

Evaluation of lysozyme, complement C3, and total protein in different developmental stages of Caspian kutum (*Rutilus frisii kutum* K.)

Razieh Abdollahi, Behrooz Heidari, Mahmoudreza Aghamaali

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Abstract. In this study, non-specific immune parameters in fertilized eggs, eyed embryos, larvae 10, 25, 50, 60, and 70 days post hatch (DPH), and female broodstock of Caspian kutum, *Rutilus frisii kutum* (Kamensky), were evaluated. The lysozyme activity, complement C3, and total protein levels were measured with the turbidimetric, immunoturbidimetric, and Bradford methods, respectively. The results showed that lysozyme levels decreased from levels noted in the fertilized eggs until the larvae were 10 days old. Subsequently, significant increases in lysozyme levels were observed until 70 DPH. An increasing trend of complement component C3 was noted from the levels in fertilized eggs to 10 DPH, following which it decreased significantly. Total protein levels differed significantly in early developmental stages of Caspian kutum. The higher values of complement component C3 than of lysozyme in the early life stages could be indicative of the former's more fundamental role.

Keywords: Caspian kutum, lysozyme, C3, development

Introduction

The eggs of most fishes are released and fertilized externally, which means that the resulting embryos and larvae are exposed to an aquatic environment full of potential pathogens (Zapata et al. 2006). At hatching, the immune system of fish larvae is still developing and lacks the functionality found in adults (Ellis 1999, Zapata et al. 1990). As a result, it is crucial for these early developmental stages to have protective immune mechanisms. They can be protected by both innate and adaptive immune substances transferred from the female broodstock to the eggs during vitellogenesis (Magnadóttir et al. 2005). Innate immune factors such as lysozyme (Yousif et al. 1991, 1994) and complement component C3 (Huttenhuis et al. 2006, Løvoll et al. 2007, Wang et al. 2008) are considered to play substantial roles in larval survival (Mulero et al. 2008, Chettri et al. 2012).

Lysozyme is an important defense molecule of the innate immune system mediating protection against microbial invasion. Lysozyme, also referred to as N-acetylmuramide glycanohydrolase or muramidase, is a well-studied bacteriolytic enzyme identified in a wide range of organisms including fish (Alexander and Ingram 1992). In the larval stage, lysozyme is one of the most important proteins involved in non-

R. Abdollahi, B. Heidari [✉], M. Aghamaali
Department of Biology, Faculty of Science, University of Guilan,
Rasht, Iran

B. Heidari
Caspian Sea Basin Research Center, Department of Marine Sciences,
University of Guilan, Rasht, Iran
email: Bheidari@guilan.ac.ir, Behrooz1072@yahoo.com

specific defenses at a very early stage (Takemura 1996) when the specific immunological response is not yet developed. Lysozyme has been detected in oocytes, fertilized eggs, and larval stages of several fish species (Kudo 1991, 1992, Brown et al. 1997).

The complement system is an important element of both the innate and adaptive immune systems (Lange et al. 2004a, b), and it participates in several defense mechanisms such as the formation of the membrane attack complex, opsonization, and the development of antibodies (Lambris et al. 1993, Mauri et al. 2011). The third complement component (C3) is a key protein of the complement system and a major humoral factor in host defense (Nakao et al. 2004). Complement component C3 is synthesized in the liver and is also expressed in other tissues such as gills, the intestine, and the skin (Engstad et al. 1992, Mauri et al. 2011). Complement component C3 was studied in Atlantic halibut, *Hippoglossus hippoglossus* (L.) larvae (Hermannsdottir et al. 2009, Lange et al. 2004a) and Atlantic cod, *Gadus morhua* L. larvae (Lange et al. 2004b), in the eggs and embryos of zebrafish, *Danio rerio* (Hamilton) (Wang et al. 2009), and in spotted wolffish, *Anarhichas minor* Olafsen (Ellingsen et al. 2005).

Caspian kutum, *Rutilus frisii kutum* (Kamensky), is an endemic species distributed in the southern Caspian Sea and the rivers that flow into it (Heidari et al. 2010). This species is one of the most valued fishes on the Iranian Caspian Sea coast (Holčík 1995). Kutum becomes sexually mature at an age of 3-4 years, and the size of mature individuals ranges from 25 to 58 cm (Bani and Vayghan 2011). The Caspian kutum is a migratory, anadromous fish that spawns on aquatic weeds, gravel and sandy substrates in rivers, and in lagoons from March to April (Gharache et al. 2013). The larvae hatch in 8.5-9 days post-fertilization at 14-16°C (Jafari et al. 2010). With the onset of exogenous feeding on the tenth day, the yolk sac is mostly absorbed and all tissues and organs are formed 10 days after hatching (Jafari et al. 2009).

Increasing fish resistance to diseases, especially in the early developmental stages of the Caspian kutum, through the detailed study of the immunity

parameters can be helpful in maintaining stock resources. Hence, the aim of the present study was to investigate variations in lysozyme activity and complement component C3 levels in the Caspian kutum from fertilized eggs to larvae (from the age of 10 to 70 days post hatch (DPH)) and in female broodstock.

Material and Methods

Sampling

Fertilized eggs (1 hour after fertilization) and eyed embryos (6 days after fertilization) were obtained from Shahid Ansari Farming Center in April and transported alive to the Marine Biology Laboratory at the University of Guilan. After unhealthy fertilized eggs and eyed embryos had been removed, the healthy ones were rinsed three times with sterile phosphate-buffered saline (PBS) at pH 7.2. The eggs were immediately homogenized with PBS (weight:volume 1:10) for 1 min and centrifuged at 6000 rpm for 20 min at 4°C. The supernatant was pooled, aliquoted, and stored at -70°C until the parameters were analyzed. Caspian kutum larvae (10, 25, 50, 60 and 70 DPH) (n = 150 at each larval stages) were obtained from Shahid Ansari Farming Center during in April, May, June, July, and August, respectively. The samples were fed twice daily with a feeding powder purchased from Isfahan Mokammel Co. (Isfahan, Iran). Whole bodied samples were immediately homogenized with PBS for 1 min, and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) was added, and then it was centrifuged at 6000 rpm for 20 min at 4°C, and finally the supernatant was frozen at -70°C until analysis. The Caspian kutum female broodstock (n = 5) was provided by the Shahid Ansari Farming Center in April, and the fish were bled from the caudal vein with a heparinized syringe. After centrifuging, the plasma was frozen until assays of lysozyme and C3.

Lysozyme assay

The lysozyme activity of the samples (the plasma from the broodstock and the prepared egg and larval

homogenates) were measured using a method based on the ability of lysozyme to lyse the bacterium *Micrococcus lysodeikticus* (Ellis 1999). Quantities of 100 μl of the samples were mixed with 200 μl of a 0.2 mg ml^{-1} suspension of *M. lysodeikticus* (Sigma-Aldrich, St. Louis, MO, USA) in a phosphate buffer (pH 6.2, M 0.1). Optical density was read at 450 nm at 0, 15, 30, and 60 min. The lysozyme values are expressed as U mg^{-1} total protein.

Complement component C3 assay

The method of immunoturbidimetry with separated Eastbiopharm ELISA kits (Hangzhou Eastbiopharm CO., LTD., Torrance, USA) was used in the assay for complement component C3 levels. The kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of complement component C3 in the samples. Complement component C3 was added to a monoclonal antibody enzyme well which was pre-coated with a fish complement component C3 monoclonal antibody. This was incubated, and then complement component C3 antibodies labeled with biotin were added and combined with Streptavidin-HRP to form an immune complex. Then the material was incubated and washed again to remove the uncombined enzyme. Then, chromogen solution A, B, was added, and the color of the liquid changed to the blue, and from the effect of sulfuric acid (M 2), the color finally became yellow. The optical density (OD) value was measured with an ELISA reader at 450 nm. The C3 values are expressed as mg ml^{-1} .

Total protein assay

A modification of the Bradford method (Bradford 1976) was used to measure total protein using Coomassie Brilliant blue G-250 as the protein indicator. The optical density was read at 595 nm for 10 μl aliquots of the diluted homogenate using bovine serum albumin as the standard. The total protein values are expressed as mg ml^{-1} .

Statistical analysis

The raw data on the lysozyme and total protein of samples were initially tested assuming normality and homogeneity of variance. In cases in which the assumptions were met, the data were analyzed in the fertilized eggs, eyed embryos, and larvae 10, 25, 50, 60, 70 DPH with one-way analysis of variance (ANOVA) in SPSS (Version 20, IBM). The Duncan post hoc test was used to identify significant differences among the various means at a confidence level of 95%. All experiments were performed in triplicate, and the values were expressed as means \pm SD.

Results

Lysozyme

There was a significant difference in lysozyme activities among various developmental stages of Caspian kutum ($P < 0.05$) (Fig. 1). The lysozyme levels from the fertilized egg to 10 DPH stages decreased ($P < 0.05$), and after this there was a significant increase in the level until 70 DPH ($P < 0.05$). There was a significant reduction between the lysozyme of the female broodstock ($13.21 \pm 2.31 \text{U mg}^{-1}$) and larvae 70 DPH ($P < 0.05$).

Complement component C3

A significant difference in complement component C3 levels was observed during various early life stages of Caspian kutum. Complement component C3 levels showed a significant increment from fertilized egg to 10 DPH ($P < 0.05$), so that the highest value of C3 was found on day 10 post hatch (0.673 ± 0.02). Then, decreasing levels of C3 were observed until the end of the experimental period ($P < 0.05$) (Fig. 2). Compared to 10 DPH, there were no significant variations in C3 from larvae 50 to 70 DPH and plasma C3 of the female broodstock ($0.4 \pm 0.02 \text{mg ml}^{-1}$) ($P > 0.05$).

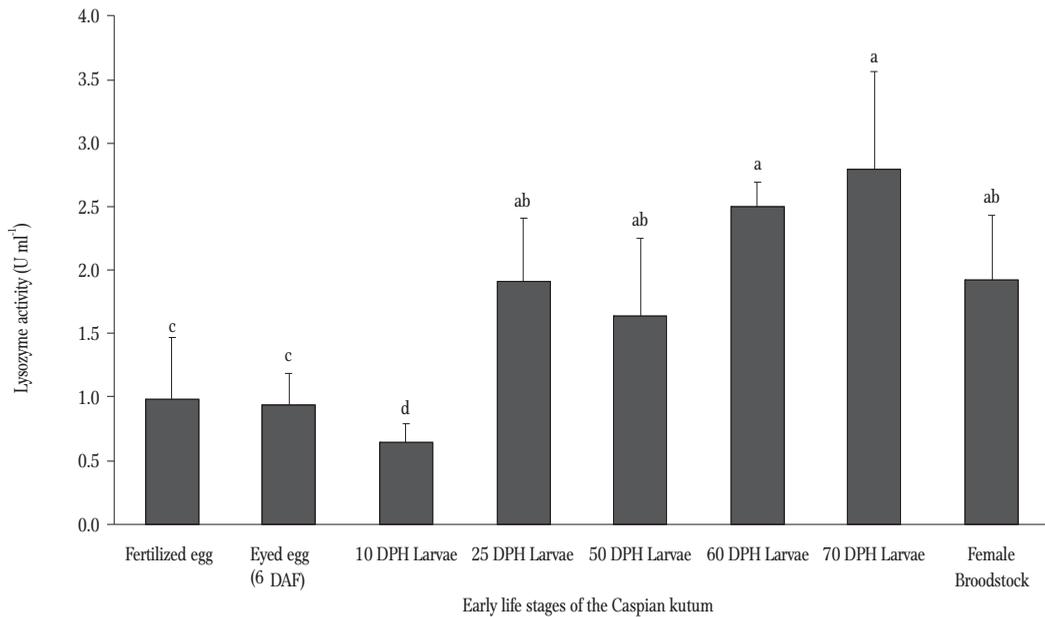


Figure 1. Comparison of lysozyme levels in early life stages (eggs and larvae) of Caspian kutum. Statistical significance determined at $P < 0.05$.

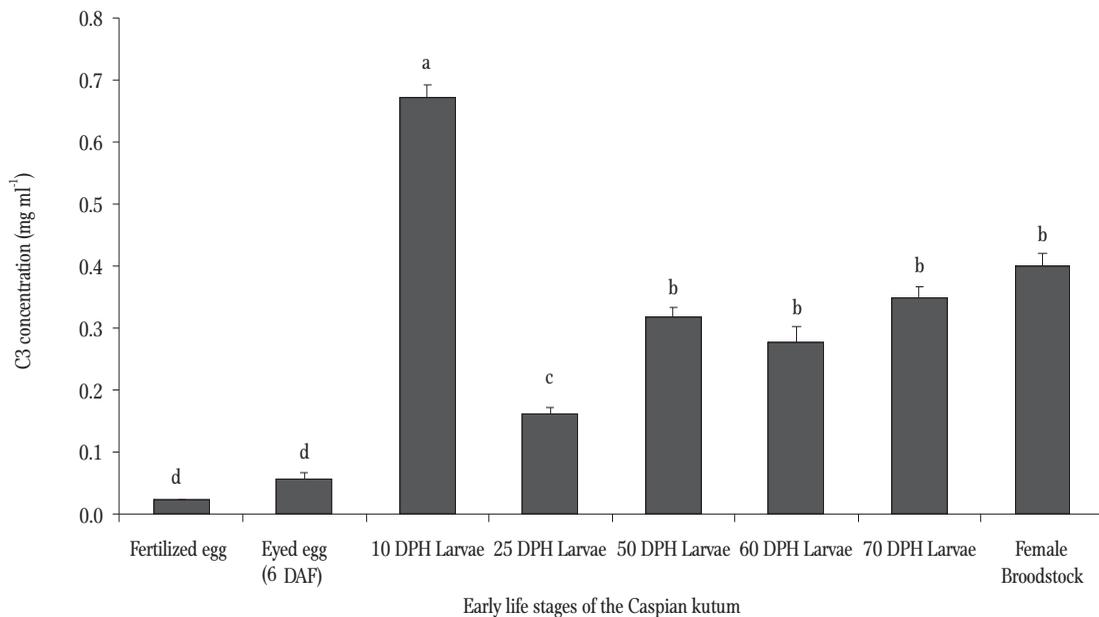


Figure 2. Comparison of complement component C3 levels in early life stages (eggs and larvae) of Caspian kutum. Statistical significance determined at $P < 0.05$.

Total protein

Significant differences in total protein levels were noted in the early life stages of Caspian kutum ($P < 0.05$). There was a significant increment in the total protein levels from fertilized egg to larvae 25 DPH ($P < 0.05$) (Fig. 3). Afterwards, there were significant fluctuations

in the total protein levels in larvae after 25 DPH to 70 DPH, so that a remarkable decreasing trend was eventually observed in larvae 60 DPH ($P < 0.05$). A significant reduction in the plasma total protein in female broodstock ($54.79 \pm 2.31 \text{ mg ml}^{-1}$) compared to larval stages of Caspian kutum was observed ($P < 0.05$).

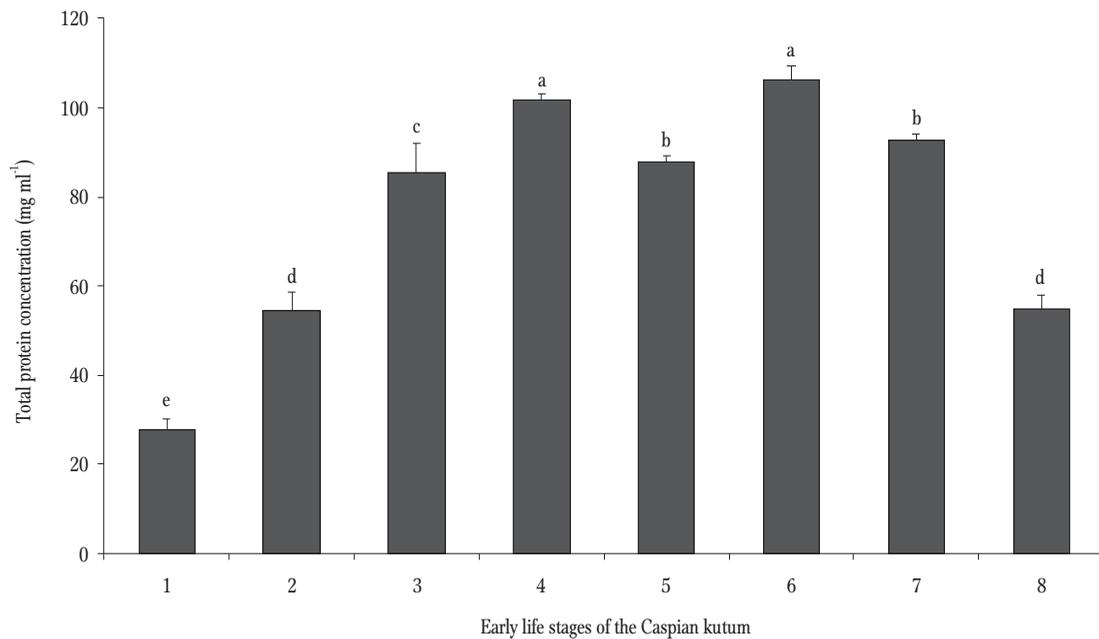


Figure 3. Comparison of total protein in early life stages (eggs and larvae) of Caspian kutum. Statistical significance determined at $P < 0.05$.

Discussion

Fish larvae must have defensive factors that play anti-infective roles before their own defense mechanisms are fully developed if they are to survive attacks by pathogens. Proteins involved in both the innate and adaptive immune responses of fish, which are transferred maternally, are such factors (Mulero et al. 2007).

Since the immune system of eggs and larvae have not completely developed, they need defensive factors such as lysozyme and complement component C3, both of which are involved in the innate and adaptive immune responses of fish. It is likely that the cooperation of lysozyme and C3 in the defense against pathogens does occur in early developmental stages of fishes (Wang and Zhang 2010).

Lysozyme has been detected in oocytes, fertilized eggs, and larval stages of several fish species, for example, in the eggs of coho salmon, *Oncorhynchus kisutch* (Walbaum), (Yousif et al. 1991) and other salmonids (Yousif et al. 1994), sea bass, *Dicentrarchus labrax* (L.), (Cecchini et al. 2000), tilapia, *Oreochromis mossambicus* (Peters), (Takemura and Takano 1995) and other species.

Lysozyme in eggs has been shown to play a role in the prevention of mother to progeny (vertical) transmission of some bacterial pathogens like *Aeromonas salmonicida* (Magnadóttir et al. 2005). A study of the lysozyme activity in developmental stages of sea bass showed higher lysozyme activity in newly fertilized eggs than in embryos and larvae 144 h old (Cecchini et al. 2000). In the present study, the lysozyme activity decreased from fertilized eggs to larvae 10 DPH in Caspian kutum. The high level of lysozyme in fertilized eggs and eyed embryos could indicate the maternal transfer of this enzyme during vitellogenesis. The decreasing trend of lysozyme levels in fertilized eggs, eyed embryos, and larvae 10 DPH larvae could be attributed to yolk absorption, which is complete at 10 DPH (Jafari et al. 2009). Takemura (1993) reported a similar decrease in maternally-derived immunoglobulin proteins in larval stages of tilapia, *O. mossambicus*, as the yolk was absorbed. After day 10, the lysozyme levels of Caspian kutum showed an increasing level in larvae older than 10 days until day 60. It seems that the reason behind increased levels of lysozyme after day 10 was the development of various organs and tissues such as the kidney, liver, spleen, alimentary tract, and gills (Jafari et al. 2009),

which play significant roles in the production of lysozyme.

The presence of C3 has been demonstrated in the eggs and embryos of *D. rerio* (Wang et al. 2009) and *A. minor* (Uribe et al. 2011). In *H. hippoglossus*, C3 was detected in larvae from days 5 to 99 after hatch (Hermannsdottir et al. 2009, Magnadóttir et al. 2005). A growing body of evidence suggests that the C3 complement might play important roles in diverse biological processes ranging from early hematopoiesis to skeletal and vascular development and normal reproduction (Rooney et al. 1993, Andrades et al. 1996, Lange et al. 2004a, b). In addition, earlier findings (Lange et al. 2004a, b) suggest that complement component C3 could play a role in the generation of cells and tissues in the early developmental stages of this fish.

In Caspian kutum, our results showed a significant increment of complement component C3 from fertilized eggs to larvae 10 DPH, so that on day 10 PH the highest levels was observed. The presence of complement component C3 in the eggs and larvae of Caspian kutum could be indicative of its defensive role against pathogens in larval stages. In addition, the high concentration of C3 in larvae compared to that in the eggs and embryos of Caspian kutum is related to the development of the adaptive immune system in fish. With regards to the diverse biological processes of complement component C3, the highest levels of C3 in larvae 10 DPH probably refers to the generation of cells and tissues at this stage of larval development. It is reported that on day 10 after hatch, the organogenesis process of the Caspian kutum larvae is completed and they started exogenous feeding (Jafari et al. 2010). In larvae older than 10 days, complement component C3 decreased, which is likely attributed to the completion of tissue generation.

Apart from lysozyme and C3, there are the other antimicrobial proteins in the non-specific immune system. Accordingly, in the present study, the total protein of the early developmental stages of Caspian kutum was measured. It was observed that total protein increased significantly with increasing age in the embryonic and larval stages of Caspian kutum. In

fertilized eggs and eyed eggs, total protein levels could come from yolk proteins that are transferred from the mother. After these stages, the yolk sac was absorbed, and the larvae began exogenous feeding. It seems that an increment of the total protein level in the larvae 10 DPH and later larval stages is related to the development of the digestive organs and immune competence including enzymes and the other immune proteins.

In conclusion, the lowest and highest values of the lysozyme and complement component C3 were recorded in larvae 10 DPH, which is the moment of the transitional period between endogenous and exogenous feeding in Caspian kutum. Attention to this larval stage could play a key role in the more efficient culture of Caspian kutum fry.

Author contributions. B.H. designed the experiment and methods; R.A. and M.A. performed the experiment procedures and analyzed the data; R.A. wrote and B.H. corrected the manuscript.

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