Study of the Activities of Digestive Enzymes, Amylase and Alkaline Phosphatase, in Kutum Larvae, *Rutilusfrisiikutum* Fed Artemia Nauplii

[1]F. Hassanatabar, [1]H. Ouraji, [1]A. Esmaeili and [2]S.S. Babaei

[1]Sari Agricultural Sciences and Natural Resources University (SANRU), Sari, Iran
[2]Fisheries Department, Faculty of Natural Resources and Marine Sciences,
Tarbiat Modares University, Mazandaran, Iran

Abstract: The purpose of this experiment to determine thegrowth rate, survival and activity of digestive enzymes infishlarvaefed Artemianauplii. The experiment was performedin 115-liter glasstanks containing 7000 pieces of kutum larvaewith an average initial weight of 5.1 mg (2days afterhatching). The results displayed that the stocking in high density, decrease growth efficiency but there was not considerable effect on survival rate of larvae. In thepresent study was observed a significant increase in activity of α-amylase from 23 to 30 dayasthe highestamylaseactivitywas found at30 days after hatching. The highcontent of glycogen and carbohydrates in live food may be to stimulate synthesis and secretion of amylase. Anearly peak of activity in alkaline phosphatase enzyme activitywas observed at day 8 after hatching the indicating the maturation process in the intestines of kutum larvae.

Key words: Kutum Larvae ⋅ Digestive Enzymes ⋅ Amylase ⋅ Alkaline Phosphatase

INTRODUCTION

One of the most important and economic aquatics of Caspian Sea is Kutum (*Rutilusfrisiikutum*) which devoted approximately 44 % of bony fish catch in 2006 (9631 tons). This species is an omnivore; however, unlike other omnivores it has a limited food supply, due to its digestive tract [1]. The efficiency of food depends on physiological capacities in fish to digest and transform ingested nutrients [2]. A comprehensive analysis of the ontogenic changes during the early life stages of fish is essential for the design of feeding strategies and formulation of dry diets [3], so digestion mechanisms in fish larvae have been particularly studied in the last two decades. The analysis of digestive enzyme activities is an easy and reliable biochemical method that can provide insight into the digestive physiology in fish larvae, their nutritional condition [4].

Carbohydrases (like amylase) are the least studied of the digestive enzyme groups in fish larvae, since there hasbeen no essential requirement found for carbohydrates in fish [5], but are nonetheless important for the development of diets for commercial purposes where one of the goals is to minimize the cost of the feed. Digestion and absorption of nutrients depend on the activity of the digestive enzymes, in particular those located in the brush border section of the intestine, which are responsible for the final stages of breaking down and assimilation of the food [6]. Alkaline phosphatase is a dominant enzyme of the intestinal brush border, and is often used as amarker of intestinal integrity [7]. Its activity is increased in a few hours in presence of its substrates. The functional significance of this enzyme is far to be fully understood, however, it hydrolyzes phosphoester bounds in various organic compounds like proteins, lipids, and carbohydrates [8].

The aim of this study was to characterize the activities of α-amylase (pancreatic enzyme) and alkaline phosphatase(intestinal enzyme) of Kutum (*Rutilusfrisiikutum*) fed with live food during larval development.

Corresponding Author: Fatemeh Hassantabar, Department of Fisheries, Faculty of Animal Sciences and Fisheries Sari Agricultural Sciences and Natural Resources University, 9 km Sea Road, Sari, Iran.
Tel: +09363758941.

MATERIALS AND METHODS

Location and Experimental Design: The study was carried out from March to June 2011 at laboratory of aquaculture located in Sari Agricultural and Natural Sciences University. Larvae of *R. frisiikutum* aged 48 hours after hatching (hah) were stocked in 115-L aquaria with little aeration at a density of 7000 larvae in each tank. The photoperiod was maintained on a 16 L:8 D cycle. Larvae were hand fed ad libitum at an approximately 4-h interval through out the day from 7:00 to 19:00 hours manually 7 days a week.

Feeding started from 72 h post-hatch when the yolk sac was not completely absorbed. Each tank was finely siphoned just before new feeding to find uneaten food and likewise remove faces and dead fish every day. After siphoning, the water volume (approximately 75%) was adjusted in each aquarium and dead fish were counted.

Sample Preparation: Samples for enzymatic assays were taken at 2, 3 (exogenous feeding day), 5, 10, 13, 17, 23, 27, 30 dph. No food was added to the rearing tank at night on the day prior to sampling, and larvae were sampled in the morning to minimize the effects of exogenous enzymes from live food in fish guts [9]. The samples immediately stored at-70 °C for further analysis. Growth measurements were obtained from a pool of 30 larvae at the sampling days.

Enzymatic Determinations: Whole larvae were homogenized at 0-4 °C in an electric homogenizer (IKA T25 digital, Ultra Turrax 169 model). For the homogenization, a 100 mMTris-HCl buffer with 0.1 mM EDTA and 0.1% Triton X-100, pH 7.8, was used at a proportion of 1 g tissue in 9 ml of buffer. The homogenates were centrifuged at 30,000 g for 30 min. at 4°C (Hettich D78532, Germany). After centrifugation, the supernatant was collected and frozen at -80 °C [2].

Determinations of the intestinal brush border membrane enzyme according to a method described by Cahu *et al*. [10] methods. The samples were homogenized in 30 v/w fractions of Tris (2 mM)-mannitol (50 mM) with a homogenizer (IKA T25 digital, Ultra Turrax 169 model) at 19,000 g for 30s. Then, tissue homogenates were centrifuged at 9000 g for 10 min. after 0.1 M $CaCl_2$ added.

Amylase (E.C.3.2.1.1) activity was assayed using the 3, 5-dinitrosalicylic acid (DNS) method at 540 nm [11, 12]. Starch was used as the substrate in the determination of amylase. Starch (250 μl) was incubated with the enzyme extract (50 μl) at 35 °C. The reaction was terminated by adding 0.5 ml of 1% dinitrosalicylic acid (DNS) and boiled for 5 min. After cooling, 5 ml of distilled water was added to the mixture. To draw the standard curve used maltose. The specific activity of α-amylase was as μmol of maltose released from starch per min per mg protein at the specified condition.

Alkaline phosphatase (AP) (E.C.3.1.3.1) activity was analyzed at 37 °C using 4 pNPP (4-nitrophenyl phosphate) 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 [13].

Statistical Analysis: Digestive enzyme activities were compared by means of a one-way ANOVA, and means were compared with a Duncan's test. The level of significance set at 0.05. Statistical analyses were done using SPSS 16.0.

RESULTS

The survival rates of larvae fed with artemianauplii was high during the experimental period (85%±2.4). The result of growth was shown in figure 1. The mean body weight of two-day larvae was 5.3±0.9 mg. According to figure 1 fish gradually grew with time, and the highest individual weight was obtained in the 4th week.

Age significantly affected amylase specific activity (P< 0.05), the lowest amylase activity observed on 3 dph (0.62± 0.15 u/mg protein) and followed by gradual increase (P<0.05) then decreased after 10 days. The activity of alpha-amylase again increased at the end of rearing period (P<0.05) (Fig. 2).

The specific activity of alkaline phosphatase (AP) significantly and abruptly increased until 8 dph, reaching maximum values of activity (116.7± 8.8 u/mg protein). After that, AP activity levels showed a general tendency to decrease until day 23 ph, between 13 and 23 dph wasn't observed any significant difference (P>0.05). Subsequently, AP activity gradually increased until the end of the study (Fig. 3).

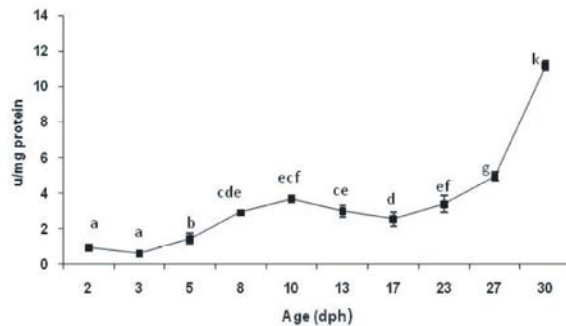Fig. 1: Growth curve of kutum larvae fed live feed during four weeks
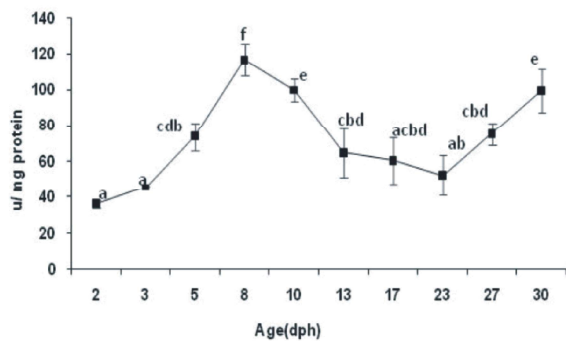

Fig. 2: The specific activity of amylase

Fig. 3: The specific activity of alkaline phosphatase

DISSCUTION

As it was shown figure.1 Kutum is gradually grew with time. The reduction in growth is probably due to stock at high densities and social interactions for food and space [14].In many cultured fish species, growth isinversely related to stocking density and this is mainlyattributed to social interactions [15-17]. Socialinteractions through competition for food and/or space can negatively affect fish growth.

The activity of amylase was found at 2 dph and after the onset of exogenous feeding the specific activity of α-amylase increased and slightly decreased in both treatments on 17 day. Highα-amylase activity values during the consumption of yolk reserves might be due to the presence of large glycogen deposits accumulated in the yolk sac in this group of chondrostean species [18]. The high activity of α-amylase during the larval stage in kutum might exhebit that dietary carbohydrates may fill the energy gap between endogenous and exogenous protein requirements in larvae as suggested by Tanaka [19]. In current study, it was observed a clear increase in α-amylase from 27 to 30 day. Also specific amylase activity in Indian carp (*Catlacatla*) showed a constant increase from day-20 onwards [20].

In addition, α-amylase activity also reflected the relatively high carbohydrate content (6–10%) in live preys [21] used for feeding larvae, which might have differentially stimulated the synthesis and secretion of α-amylase during larval development. In general, the amylase activity is higher in omnivorous fish than in carnivorous fish [22]. Kutum larvae exhibit a higher amylase activity in comparison to some carnivorous such as pike perch (*Sander lucioperca*) [23]. Amylase activity has been reported in various herbivorous and omnivorous species dueto need to break down polysaccharides into short-chain sugars [22].

Two groups of digestive enzymes are found in enterocytes: cytosolic enzymes (mainly peptidases) found in the cytoplasm, andbrush border membrane enzymes(alkaline phosphatase and aminopeptidase-N.), which are linked to the cell membrane [24]. The development of a functional intestine implies different maturational and morphological events that are very well preserved among vertebrates [25].

According to figure3, AP activity exhibited a same trend. In our study, at 8 dphAP activity reached theirs maximum value. For some speciesan earlier peak of activity was described for these enzymes, namely at 6 dphfor *D. dentex*[26] and at 9 dphfor *D. sargus*[27]. After 8 day the activity of APtend to decrease and from third week after hatching it increased again. It is found in a morphological study on kutum larvae, about 20 dph,as the yolk sac was completely exhausted, the gut folds were more developed [28]. 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 [29]. According to previous studies, this change has been reported to occur around the 3rd and 4th week after hatching in temperate marine teleost fish species. Also, in theperesent study, the AP activity in larvae fed live feed increased 23 days that it is possible to show development of brush border membrane.

CONCLUSION

Considering data of digestive enzymes from the α-amylase and alkaline phosphatase activity, kutum larvae could be progressively weaned around 8–10 dph. Considering the amylase activity, the present results suggest that the digestive tract of kutumis well adapted to carbohydrates digestion.

ACKNOWLEDGMENTS

REFERENCES

1. Khanipour, A.A and A.R. Vali pour, 2009. Kutum, Jewel of the Caspian Sea. Fisheries Iran, Tehran, pp: 84.

2. Furne, M., M. García-Gallego, M.C. Hidalgo, A.E. Morales, A. Domezain, J. Domezain and A. Sanz, 2008. Effect of starvation and refeeding on digestive enzyme activities in sturgeon (*Acipensernaccarii*) and trout (*Oncorhynchusmykiss*). Comp. Biochem. Physiol., 149: 420-425.

3. Verreth, J. and H. Segner, 1995. The impact of development on larval nutrition. In: Lavens, P., Jasper, E., Roelants, I. (Eds.), Larvi' 95, Fish and Shellfish Larviculture Symposium, Europe. Aquacult. Society, Special publication, 24, Ghent, Belgium.

4. Bolasina, S., A. Perez and Y. Yamashita, 2006. Digestive enzymes activity during ontogenetic developmentand effect of starvation in Japanese flounder, *Paralichthysolivaceus*. Aquaculture, 252: 503-515.

5. Rust, M.B., 2002. Nutritional Physiology in Fish Nutrition. In: Halver, J.E., Hardy, R.W. (Eds.), Academic Press, California, U.S.A, pp: 367-452.

6. Klein, S., S.M. Cohn and D.H. Alpers, 1998. The alimentary tract in nutrition. In: Shils,M.E., Olson, A.J., Shike, M., Ross, A.C. (Eds.), Modern Nutrition in Health and Disease, pp: 605-630.

7. Wahnon, R., U. Cogan and S. Mokady, 1992. Dietary fish oil modulates the alkaline phosphatase activity and not the fluidity of rat intestinal microvillus membrane. J. Nutr., 122: 1077-1084.

8. Nikawa, T., K. Rokutan, K. Nanba, K. Tokuoka, S. Teshima, M.J. Engle, D.H. Alpers and K. Kishi, 1998. Vitamin A up-regulates expression of bone-type alkaline phosphatase in rat small intestinal crypt cell line and fetal rat small intestine. J. Nutr, 128: 1869-1877.

9. Kolkovski, S., 2001. Digestive enzymes in fish larvae and juveniles- implications and applications to formulated diets. Aquaculture, 200: 181-201.

10. Cahu, C.L., J.L. ZamboninoInfante, A.M. Escaffre, P. Bergot and S. Kaushik, 1998. Preliminary results on sea bass (*Dicentrarchuslabrax*) larvae rearing with compound diet from first feeding,comparison with carp (*Cyprinuscarpio*) larvae. Aquaculture, 169: 1-7.

11. Bernfeld, P., 1951. Amylases α and β. In: Colowick, P., Kaplan, N.O. (Eds.), Methods in Enzymology. Academic Press, New York, pp: 149-157.

12. Worthington, C.C., 1991. Worthigton Enzyme Manual Related Biochemical, 3th Edition. Freehold, New Jersey.

13. Bessey, O.A., O.H. Lowry and M.J. Brock, 1946. Rapid coloric method for determination of alkaline phosphatase in five cubic millimeters of serum. J. Biol. Chem., 164: 321-329.

14. Imorou Toko I., E.D. Fiogb` and P. Kestemont, 2008. Determination of appropriate age and stocking density of vundalarvea, *Heterobranchuslongifilis* (Valenciennes 1840), at the weaning time. Aquaculture Research, 39: 24-32.

15. Holm, J.C., T. Refstie and S. Bo, 1990. The effect of fish density and feeding regimes on individual growth rate and mortality in rainbow trout (*Oncorhynchusmykiss*). Aquaculture, 89: 225-232.

16. Irwin, S., J.O. Halloran and R.O. Fitz Gerald, 1999. Stocking density, growth and growth variation injuvenile turbot (*Scophthalmusmaximus*). Aquaculture, 178: 77-88.

17. Ma, A., C. Chen, J. Lei, S. Chen, Z. Zhuang and Y. Wang, 2006. Turbot *Scophthalmusmaximus*: stockingdensity on growth, pigmentation and feed conversion.(Abstract) Chinese J. of Oceanology and Limnology, 24: 307-312.

18. Gisbert, E. and S.I. Doroshov, 2006. Allometric growth in green sturgeon larvae. J. Appl. Ichthyol., 22: 202-207.

19. Tanaka, M., 1973. Studies on the structure and function of the digestive system of teleost larvae. Ph.D. Dissertation, Kyoto University, Japan, pp: 136.

20. Rathore, R.M., S. Kumar and R. Chakrabarti, 2005. Digestive enzyme patterns and evaluation of protease classes in *Catlacatla* (Family: Cyprinidae) during early developmental stages. Comparative Biochemistry and Physiology, 142: 98-106.

21. Ma, H., C. Cahu, J.L. Zambonino-Infante, H. Yu, Q. Duan, M.M. Le Gall and K. Mai, 2005. Activities of selected digestive enzymes during larval development of large yellow croaker (*Pseudosciaenacrocea*). Aquaculture, 245: 239-248.

22. Hidalgo, M.C., E. Urea and A. Sanz, 1999. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. Aquaculture, 170: 267-283.

23. Hamza, N., M. Mhetli and P. Kestemont, 2007. Effects of weaning age and diets on ontogeny of digestive activities and structures of pikeperch (*Sander lucioperca*) larvae. Fish Physiol. Biochem., 33: 121-133.

24. Zambonino-Infante J.L. and C.L. Cahu, 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comparative Biochemistry and Physiology, 130: 477-487.

25. Henning, S.J., 1987. Functional development of the gastrointestinal tract, In: Johnson, L.R. (Ed.), Physiology of the Gastrointestinal Tract, 2nd edition. Raven Press, New York, pp: 285-300.

26. Gisbert, E., G. Giménez, I. Fernαndez, Y. Kotzamanis and A. Estévez, 2009. Development ofdigestive enzymes in common dentex, *Dentexdentex*, during early ontogeny.Aquaculture, 287: 381-387.

27. Cara, J.B., F.J. Moyano, S. Cαrdenas, C. Fernαndez-Dias and M. Yúferas, 2003. Assessment ofdigestive enzyme activities during larval development of white bream. J. Fish Biol., 63: 48-58.

28. Jafari, M., M.S. Kamarudin, C.H. Saad, A. Arshad, S. Oryan and M. Bahmani, 2009. Develop- ment of morphology inhatchery-reared *Rutilusfrisiikutum* larvae. Eur. J. Sci. Res., 38: 296-305.

29. Zambonino-Infante, J., E. Gisbert, C. Sarasquete, I. Navarro, J. Gutiérrez and C.L. Cahu, 2009. Ontogeny and physiology of the digestive system of marine fish larvae. In: Cyrino, J.E.O., Bureau, D., Kapoor, B.G. (Eds.), Feeding and Digestive Functions of Fish. Science Publishers, Inc, Enfield, USA, pp: 277-344.