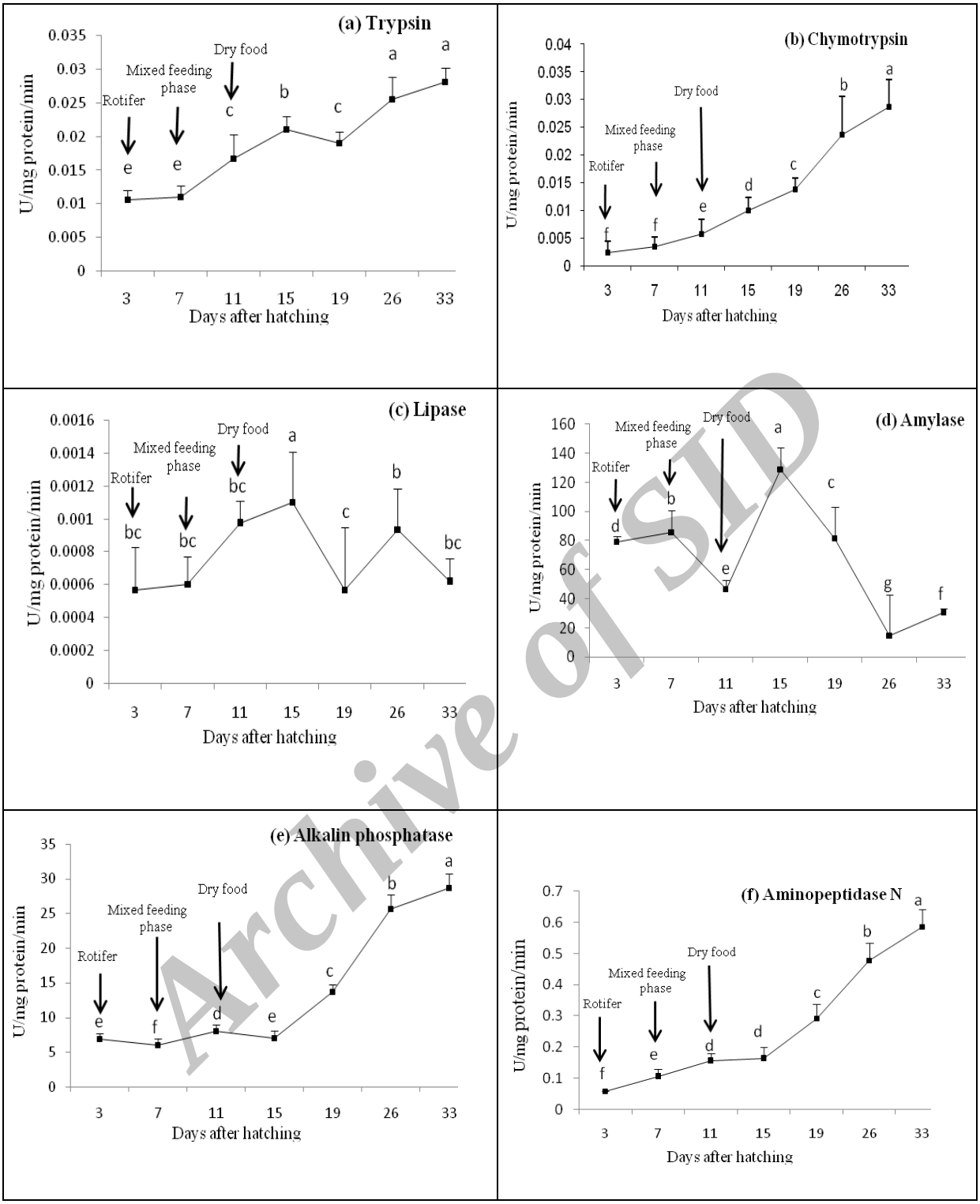

Figure 2. Specific activity of different digestive enzymes during the larval development of common carp. (a) Trypsin (U/mg protein/min); (b) Chymotrypsin (U/mg protein/min); (c) Lipase (U/mg protein/min); (d) Amylase (U/mg protein/min); (e) Alkaline phosphatase (U/mg protein/min); (f) Aminopeptidase N (U/mg protein/min). Each value represents mean ± SD (n=3). Data with *different letters* are significantly different (ANOVA, P<0.05). The arrows indicate the onset of exogenous feeding with rotifer and dry food.

Discussion

The present study described the development of some digestive enzymes in common carp larvae fed on live prey and dry food. Three main steps are considered in maturation of digestive system in fish larvae: the first step is pancreas enzymes, the second step is beginning of the intestinal enzymes secretion and third step is the development of the stomach and the onset of pepsin secretion (Gisbert et al., 2009), due to lack of stomach and acidic digestion, third step is not considered in carps (Chakrabarti et al., 2006).

In the present study all the enzymes studied were detected at the first feeding of common carp. Trypsin and chymotrypsin play a major role in the early development in all cyprinids that lack a true stomach. The activity of these proteases is associated with pancreatic secretions (Chakrabarti and Rathore, 2009). Generally, at the beginning of first feeding until the end of the experiment, activity of trypsin and chymotrypsin increased in common carp larvae.

The increase of trypsin and chymotrypsin activity have been reported in silver carp (♂) and bighead carp (♀) hybrid and *Labeo rohita* larvae fed live prey and dry food by Chakrabarti et al. (2006b) and Chakrabarti et al., 2006a), respectively, which is in agreed with our results. The significant increase of trypsin and chymotrypsin activities from 7-day onwards in the present study can be attributed to the adaptation of larvae to digest greater protein content in the food. Applebaum and Holt (2003) suggested that activity of chymotrypsin and trypsin was very much dependent on the nutritional conditions of the fish and cited an increase in chymotrypsin activity with age in properly fed *Sciaenops ocellatus* larvae. Similar results were reported by Rathore et al. (2005) and Ma et al. (2005) for *Catla catla* and *Pseudosciaena crocea* larvae, respectively.

Chakrabarti and Rathore (2009) and Chakrabarti et al. (2006 b) reported that the activity of trypsin decreased in *Cirrhinus mrigala* and silver carp (♂) and bighead carp (♀) hybrid on day 26 and 34, respectively. Similar to previous studies, in this study, decrease in trypsin activity observed from day 15 to day 19. The decrease in trypsin activity might be related to development of new organs and an increase in tissue proteins and dose not relate to decline in activity of digestive enzymes (Zambonino-Infante et al., 2009).

Oozeki and Bailey (1995) suggested that two forms of lipase exist in fish larvae, one related to yolk-sac absorption and the other related to digestion of exogenous lipids. It seems that with the onset of the exogenous feeding, changes in lipase activity during larval ontogeny are species-specific. Lipase total activity in *Dentex dentex* peaked at 35 dph and decreased after weaning (Gisbert et al., 2009). In contrast, lipase activity in *Sciaenops ocellatus* fed live prey decreased after

first feeding and peaked between 17 and 22 dph (Lazo et al., 2007).

Lipase activity in silver carp (♂) and bighead carp (♀) hybrid and *Cirrhinus mrigala* larvae increased during whole larval period (Chakrabarti et al., 2006b ; Chakrabarti and Rathore, 2009). These results are somehow different from those reported in the present study with common carp, wherein lipase was detected at the first feeding of common carp, peaking at 15 dph, then the activity decreased during larval development. The significant increase in lipase activity from day 3 to day 15 in this study may be attributed to the adaptive changes in fish for better utilization of dietary lipids. Martínez et al. (1999) reported maximal lipase activity in *Solea senegalensis* larvae on day 10, probably due to the development of exocrine pancreas. Similar to previous studies, in this study, highest level of lipase activity on day 15 may be related to development of exocrine pancreas.

Decrease in the lipase activity in later stages might be related to increase of tissue protein in larvae and dose not relate to decline in activity of digestive enzymes (Cuvier-Peres and Kestemont, 2002).

In common carp, high α-amylase activity observed at early-life stages that decreased during larval development. The same has been reported in silver carp (♂) and bighead carp (♀) hybrid by Chakrabarti et al. (2006). With first feeding on rotifer, from day 3 until day 7, the specific activity increased. The high activity of α-amylase during early-life stages demonstrates the importance of carbohydrate metabolism at the first feeding of common carp larvae. Moyano et al. (1996) also reported that in gilthead sea bream, enhancement in α-amylase activity after first feeding is related to formation of exocrine pancreas. From day 7 to day 11, when dry food was provided, the α-amylase activity decreased. This may be because of delay in modulation of digestive system for carbohydrate metabolism during early development. Shan et al. (2009) found that in croaker larvae, after the larvae started feeding on rotifers, α-amylase activity increased, but when copepods were offered, α-amylase activity decreased. In large yellow croaker larvae, activity of α-amylase was lower when the larvae fed on rotifers (4% glycogen) than that fed on frozen copepods (7.4% glycogen). This phenomenon may be related to the high concentration of glycogen in frozen copepods (Ma et al., 2005). In common carp larvae, maximum activity of α-amylase observed on day 15. Cahu et al. (2004) suggested that the level of α-amylase activity as an indicator of the pancreas maturation. In common carp larvae, from day 15 to day 33, the specific activity of amylase decreased. Decline in amylase activity when the juvenile stage is reached, irrespective of the dietary carbohydrate level, may be due to lower amylase mRNA levels (Douglas et al., 2000; Zambonino Infante and Cahu, 2001).

Two groups of digestive enzymes are found in enterocytes: cytosolic

enzymes (mainly peptidases) found in the cytoplasm, and brush border membrane enzymes, which are linked to the cell membrane. Different types of membranous enzymes are detected such as alkaline phosphatase and aminopeptidase-N. Generally, at the beginning of first feeding until the end of the experiment, activity of brush border membrane enzymes increased in common carp larvae. In this work, the presence of brush border membrane enzymes at the early stages of larval development indicates that the larvae of this species are capable of nutrients absorption. In common carp larvae, specific activity of alkaline phosphatase and aminopeptidase N increased sharply during third week of life, from day 15 to day 33. Increase in enzymes of the brush border membranes corresponds to maturation process of enterocystes (Moyano et al., 1996). In *Gadus morhua* and *Sciaenops ocellatus*, the activity of brush border membrane enzymes were lowest at first feeding and subsequently increased with age, during the third week (Kvåle et al., 2007; Lazo et al., 2007).

The appearance of a functional microvillus membrane in enterocytes constitutes a crucial step during larval development of fish for the acquisition of an adult mode of digestion (see review in Zambonino-Infante et al., 2009). According to the former authors, this change has been observed to occur around the 3^{th} and 4^{th} week after hatching in temperate marine fish species.

In conclusion, the elevated alkaline proteases (trypsin and chymotrypsin) activity on 3-day onwards can be related to adaptation of larvae to digest protein content in the food. The maximum activity of α-amylase and lipase on day 15 may be related to development of exocrine pancreas. Regarding intestinal enzymes, alkaline phosphatase and aminopeptidase N were found at day 3, although the achievement of an efficient brush border membrane digestion take place between days 15 and 33. This developmental process, and particularly for the digestive functions can be considered in the feed formulation of the common carp larvae.

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