Changes of digestive enzymes activity in Caspian Kutum (*Rutilus frisii kutum*) during larval developmental stages

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Abstract

The ontogenesis and specific activities of pancreatic (trypsin, chymotrypsin, amylase and lipase) and intestinal enzymes (alkaline phosphatase, aminopeptidase N) were investigated in Kutum (*Rutilus frisii Kutum*) from the onset of exogenous feeding (3 day after hatching, DAH) to the juvenile stage at 50 DAH. Trypsin- and chymotrypsin-specific activity showed similar patterns and increased with larval development and age. After the first feeding, specific activity of amylase and lipase is increased and reach a peak at 10 DAH, followed by a sharp decrease until 25 DAH, after which it is increased again. Carbohydrate and lipid content changes in diet have led to fluctuations (increases or decreases) in amylase and lipase activities. Alkaline phosphatase and aminopeptidase N specific activity had similar patterns and showed increased trend with age. Sharp increases in activity for both enzymes from 7-10 DAH indicated maturation of the enterocytes and the achievement of adult-like mode of digestion. Our results suggest that Kutum is capable of digesting protein, lipid and carbohydrates at early stages of growth. However, due to low level of lipase-specific activity compared to other enzymes, it seems that Kutum larvae prefer diets containing higher protein levels than diets with higher lipid content. Therefore, for this species lipid component should remain at low level in formulated diets. In this study, specific activity of most digestive enzymes exhibited a sharp increase when co-feeding on rotifers and formulated diet started from 7-10 DAH. The increase in activities of pancreatic and intestinal enzymes in this period can be due to maturation of exocrine pancreas and brush border enterocytes, respectively and showed that this fish has ability to digest formulated diets.

Keywords: *Rutilus frisii Kutum*, Larviculture, Pancreatic enzymes, Intestinal enzymes, Ontogeny.

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Introduction

Kutum, *R. frisii kutum* (Kamenskii, 1901; family Cyprinidae), is an endemic fish of Caspian Sea. It is one commercially important bony fish in the south the Caspian Sea and has a great demand, due to high nutritional value and good taste. This species was threatened due to increased pollution, construction of bridges and dams, rivers' ecological changes, overfishing and destruction of spawning areas, which caused a sharp decline in stocks of this species in 1970s and early 1980s (Ghaninejad and Abdulmaleki, 2007). For these reasons, Iran Fisheries Organization began the production of fingerlings for restocking and rearing this valuable species to market size to reduce pressure on natural Caspian Sea stocks (Keyvanshokooh *et al.,* 2007).

Rearing fish larvae is a critical issue in aquaculture. In rearing ponds, there is high mortality especially during weaning, the onset of exogenous feeding. This could occur if the digestive system is not yet fully developed and differentiated (Govoni *et al.,* 1986; Yang *et al*., 2010). Transition from endogenous feeding to exogenous feeding is an extremely sensitive period in the life of fish larvae, during which series of hormonal and digestive processes take place to help ensure the survival and growth (Buentello *et al.,* 2011). Weaning of larvae from live to formulated feeds is the bottleneck of commercial aquaculture for many species. Knowledge of digestive tract developmental changes associated with food absorption process may help identify limiting factors during larvae rearing and reduce bottlenecks in the weaning process (Hamlin *et al.,* 2000). Kutum larvae are poorly developed at early stages of life and undergo important functional and morphological changes during early larval period. The main factors in improved larval growth are preparation of nutrient particles suitable to the larval developing gut, synchronization of feeding protocols with physiological stages of larvae, and their digestion and absorption capabilities (Lazo *et al.,* 2007; Guerreiro *et al.,* 2010).

Digestion process has the primary role in animal metabolism, since it determines the availability of nutrients needed for all biological functions (Gisbert *et al.,* 2009). Therefore, investigation of the ontogenetic patterns of digestive enzyme activities is an important tool to know better the capabilities of larvae in digestion and absorption of various nutrients, such as live food and/or compound microdiets, and establishing feeding protocols for optimizing larval rearing production (Ueberschär, 1993, 1995; Diaz *et al.,* 1997; Zambonino-Infante and Cahu, 2001; Suzer *et al.*, 2007).

In addition, the study of digestive enzyme activities is an index of digestive processes and nutritional status of fish larvae (Ueberschär, 1988). Inadequate knowledge of nutritional requirements and digestive physiology during larval period of Kutum motivated us to contribute in increasing the knowledge

about this important species in order to develop improved rearing methods.

In last decades, several studies have analyzed ontogeny and digestive enzyme activities in many marine and freshwater fish species (see review in Zambonino-Infante *et al.,* 2008). Also we carried out studies on developmental changes of digestive enzymes in Persian sturgeon (Babaei *et al.,* 2011) and common carp (Farhoudi *et al.,* 2013). To date, no study has been carried out on the ontogenetic development of digestive enzymes in Kutum larvae. In order to rear larvae more successfully, it is essential to understand the ontogeny of its digestive system. Therefore, the objective of the present study was to evaluate the ontogenetic development and pattern of main digestive enzyme activities in *R. frisii kutum* larvae fed with live food and compound microdiet from the first feeding (first exogenous feeding) until the end of the experiment at 50 DAH. This information will be useful for larvae rearing management and improving formulated compound diets specific to this species.

Materials and methods

Broodstock, egg incubation and larval rearing

Caspian Kutum larval culture was carried out in May 2010 at the Shahid Rajaee restocking center in Sari, Mazandaran Province, Iran (lat 36˚37′ N, long 53˚05′ E). In April and May 2010, broodstock were selected from wild breeders which were captured from the following four rivers: Shirod, Tonekabon, Tajan and Goharbaran. For the purpose of propagating this species, caught brood stocks were stripped for their eggs. Ripe eggs from each female were fertilized by sperm stripped from 2 to 3 males. Mean weight of male and female broodstocks, were 0.68-0.7 kg and 1.18-1.20 kg, respectively. Fertilized eggs were placed in Seth Green incubators in the river.

During propagation, temperature was 24-25ºC. After a few days of incubation, developing embryos were transferred to vase incubator in Shahid Rajaee restocking center, and egg incubation and larval rearing were conducted there. Water temperature, oxygen level and pH in vase incubators were maintained at 18-19ºC, >5.1 mg l^{-1} and 7.9 respectively, during the study period.

Buoyant viable eggs were separated from sinking dead eggs daily. Hatching occurred after 5-7 days. When yolk sacs were almost completely absorbed, marking the onset of exogenous feeding, newly hatched larvae were transferred from incubators to the one-hectare ponds at 3 DAH at a density of 100 larvae/m^2. Ponds were supplied with freshwater. Water temperature, oxygen level and pH in the ponds were maintained at 24-26ºC, 5.5-7 mg l^{-1} and 8, respectively.

After yolk sac absorption (third day), rotifer (with a composition of 57.48% protein, 11.65 % lipid, 10.60% ash and 20.27% carbohydrates) was introduced to larvae from day 3 to day 7. Co-feeding based on rotifer and a commercial formulated diet (SFK, Dam Mazandaran Co. Sari, Iran) was offered form 7 to 10th DAH, while from then to the end of

the study (50 DAH) larvae were fed with the commercial formulated diet (SFK) containing 38.54% protein, 12.30% lipid, 11.60% ash, and 37.56% of carbohydrates.

Sample collection and growth analysis

Larvae samples were randomly collected from vase incubators and ponds at 3 (onset of exogenous feeding), 7, 10, 15, 20, 25, 30, 35, 40 and 50 DAH for digestive enzyme activity analyses. Samples were collected in morning. No feed was added to the rearing tank at the night prior to sampling, to minimize effects of exogenous enzymes from undigested live food in fish guts (Kolkovski, 2001).

A 1-gram sample of larvae for each larval stage was washed with distilled water and dried with a filter paper. Samples were frozen in liquid nitrogen at -196 °C and stored at -80 °C until enzymatic analyses.

Thirty larvae samples were randomly collected from ponds to monitor fish growth (measurements of total length and wet weight of larvae) on the sampling days.

Total length, from tip of the snout to posterior margin of the body of larvae was measured in each period. Wet body weight of larvae was measured after removing water with filter paper. The total length was measured to the nearest 0.1 mm using a dial caliper and individual wet weight was determined by precision balance (0.1 mg sensitivity).

Evaluation of activity of digestive enzymes

As described in our previous study (Babaei *et al.,* 2011), whole larvae were homogenized at 0-4°C in 100-mMTris-HCl buffer with 0.1 mM EDTA and 0.1% Triton X-100, pH 7.8, with a ratio of 1 g tissue to 9 ml of buffer, with an electric homogenizer (Wiggen Hauser D500 homogenizer, Berlin, Germany). Supernatants obtained after centrifugation (30,000×g for 30 min at 4°C) were stored at -80°C before the enzyme analyses (Furné *et al.,* 2008). Concentration of soluble protein in pond samples was determined by the method described by Bradford (1976), using bovine serum albumin as standard. Enzyme activities were expressed as specific activity (U mg protein^{-1}). All assays were carried out in triplicate.

According to Erlanger *et al*. (1961) Trypsin (E.C.3.4.21.4) activity was assayed at 37°C using N-α-benzoyl-DL-arginine p-nitroanilide (BAPNA) as substrate in 50 mM Tris-HCl, with a pH of 7.5, containing 20 mM $CaCl_2$. One unit of Trypsin per ml (U) was defined as 1 μmol BAPNA hydrolyzed per min per ml of enzyme extract at 410 nm.

Chymotrypsin (EC. 3.4.21.1) activity was measured at 37°C according to Erlanger et al. (1961), using 0.1 mM Suc-Ala-Ala-Pro-Phe-p-nitroanilide (SAPNA) as substrate in 50 mM Tris-HCl, with 20 mM $CaCl_2$, in pH 7.5, and monitored at 410 nm for 3 min. The activity unit (U) was defined as 1 μmol of SAPNA, also molar extinction coefficient for trypsin and chymotrypsin was 8800 cm^2 mg^{-1}. Amylase

(E.C.3.2.1.1) activity was quantified according to Bernfeld (1951) and Worthington (1991) using (1%w:v) soluble starch dissolved in 0.02 M Na_2HPO_4 buffer containing 0.006 M NaCl with a pH of 6.9 as substrate. Incubation was carried out for 3-5 min at 25°C. Then 0.5 ml of 1% dinitrosalicylic acid (DNS) solution was added to reveal the reaction, and then boiled for 5 min. After boiling, a solution of 5 ml of distilled water was added to the mixture and the absorbance of the cooled solution was recorded at 540 nm. Maltose (0.3–5 μMml−1) was used for preparation of the standard curve. The α-amylase specific activity was defined by μmol of maltose produced per min per mg protein at the specified condition.

Lipase (E.C.3.1.1) activity was determined at 30°C according to (Iijima *et al.*, 1998) using hydrolysis of n-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) and the incubation time of the samples was 15 min. The reaction was terminated by adding 0.7 ml of acetone n-heptane (5:2, v.v). The activity unit (U) was defined as 1 μmol of n-nitrophenol released at 405 nm per min, with extinction coefficient of 16,500 M^{-1} cm^{-1} l^{-1} (Iijima *et al.*, 1998)

Alkaline phosphatases (AP) (E.C.3.1.3.1) activity was assayed at 37°C according to Bessey et al. (1946) using 4-nitrophenyl phosphate (PNPP) as substrate in 30 mM Na_2CO_3 buffer (pH 9.8). One unit was defined as 1 μg N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE) released per min at ml of brush border homogenate at 407 nm.

Aminopeptidase (AN) (E.C.3.4.11.2) activity was determined at 25°C according to Prescott and Wilkes (1976), and Spungin and Blumberg (1989), using l-leucine p-nitroanilide as substrate and 50 mM Tris-HCl at the pH 8.0. One unit of enzyme activity (U) was defined as 1 μg nitroanilide released per min per ml of brush border homogenate at 405 nm with an extinction coefficient of 10,600 M^{-1} cm^{-1}.

Statistical analyses

Data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Bartlett's test) prior to their comparison. Results are given as mean ±S.D. (n=3 for enzymes; n=3 for body weight of 30 larvae; n=3 for total length of 30 larvae). Data of body weight and length were analyzed by using appropriate regression model analysis. Enzymatic activity data were compared by one-way ANOVA, and mean comparisons were performed with a Duncan's test. The level of significant difference was set at $p<0.05$. Statistical analyses were performed using SPSS 11.5 (SPSS Inc., Chicago, IL, USA).

Results

Growth in terms of length and weight of Kutum larvae during the period of study is displayed in Fig. 1. An exponential relationship (R^2=0.95, Y=2.773x+ 9.038) and (R^2=0.98, Y=53.21x-86.79)

described Kutum growth, and is expressed in total length (TL) and wet body weight, respectively. Resulting Kutum larvae growth measurement in weight and length showed a rapid exponential growth from hatching to 50 DAH. Mean initial weight, length (1 DAH), and final weight and length (50 DAH) of this fish were 4.02±0.01mg, 8.47±0.02mm, and 483.33±1.11mg, 35.34±0.41mm, respectively. The percentage weight gain over 50 DAH from 1 DAH was 11923%.

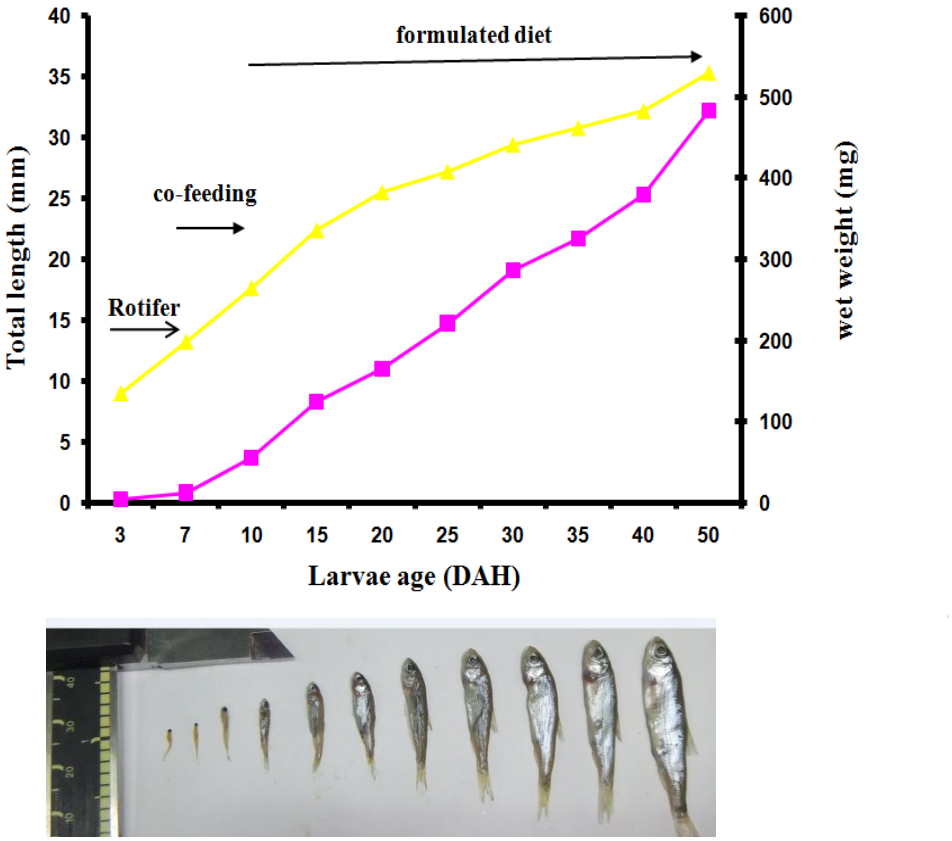

Figure 1: Growth in wet weight (■) and total length (▲) of Kutum *(Rutilus frisii Kutum)* during 50 days after hatching (DAH).

Activity of pancreatic enzymes

Specific activity of trypsin in Kutum larvae increased steadily with larval age. The specific activity of this enzyme was detected from onset of exogenous feeding (3 DAH) to be 0.0049±0.00 u mg protein^{-1}. Initially it was very low and increased gradually with larval development until last day of the experiment (50 DAH), when it reached a maximum of 0.017±0.00 u mg protein^{-1} (p<0.05, Fig. 2A).

At the onset of exogenous feeding (3 DAH), specific activity of chymotrypsin was low (0.010±0.00 u mg protein^{-1}) and increased gradually until 25 DAH. Thereafter, chymotrypsin activity

increased rapidly and reached a maximum at 50 DAH (0.039 ± 0.00 u mg protein^{-1}, p<0.05, Fig. 2B). Trypsin and chymotrypsin specific activity (Fig. 2A, B) showed similar patterns and increased with larval development and age.

The specific activity of amylase was detected at the time of first feeding (3 DAH, 7.87 ± 0.10 u mg protein^{-1}), and sharply increased at 7 DAH. This enzyme activity continued rising gradually to 10 DAH, when the peak of amylase-specific activity was measured (12.96± 0.01 u mg protein^{-1}). Then this activity suddenly decreased to 15 DAH, and continued to decrease, reaching minimum activity at 25 DAH (6.37± 0.01 u mg protein^{-1}, p<0.05, Fig. 2C). Afterwards, amylase activity progressively increased until 40 DAH and decreased again at 50 DAH (p<0.05).

The specific activity of lipase was lower than activity of other enzymes. The activity of this enzyme was detected at 3 DAH (onset of exogenous feeding, 0.0011±0.00 u mg protein^{-1}), and suddenly increased until 10 DAH, and reached a maximum peak on this day (0.0023±0.00 u mg protein^{-1}, p<0.05, Fig. 2D). After that (After day 10), the specific activity of lipase decreased and fluctuated until end of the experiment (50 DAH), however, this was not significantly different (p>0.05).

Activity of intestinal enzymes

Developmental patterns of intestinal enzymes were similar. The specific activity of alkaline phosphatase (Fig. 2E) and aminopeptidase N (Fig. 2F) was detected at the onset of the first feeding (3DAH, 2.29± 0.18 and 0.07±0.00 u mg protein^{-1}, respectively), increased with larval development, and increased sharply between 7-10 DAH, coinciding with fish co-feeding on rotifer and commercial formulated diet (p<0.05). They continued to increase until 20 DAH, and then decreased gradually on 25 and 30 DAH. After this date, specific activities of the brush border membrane (BBM) enzymes, AP and AN, showed a progressive increase again until the end of the experiment, reaching a peak at 50 DAH (12.25±0.53 and 0.34±0.02 u mg protein^{-1}, respectively,-p<0.05).

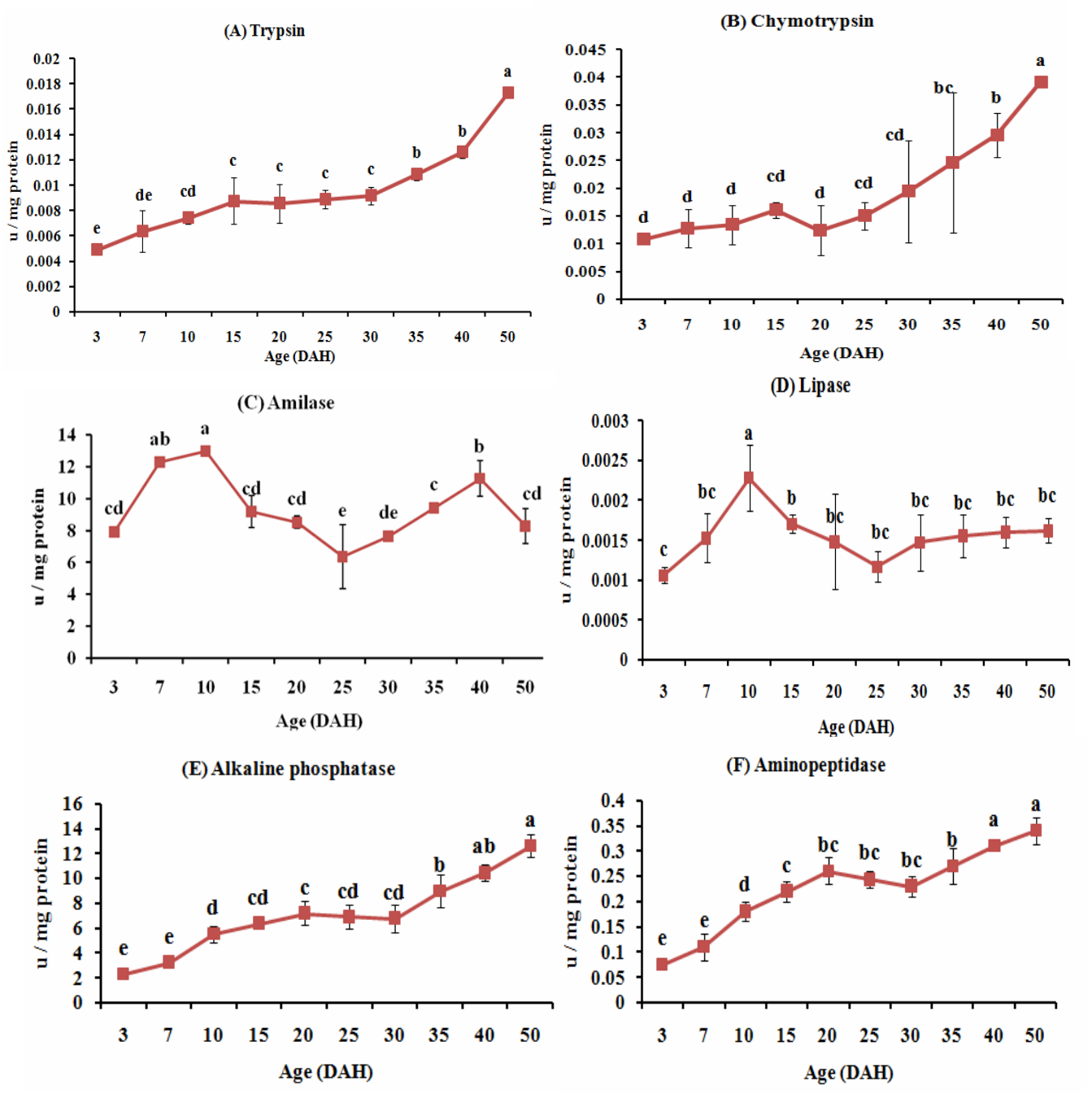

Figure 2: Specific activity (U mg protein^{-1}) of trypsin (A), chymotrypsin (B), amylase (C), lipase (D), alkaline phosphatase (E) and aminopeptidase (F) in *Rutilus frisii Kutum* from onset of exogenous feeding to 50 DAH. Different values of enzyme activity (mean ± SD, n=3), different superscript letters are statistically significant (p<0.05).

Discussion

Growth rate (length and weight) showed an increasing trend in this study. The average larval weight and length with feeding based on co-feeding from 7 DAH increased rapidly until 15 DAH, indicating that larval growth was influenced significantly by diet. After 15 DAH to the end of the experiment, a substantial increase in weight and length occurred. During this experiment, growth trend of Kutum larvae was

similar with other farmed marine fish species such as European sea bass (Zambonino-Infante and Cahu, 1994) large yellow croaker (Ma *et al.,* 2005), common dentex (Gisbert *et al.,* 2009), Persian sturgeon (Babaei *et al.,* 2011) and in particular common carp (Farhoudi *et al.,* 2013).

Ontogeny and regulation of the digestive enzymes are affected by morphological and functional changes in the digestive tract and composition of diets, genetically programmed and induced by the specific substrate in the food (Gruzdkov *et al.,* 1986). Zambonino-Infante *et al.* (2008) pointed out that fluctuations in the specific activity of digestive enzymes during fish larvae development can occur because of different metabolic and physiological processes.

At first exogenous feeding, the digestive tract in most species has enzymes involved in the digestion of protein, lipid and carbohydrate (Kolkovski, 2001). The level of enzymatic activity in fish larvae in this early stage is lower than in next stages (Cousin *et al.,* 1987; Rathore *et al.,* 2005).

In Kutum larvae, development of all pancreatic and intestinal enzymes was determined at the time of first external food. The presence of these enzymes at first feeding of some fish larvae is reported by several authors (Chakrabarti and Sharma, 1997; Kumar and Chakrabarti, 1998; Kumar *et al.,* 2000; Rathore *et al.,* 2005; Chakrabarti *et al.,* 2006; Babaei *et al.,* 2011 and Farhoudi *et al*., 2013). Pancreatic and intestinal enzyme activities were detected to be mostly low at first feeding (Cousin *et al*., 1987).

Certain fish species lack a distinct stomach, e.g., the carp family, such that no acid secretion or pepsin digestion occurs (Govoni *et al*., 1986), and pancreatic secretions such as trypsin and chymotrypsin play an important role in protein digestion (Zambonino-Infante and Cahu, 2001; Lazo *et al.,* 2007).

In the present study, the specific activity of proteolytic enzymes was detected at first feeding (3DAH), and then showed a continuous increase with age until the last days of the experiment, reaching a peak at 50 DAH. This increase coincided with the development of pancreas and intestine. Similar results were also found in some species such as *Cyprinus carpio* (Farhoudi *et al.,* 2013), silver carp (♂) and bighead carp (♀) hybrid (Chakrabarti *et al.,* 2006) and *Cirrhinus mrigala* (Chakrabarti and Rathore, 2009).

The increase in specific activity of trypsin and chymotrypsin observed during larvae growth can be influenced by two major factors. The first is a rapid increase in growth from 7 DAH onwards because trypsin is a growth indicator in fish larvae (Rungruangsak-Torrissen *et al.,* 2006). The second factor is related to the feeding of rotifer and dry food. Hence, the quantity of protein in food and different nutritional composition of rotifer and microdiet can modulate the activity of this enzyme (Moyano *et al.,* 1996; Cahu *et al.,* 2004; Morais *et al*., 2004). According to Zambonino-Infante *et al.* (1996) and Pedersen *et al*. (2003),

the trypsin enzyme reacts to the protein ratio in the diet. In fish larvae such as *Dicentrarchus labrax* (Péres *et al.*, 1998; Cahu *et al.,* 2004) and *Chelon labrosus* (Zouiten *et al.,* 2008), trypsin activity should be related to dietary protein concentration. The increasing trend of the proteolytic enzymes in Kutum larvae can be attributed to adaptation of larvae for better digestion of dietary proteins. Applebaum and Holt (2003) suggested that activity of proteolytic enzymes is greatly influenced by nutritional conditions; in red drum larvae proper nutrition led to an increase in chymotrypsin activity with age.

In Kutum larvae, an abrupt increase in amylase-specific activity was observed from the onset of exogenous feeding until 10 DAH which could be related to modulation of the digestive system for carbohydrate metabolism (Chakrabarti *et al.,* 2006). Moyano *et al.* (1996) also reported that in gilthead sea bream, enhancement in amylase activity after first feeding is related to formation of exocrine pancreas.

In addition, synthesis and secretion of amylase usually is stimulated by presence of carbohydrates in the dietary and feeding regime of larvae, affecting the activity of this enzyme in larval body (Zambonino-Infante and Cahu, 1994; Péres *et al.,* 1998; Corrêa *et al.,* 2007).

Different contents of carbohydrate (high or low amount of carbohydrate) in diets (live food and/or compound food) offered to larvae might lead to a change (increase or decrease) in this enzyme activity. For example, in *C. carpio*, high amylase activity was related to the presence of many fruits in the diet of this species (Corrêa *et al.,* 2007).

Thus, amylase-specific activity during early stages until 10 DAH reflected relatively high carbohydrate content in live food (Ma *et al*., 2005) and dry food (Suzer *et al.,* 2007).

The decrease in specific activity of amylase during 10-25 DAH can be attributed to an increase in body protein (as specific activity is defined as the ratio of activity per mg protein, Chakrabarti *et al*., 2006). Moreover, another possible explanation for decrease in amylase activity in later stages, regardless of the diet carbohydrate content, could be as a result of lower amylase mRNA levels (Douglas *et al.,* 2000; Ma *et al.,* 2001).

Decrease in amylase activity in silver carp (♂) and bighead carp (♀) hybrid from day 10 to day 16 is due to developmental changes in the gut morphology (Chakrabarti *et al*., 2006).

Zambonino-Infante and Cahu (2001) suggested that the decrease of specific enzyme activity is due to the allometric growth of fish biomass instead of a reduction in enzyme synthesis.

Also the increase in amylase activity from day 25 onwards might be due to high level of carbohydrate in compound diet. Similar results were found in some species such as *D. labrax* (Zambonino-Infante and Cahu, 1994), *S. ocellatus* (Lazo *et al.,* 2000), large yellow croaker, *Pseudosciaena crocea*, (Ma *et al.,* 2005), *P. erythrinus* (Suzer *et al.,* 2006) and *D puntazzo* (Suzer *et al.,* 2007) larvae.

In general, the decline in trypsin, chymotrypsin, amylase and lipase activities during the experiment may be due to increase of protein level in the tissue in growing larvae and not related to decline in activity of digestive enzymes. Increase in tissue protein may be attributed to anatomical and physiological changes resulting before the adult mode of digestion (Cuvier-Péres and Kestemont, 2002; Chakrabarti *et al.,* 2006).

Lipase activity was detected from the beginning of the experiment (3 DAH).

In common carp, Lipase activity was detected at the first feeding and peaked at 15 DAH, and then the activity decreased during larval development (Farhoudi *et al.,* 2013).

Exogenous feeding with live food such as rotifers increased the activity of this enzyme in Kutum larvae. This may be because the large amount of wax esters, phospholipids and triacylglycerols of zooplanktonic prey (Shields *et al.,* 1999) might have stimulated the production of lipase in the pancreas of fish larvae (Rønnestad and Morais, 2007; Babaei *et al.,* 2011). With the beginning of co-feeding from 7 DAH, lipase reached a maximum activity at 10 DAH. One possible reason for lipase activity peaking on 10 DAH could be related to the higher lipid content of co-feeding (rotifer 11.65% and formulated diet 12.30%). Maximum lipase activity in Kutum larvae at 10 DAH can be indicative of the development of pancreas. High level of this enzyme found in *S. senegalensis* (Martínez *et al.,* 1999) and *S. maximus* (Cousin *et al.,* 1987) is attributed to this cause. From 10 DAH, the diet changed from co-feeding to a formulated diet led to a decrease in lipase activity until 25 DAH. Thus, lipid content in diets might have modified activity of the enzyme greatly (Zambonino-Infante and Cahu, 1999, 2007). The increase in lipase activity from day 25 after hatching can be as a result of better adaptation to digestion and utilization of lipids present in the diet.

Importance of the parietal digestive enzymes (alkaline phosphatase and aminopeptidase N) is due to mature enterocytes. The sudden increase in the activity of brush border membrane enzymes is associated with a progressive maturation of enterocytes and attains a more adult character with improved luminal digestive capacity (Ribeiro *et al.,* 2002; Zambonino-Infante and Cahu, 2007; Alvarez-González *et al*., 2008).

Alkaline phosphatase and aminopeptidase N specific activity was detected in Kutum larvae at the onset of feeding. Both enzymes had similar patterns and showed abrupt increases with age. At first, aminopeptidase N activity was lower than alkaline phosphatase, but the activity of this enzyme increased in response to the presence of food. In this study, the presence of brush border membrane enzymes at the early stages of larval development showed that Kutum larvae are capable of nutrients absorption.

In Kutum larvae, Morpho-physiological changes and a sudden increase in activity of brush border

membrane enzymes AP and AN from 7 DAH indicated the presence of more functionally developed enterocytes and maturation of enterocystes. Although in the present study we did not analyze the activity of intestinal cytosolic enzyme, it seems acceptable that the observed increase in brush border membrane enzymes, AP and AN, was coincided with a sharp decline of cytosolic enzyme activities, as this process characterizes the normal development of intestine and maturation of the enterocytes in vertebrates (Zambonino-Infante *et al.*, 2008) and transition from a primary to an adult mode of digestion (Ma *et al*., 2005; Suzer *et al*., 2007). Achieving this process properly determines larval survival (Cahu and Zambonino-Infante, 1995).

In gilthead sea bream, the increase in activities of pancreatic enzymes (trypsin and amylase) and intestinal enzyme (alkaline phosphatase) after first feeding is caused by formation of exocrine pancreatic and brush border enterocytes, respectively (Moyano *et al.,* 1996).

In this study, the specific activity of most digestive enzymes exhibited a sharp increase when co-feeding on rotifers and formulated diet started during 7-10 DAH. Increasing pancreatic enzyme activity in this period can be due to maturation of the exocrine pancreas, while the intestinal enzyme can be resulted from maturation of brush border enterocytes.

This study presents data to determine ontogenic development of pancreatic and intestinal enzymes in Kutum, *R. frisii kutum,* larvae. Results indicate that Kutum is capable of digesting protein, lipid and carbohydrates from onset of exogenous feeding. Coinciding with growth of Kutum larvae we observed continuous increase in activity of proteolytic and parietal enzymes, while this trend was not observed in lipase and amylase enzymes activity.

Low specific activity of lipase compared with other enzymes reflects the limited ability of larvae in lipid digestion. Consequently, Kutum prefers diets with high protein levels over diets with lower protein and higher lipid levels. Therefore, lipid content should remain at a low level in formulated diets. A sharp increase during 7-10 DAH was observed in the specific activity of most digestive enzymes during co-feeding. This infers that the Kutum digestive enzymatic equipment is complete at 7-10 DAH, when brush border enterocytes mature and morpho-physiological changes occur, achieving an adult mode of digestion. Changes in enzyme activity during larval development seemed to be more dependent on digestive system development and composition of the diet components. Our findings on ontogenetic changes of digestive tract in *R. frisii kutum* could result in better understanding of the ontogeny of fast-growing fish larvae and improvements in feed formulation for commercial larval rearing.

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