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RESEARCH PAPER

Variations of Immune Parameters in the Early Life Stages and the Female Broodstock of the Cultured Persian Sturgeon, *Acipenser persicus* (Borodin 1897) and the Sterlet, *Acipenser ruthenus* (Linnaeus, 1758)

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Abstract

In this study, some important immune parameters in the unfertilized and fertilized eggs, larvae of 10 days post hatching (DPH), juveniles of 1 to 12 month post fertilization (MPF) and the female broodstock were evaluated. In the Persian sturgeon, there was a significant decrement in the lysozyme activity from the unfertilized eggs to the 10 DPH larvae and after that, the remarkable variations were not observed. The C3 concentration did not show any significant variations from the unfertilized eggs to the 1 MPF and after that, it significantly decreased. Apart from the fertilized eggs, the variations of the IgM level were similar to the C3 level in the early life stages of the Persian sturgeon. In the Sterlet, the lysozyme level was decreased significantly from the unfertilized eggs to 10 DPH larvae, then it was increased in 1 MPF and finally, a significant difference was not observed from 2 to 12 MPF. The variations of both the IgM and the C3 levels were similar from the fertilized eggs to 12 MPF. In conclusion, the considerable fluctuations of the immune parameters were observed in early life stages especially under 1 MPF which indicates immunologically sensitivity of the fish in these stages.

Keywords: Sturgeon, IgM, C3, lysozyme, immunity.

Introduction

The sturgeons (family: Acipenseridae) are an ancient group of chondrostean fishes which are considered to be 'living fossils' appeared approximately 150-200 million years ago (Bemis, Findeis, & Grande, 1997). The anadromous and potamodromous sturgeons are 28 species, six of which inhabit the Caspian Sea basin (Bahmani, Kazemi, & Donskaya, 2001) including the Persian sturgeon, *Acipenser persicus* Borodin, 1897 and the Sterlet, *Acipenser ruthenus* Linnaeus, 1758. The Sterlet is a freshwater species that is the smallest species of sturgeon. The large population of Sterlet has decreased, but local populations are still living in most parts of rivers draining to Black, Azov and Caspian Seas; Siberia from Ob eastward to Yenisei drainages. The catch statistics in the Russian Federation show that the Sterlet catch has declined by nearly 40% between 1990 (116 tones) and 1996 (80.6 tones) (Gesner, Freyhof, & Kottelat, 2010a).

The Persian sturgeon is a native species of the Caspian Sea, but it mainly inhabits in the southern Caspian along the Iranian coast (Moghim, Vajhi, Veshkini, & Masoudifard, 2002). In recent years, the Persian sturgeon contains the largest ratio of the total Iranian commercial catch (Moghim, Kor, Tavakolieshkalak, & Khoshghalb, 2006). Unfortunately, the Persian sturgeon and the Sterlet are critically endangered and vulnerable, respectively (Gesner *et al*., 2010a; Gesner, Freyof, & Kottelat, 2010b) due to over harvesting, illegal praying, spawning grounds loss and essential chemical pollution (Ivanov, Vlasenko, Khodorevskaya, & Raspopov, 1999).

The larval culture is one of the most sensitive stages in intensive sturgeon farming, which is often accompanied with a high mortality rate during and after yolk sac absorption (Mohler, King, & Farrell, 2000). For increasing the survival rate, it is necessary to study the potential factors affecting the immunity of the eggs and larvae in the fish. So, substantial efforts have been focused on determination of the most sensitive early life stages immunologically in order to understand how to increase their survivorship and hatchery efficiency.

Immune system exists in the animals and plants to protect against a variety of microorganisms (Janeway & Medzhitov, 2002). The immune system is divided into non-specific (innate) and adaptive which includes cellular and humoral factors.

In comparison to the higher vertebrates, the fish as free-living organisms depend on their innate immune system for survival from early life stages

(Rombout, Huttenhuis, Picchietti, & Scapigliati, 2005). Non-specific immunity is a fundamental defense mechanism that plays a key role in the acquired immune response in fish (Uribe, Folch, Enriquez, & Moran, 2011).

Lysozyme is an important innate defense parameter and is widely distributed in the invertebrates and vertebrates (Saurabh & Sahoo, 2008). The lysozyme is a bactericidal enzyme, involved in the hydrolysis of the b-(1, 4) linked glycoside bonds of bacterial cell wall peptidoglycans. This parameter has been found in the mucus, lymphoid tissue, serum of the most fish (Saurabh & Sahoo, 2008) as well as the oocytes, fertilized eggs and larval stages of several fish species including eggs of Coho salmon (*Oncorhynchus kisutch*), sea bass (*Dicentrarchus labrax*) and tilapia (*Oreochromis niloticus*) (Yousif, Albright, & Evelyn, 1991).

In the vertebrates, the complement system not only helps to the pathogen deleting, but also serves as a bridge between the non-specific and required response. In fish like the other vertebrates, complement system is triggered through three pathways entitled 1- the classical, 2- alternative and 3- lectin pathways (Claire, Holland, & Lambris, 2002). The C3 is a central protein of the complement system (Magnadottir, Lange, Gudmundsdottir, Bogwald, & Dalmo, 2005) that have a major role in activation of both the classical and alternative complement system. The maternal transfer of the C3 to the eggs have been reported in the spotted wolffish (*Anarhichas minor*) (Ellingsen, Strand, Monsen, Bogwald, & Dalmo, 2005).

Immunoglobulins (Ig) are the primary humoral components of the acquired immune system (Magnadottir *et al*., 2005). The IgM is the most important type of antibodies and is known as the first appeared Ig on the early life stages of the fish (Boes, 2000). The IgM has been recorded in the eggs of several fish species including carp (*Cyprinus carpio*) (Suzuki, Orito, Furukawa, & Aida, 1994), sea bass (Picchietti *et al*., 2004) and rainbow trout (*Oncorhynchus mykiss*) (Castillo, Sanchez, Dimiguez, Kaattari, & Villena, 1993). Hence the objective of this work is to study the variations of the immune parameters including lysozyme, IgM and C3 among unfertilized and fertilized eggs, larvae and juveniles as well as the female broodstock in the Persian sturgeon and the Sterlet.

Material and Methods

Animals

The animals examined in the present study were produced by artificial propagation of the broodstocks at the Shahid Dr. Beheshti Sturgeon Fish Propagating and Rearing Complex, Rasht, Guilan.

Broodstock and Eggs

The season of the sturgeon breeding and fingerling production is restricted to the period from March to July in the hatcheries of the southern Caspian Sea. Hence, the broodstocks of the Persian sturgeon and the Sterlet (n=5) were collected from the breeding ponds (diameter 5 m and water depth 1.3 m). For blooding and biometry, the female broodstock were anesthetized by the clove oil (150-200 ppm) for 10 min. The blood samples were taken from the caudal vein by 5 CC heparinized disposable syringe with 23G needle and then centrifuged at 906 rcf for 10 min at 4°C and finally the plasma was frozen at –70°C until the parameter analysis. The length and weight of the broodstocks were measured by a tape meter and a digital scale, respectively.

For preparing the fertilization, the broodstocks were injected by LHRH-A2 (4 mg per kg of body weight) in two times. Based on the position of the germinal vesicle (GV), the ripe eggs were collected by cutting the end of the oviduct of the broodstock with a specially deviced surgical scalpel (Bani & Banan, 2010). A certain volume of the unfertilized eggs (5 g per individual; n=5) were separated for the immune parameter analysis and the others were fertilized with the semi-dry method by adding the sperms. After 24 hours post fertilization (hpf), a certain amount of the fertilized eggs (5 g per individual; n=5) was collected for the parameter analysis.

After transferring the sampled unfertilized and fertilized eggs to the Marine Biology Laboratory at the University of Guilan, they were rinsed three times with sterile phosphate-buffered saline (PBS; pH 7.2). Then, after biometry (measuring the diameter and weight of egg by the coulisse and a digital scale, respectively), the eggs were immediately homogenized with PBS (W1:V10) for 1 min and centrifuged at 3622 rcf for 20 min at 4°C. The supernatant was pooled, aliquoted and stored at –70°C until analysis of the immune parameters.

Larvae and Juveniles

The fertilized eggs of the Persian sturgeon and the Sterlet were maintained in the incubators (water volume 3 L; water depth 15 cm). After hatching the eggs (after 96 h), the larvae having a yolk sac were transported to the fiberglass vase (water volume 3.5 L; water depth 20 cm). After yolk absorption (9-10 dpf), these larvae were fed by the artemia, daphnia, chironomid larvae and concentrated pellets 4 times per day by 15-20% of body weight.

The larvae and juveniles with an age of 2 weeks post fertilization (WPF) and 1, 2, 4, 8, 12-month post fertilization (MPF) were randomly collected (n=5 at each stage) from the rearing tanks and frozen immediately at –70°C until analysis of the immune parameters. After biometry of the samples, the whole

body was homogenized with PBS (pH 7.2, 0.1 M) (W1:V10) for 1 min and then centrifuged at 3622 rcf for 20 min at 4°C and finally the supernatant was frozen at –70°C until analysis.

The water-quality parameters including temperature (17-20°C), dissolved oxygen (5.5-7 mg/L), ammonia (0.1±0.001 mg L^{-1}) and pH (7.8±0.4) were monitored during the experiment.

Lysozyme Activity

The lysozyme activity of the samples (supernatant of the homogenized samples and the broodstock plasma) was measured using a turbidimetric method based on the ability of lysozyme to lyses the bacterium, *Micrococcus luteus* (Ellis, 1990) with some modifications. The 100 µl of samples were mixed with 300 µl of a 0.2 mg mL^{-1} suspension of *Micrococcus luteus* (Sigma-Aldrich, St. Louis, MO, USA) in 200 µl phosphate of 0.1M PBS, pH 6.2. Optical density (OD) was read in 450 nm every 15 second to 180 second by a spectrophotometer (Ultraspect 3000, Pharmacia Biotech). One unit of the lysozyme activity was equal to a decrement in turbidity of 0.001 per minute at 450 nm at pH 6.2 and 25°C. The lysozyme activity was obtained using the following formula *(Chakraborti et al*., 2010).

$$\text{Lysozyme Activity (Units mg}^{-1}) = \Delta OD \times Df / (\min \times 0.001 \times V_S)$$

ΔOD= decrease of optical density
Df= dilution factor
V_S= enzyme volume
0.001= one unit of lysozyme activity

The lysozyme activity expressed as U per milligram (U mg^{-1}) of total protein, which was measured according to the Bradford method (Bradford, 1976).

Complement Component C3 Assay

The concentration of C3 was measured using a sandwich ELISA (enzyme-linked immunosorbent assay) with the Fish ELISA kit (HANGZHOU EASTBIOPHARM CO., LTD.). Briefly, the 40 µl of samples was added to the well, which was pre-coated with Fish complement component C3 monoclonal antibody. Then, 10 µl of the C3 antibodies labeled with biotin was added and combined with 50 µl of Streptavidin-HRP to form the immune complexes after incubation at temperature 37°C for 1 h. After washing to remove the uncombined enzyme, 50 µl of Chromogen Solution A and B was added and incubated at temperature 37°C for 10 min. The color of liquid changed into blue and finally under the effect of the stopping solution (50 µl of sulfuric acid), the color became yellow. The OD was measured using an ELISA reader (ELX800 Absorbance Reader, BioTek, USA) at 450 nm. According to the standard concentration and the corresponding OD values, the standard curve linear regression equation was calculated out and then applied the OD values of the samples on the regression equation to calculate the corresponding sample concentrations. The blank well (containing the only Chromogen Solution A and B and stop solution) and standard well (containing only Streptavidin-HRP) were used as a negative and positive controls, respectively. Inter- and intra-assay coefficients of variation (CV) were <12% and <10%, respectively. The C3 values expressed as mg dL^{-1}.

IgM assay

The IgM like the complement component C3 was measured with a separated Eastbiopharm ELISA kit based on a sandwich ELISA method.

Statistical Analysis

The normality test (Kolmogorov–Smirnov) was initially done on the raw data of the lysozyme, IgM and complement component C3 of the samples. In cases that the normal distribution was found, the data in different life stages (the unfertilized and fertilized eggs, 10 DPH and 1, 2, 4, 8, 12 MPF) were analyzed by 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 with a confidence level of 95%. All the values were expressed as a Mean±SD.

Results

The biometric data of the Persian sturgeon and the Sterlet at different life stages as well as broodstock were presented in Table 1.

Lysozyme

There were the significant variations of the lysozyme activity during early life stages of the Persian sturgeon ($P<0.05$). The lysozyme activity was significantly decreased from the unfertilized eggs to the 10 DPH larvae ($P<0.05$) (Figure 1). Then, a significant increment was observed until 1 MPF ($P<0.05$) and finally, the juveniles older than 1 MPF did not show any significant variations until 12 MPF ($P>0.05$) (Figure 1). In general, the lowest and the highest activity of the lysozyme level was observed in 10 DPH (45.02±12.39 U mg^{-1}) and unfertilized eggs (212.68±17.46 U mg^{-1}), respectively. In addition, there was a remarkable increment of the lysozyme activity in the female broodstock (207.27±49.048) rather than the 12M old juvenile (86.20±14.00) ($P<0.05$).

In the Sterlet (*Acipenser ruthenus*), the lysozyme activity was significantly decreased from the unfertilized eggs to 10 DPH ($P<0.05$) (Figure 1). The

Table 1. The biometric data of the Persian sturgeon and the Sterlet sampled at different life stages

Sampling time	Samples	Weight (g)		Length (mm)	
		A. persicus	*A. ruthenus*	*A. persicus*	*A. ruthenus*
5-Apr-14	Unfertilized eggs	0.02±0.00	0.00 ±0.01	3.2±0.25	0.2 ± 2.1
6-Apr-14	Fertilized eggs	0.03±0.00	0.00 ± 0.01	3.7±0.1	0.2 ± 1.5
20-Apr-14	10 DPH*	1.69±0.12	0.00 ± 0.11	18±0.7	0.35 ± 17.96
6-May-14	1 MPF#	2.37±0.14	0.69 ± 2.64	57±1.52	80.00 ± 5.00
6-Jun-14	2 MPF	4.27±0.41	0.88 ± 6.47	91±0.1.52	123.3 ±5.77
6-Aug-14	4 MPF	12.1±0.27	1.05 ± 16.6	148.3±0.62	173.33 ±7.63
7-Dec-14	8 MPF	248±5.56	7.67 ± 46.86	391±4.16	241.66 ± 7.63
6-Apr-15	12 MPF	386±7.28	22.21 ± 71.03	456±6.082	277.09 ±14.54
16-Mar-15	Broodstock fish	30000±99.73	875.66 ± 0.57	1490±769.4	383.30 ±15.27

* Days Post Hatching
#Month Post Fertilization

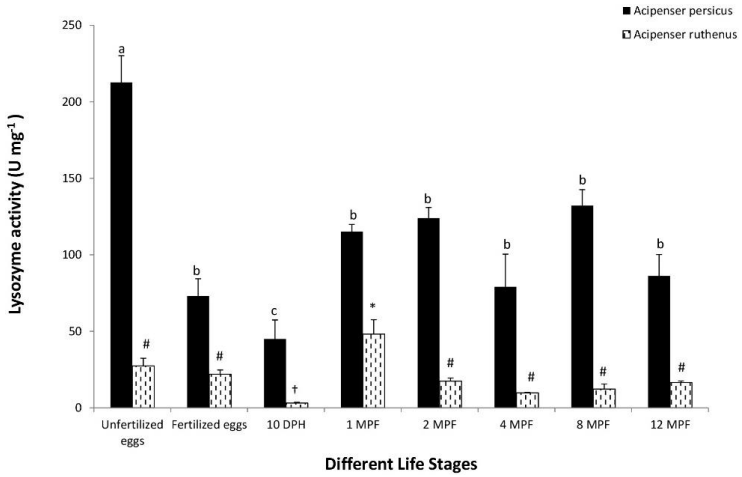

Figure 1. Variations of the lysozyme activity in the Persian sturgeon and the Sterlet from the unfertilized eggs to 12 MPF. Data are presented as mean±SD (n=5). Mean values bearing different superscripts are significantly different (P<0.05).

highest activity of the lysozyme was observed in the 1 MPF juvenile (48.15±9.41 U mg^{-1}). The significant changes of the lysozyme activity were not recorded from the juveniles with age of 2-12 MPF to the female broodstock, (P>0.05).

Complement Component C3

No significant differences were found between the complement component C3 concentration from the unfertilized eggs to the 1M old juvenile of the Persian sturgeon (P>0.05) (Figure 2). The decreasing changes of the C3 level was significantly observed in the juveniles with an age of 1 to 12 MPF (P<0.05) (Figure 2). There were no significant variations between the C3 concentration in 12 M old juvenile (14.00±2.00 mg dL^{-1}) and the female broodstock (16.1±3.85 mg dL^{-1}) (P>0.05).

From the unfertilized eggs to the 1 MPF juvenile, there was a decreasing change in the complement component C3 of the Sterlet (P<0.05) (Figure 2). The highest concentration of the C3 was significantly measured in the 2M old juvenile (125±13.23 mg dL^{-1}). The remarkable changes of the C3 were not observed between the juveniles with an age with 4 to 12 MPF and the female broodstock of the Sterlet (P>0.05).

Immunoglobulin M (IgM)

From the unfertilized eggs to the fertilized eggs of the Persian sturgeon, the IgM level showed a significant decrement (P<0.05) (Figure 3). Then, there were a remarkable increment of the IgM concentration from the fertilized eggs to the 1M old juvenile (P<0.05) and finally the significant decreasing changes was recorded in the juveniles from 1 to 12 MPF (P<0.05) (Figure 3). The significant variations were not observed between the C3 concentration in the 12M old juvenile (6.06±1.17) and the female broodstock (4.60±0.70) (P>0.05).

In the Sterlet, a significant variation of the IgM level was observed from the unfertilized eggs to the fertilized eggs (P<0.05) (Figure 3). The decreasing changes of the IgM level were significantly recorded from the fertilized eggs to the 1 MPF old juvenile

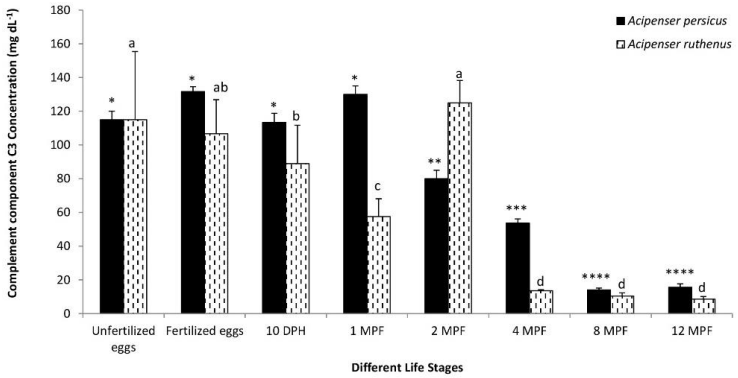

Figure 2. Variations of the Complement component C3 concentration in the Persian sturgeon and the Sterlet from the unfertilized eggs to 12 MPF. Data are presented as mean±SD (n=5). Mean values bearing different superscripts are significantly different (P<0.05).

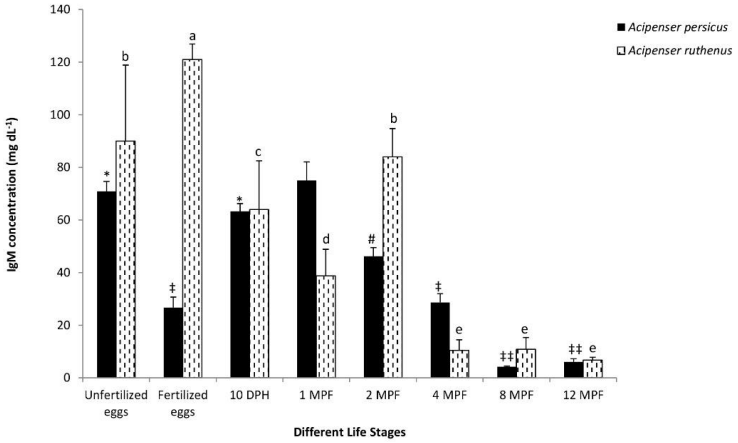

Figure 3. Variations of the IgM concentration in the Persian sturgeon and the Sterlet from the unfertilized eggs to 12 MPF. Data are presented as mean±SD (n=5). Mean values bearing different superscripts are significantly different (P<0.05).

(P<0.05). After a significant increment of the IgM level in the juvenile with an age 2 MPF (P<0.05), no the remarkable changes were found from the 4 MPF to 12 MPF juveniles (P>0.05) (Figure 3). There were no significant variations of the IgM level between 12 MPF juvenile (6.73±1.08) and the female broodstock (12±1.41 mg dL^{-1}) (P>0.05). In addition, the mean concentration of C3 from unfertilized eggs to 12 MPF juvenile was significantly higher than the IgM in both sturgeon (P<0.05) (Figure 4).

Discussion

The fish eggs are often released into the water and fertilized externally, and the resulting embryos and larvae are therefore exposed to an aquatic ecosystem full of potential pathogens. The immune system in the fish embryos and newly hatched larvae is not fully developed; their immunological capacity is very limited at these stages (Ellis, 1988) and has been little bounded ability to synthesize endogenously immune-relevant molecules and their lymphoid organs are not yet fully matured (Zapata, Diez, Cejalvo, Frias, & Cortes, 2006).

Although, the variations of the immune parameters such as the lysozyme, C3 and IgM in the larval stages of some teleost species have been studied, there is no such information for the sturgeons. The existence of maternal lysozyme has recently been confirmed in the oocytes, fertilized eggs and larval stages of Coho salmon (*Oncorhynchus kisutch*) (Yousif, Albright, & Evelyn, 1994), red sea bream (*Pagrus major*) (Kanlis, Suzuki, Tauchi, Numata, & Shirojo, 1995) and tilapia (*Oreochromis niloticus*) (Sin, Ling, & Lam, 1994). It had been shown that the lysozyme in the unfertilized eggs had higher level than the fertilized eggs (1 HPF) and 6 DPH larvae (Cecchini, Terova, Caricato, & Saroglia, 2000). A similar reduction in the lysozyme level was observed in the tilapia larvae after absorbing the yolk sac and starting first feeding (Takemura, 1993). In the Persian sturgeon (*Acipenser persicus*) and the Sterlet (*A. ruthenus*), the lysozyme activity was decreased significantly from the unfertilized eggs to the 10 DPH larvae, which it may be because of complete absorption of the yolk proteins by the embryo and larvae. It has been reported that the yolk sac in the Atlantic sturgeon disappears during the feeding of the environment, 14 days after hatching (Gradil, Wright,

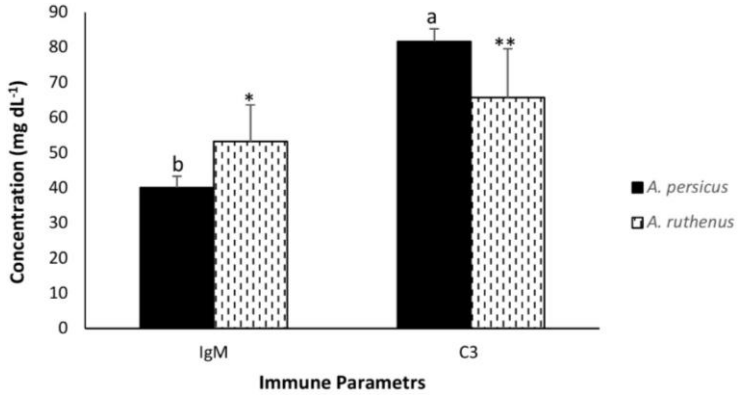

Figure 4. The mean concentration of C3 and IgM from unfertilized eggs to 12 MPF juvenile in the Persian sturgeon and the Sterlet. Data are presented as mean±SD (n=5). Mean values bearing different superscripts are significantly different ($P<0.05$).

Wadowska, & Fast, 2014). Based on our results, the yolk sac of the eggs in the Persian sturgeon and the Sterlet was consumed during 14 days after hatching. The increment of the lysozyme activity after 10 DPH in the Persian sturgeon and the Sterlet could be due to forming of the lysozyme-producing organs such as the kidney, spleen and gastrointestinal tract as well as exogenous feeding. The digestive system of the Siberian sturgeon is completed 16-18 DPF and started feeding from the environment (Gisbert & Williot, 1997). In addition, the appearance of meningeal myeloid tissue, spleen and thymus in Acipenserid juveniles are completed in 48, 33 and 48 DPH, respectively (Gradil *et al*., 2014).

The complement system is a key component of the innate immunity and it plays a role in adaptive immunity (Morgan, Marchbank, Longhi, Harris, & Gallimore, 2005). In the Atlantic halibut (*Acipenser oxyrinchus*), the C3 was detected in several cells and tissues at developmental stages from 5 to 99 DPH (Lange, Bambir, Dodds, & Magnadottir, 2004a). Also 50 days after hatching, the complement component C3 was measured in hepatocytes and liver endothelial cells of the spotted wolffish (Ellingsen *et al*., 2005). Recent findings suggest that complement system has important roles in diverse biological processes and generation of cells and tissues in the early developmental stages of fish *(Lange et al*., 2004a; Lange, Bambir, Dodds, & Magnadottir, 2004b; Mastellos & Lambris, 2002). Hence, the presence of higher value of the complement component C3 than the IgM in the early life stages (from the unfertilized eggs to 1 MPF) of the Persian sturgeon and the Sterlet could be attributed to the role of this protein in the generation of cells and tissues. From 2 to 12 MPF, the C3 was significantly decreased in the Persian sturgeon and the Sterlet. It seems that the reduction refers to the animal transition from the sensitive early life stages and also developing the innate and adaptive immune system in two sturgeons.

The maternal transfer of the IgM to the embryo has been demonstrated in several species such as plaice (Bly, Grimm, & Morris, 1986), tilapia (Takemura & Takano, 1997) and red sea bream (Kanlis *et al*., 1995). In sea bass (*Dicentrarchus labrax*), for example, the maternal IgM had been deposited in the ovary during vitellogenesis and reached a maximum concentration during ovulation. At hatching time, this level had been reduced by about 100-fold and no maternal IgM had not been detected in 5 DPH larvae of the sea bass (Breuil, Vassiloglou, Pepin, & Romestand, 1997). A similar situation had been demonstrated in tilapia (Mor & Avtalion, 1990) and the Atlantic salmon (*Salmo salar*) (Olsen & Press, 1997). Pre-larval stages of the tilapia (0 to 12 DPH) showed relatively high maternal value of the IgM level, which gradually decreased to a minimum level at 12 DPH just prior to the development of the larval lymphoid organs and the autologous IgM production (Takemura, 1993). In the salmon, the maternal IgM level was abruptly decreased after hatching, reaching minimum levels just prior to the onset of feeding and the autologous IgM production (Olsen & Press, 1997). In the Persian sturgeon, the IgM concentration was significantly decreased in the fertilized eggs that may be attributed to consumption of the yolk nutrients by the embryo and also lack of exogenous feeding as well as autologous IgM production. On the contrary, the IgM concentration of the Sterlet reached to the maximum level in the fertilized eggs. Magnadottir *et al*. (2005) suggest that the primary role of maternal antibodies is to support the eggs against direct transfer of certain pathogens or that maternal IgM may help phagocytosis or the activation of complement system approaches in early developmental stages; the IgM may even function simply as a nutritional yolk protein. An increment of the IgM concentration in the Persian sturgeon and the Sterlet was observed in 1 MPF and 2 MPF, respectively. It appears that the complete development of the haematopoiesis tissues including the kidney, thymus and spleen could be effective in this increment of the IgM. After 4 MPF, the IgM level was significantly decreased which probably due to production of the other immunoglobulins such as IgD (Uribe *et al*., 2011) by

lymphoid organs and their cooperation in the body immunity.

In the present study, there was no significant difference between the immune parameters (C3 and IgM) of the juveniles older than 8M and the plasma of the female broodstock. It seems that the Persian sturgeon and the Sterlet immunologically obtains the stable conditions after 8 MPF and the most fluctuation of the immune parameters occurs up to 8 month post fertilization.

Conclusion

In conclusion, higher value of the complement component C3 was found in the early life stages than the other immune parameters that can be indicative of its important role in these stages. The most fluctuations of the lysozyme, C3 and IgM were observed in the early life stages which was a transitional period between endogenous and exogenous feeding. Attention to these larval stages can play a key role in the more efficient culture of the Persian sturgeon larvae.

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