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Effects of short term fasting and refeeding on some hematological and immune parameters in *Mesopotamichthys sharpeyi* (Günther, 1874) fingerlings

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Abstract

This study investigated the effects of short-term fasting and re-feeding on hematological and immunological parameters of *Mesopotamichthys sharpeyi* (Günther, 1874) fingerlings. After 2 weeks of adaptation, 360 fish with an average initial body weight of 6.13±0.40g were distributed in nine 300-L circular fiberglass tanks provided with aeration system following a completely randomized design. The fish were divided in 3 experimental groups (each in triplicate) that included 4, 8 and 16 days of fasting. After food deprivation periods, fish in all the experimental groups were fed to satiation two times a day for a period of 32 days. Blood samples were collected from 15 fish in each treatment (5 fish from each replicate) at the initial and the end of fasting and also re-feeding periods. Blood samples were collected from the caudal vasculature vein using heparinized syringes. Results showed that the numbers of red blood cells, hemoglobin and hematocrit were significantly lower ($P<0.05$) in 16-days starved group than the control group. Experimental fish showed no significant change ($P>0.05$) in the number of white blood cells, though their number increased significantly after refeeding period ($P<0.05$). Lysozyme and total immunoglobulins of the experimental fish were not affected by fasting and refeeding ($P>0.05$). The results of the study showed that food deprivation up to 16 days and subsequent refeeding did not have a significant effect on hematologic and immune indices in *M. sharpeyi fingerlings.*

Keywords: Fasting; refeeding; hematology; immune parameters; *Mesopotamichthys sharpie*

1. Introduction

Fasting or food deprivation is a normal phenomenon that many species of fish may experience in the natural habitats and under culture conditions (Barcellose et al., 2010). In nature, fish often undergo periods of poor food supply during their life-cycle, as a consequence of seasonal changes in food availability or migration (Sridee and Boonanuntanasarn, 2012). Unlike mammals, fish could survive over long periods of fasting, so fasting is used as a technique to reduce flesh lipid content and therefore improve product quality (Rasmussen et al., 2000). Optimizing the feeding strategy to decrease feed costs is a crucial step in the management of intensive fish culture (Lovell, 1998 Before application of food deprivation in aquaculture, it is necessary to determine the fish response to feeding regimes in order to characterize the optimal duration of feed deprivation for each species to induce growth compensation.

The blood is a fluid vital tissue and one of the important factors to determine the physiological status of the organisms (Schuett et al., 1997). Blood, because of having various compounds, plays a critical role in creating certain immune response, buffering mode against changes in pH and maintenance of osmotic pressure. Blood cells like erythrocytes are important in the transport of nutrients and gases, whereas white blood cells (WBCs) have importance in producing antibodies, phagocytosis of bacteria, (Morshedi et al., 2011). Hematological parameters are used as appropriate indicators of nutritional status (Sridee and Boonanuntanasarn, 2012). Some studies reported controversial results (Kawatsa, 1966, Larsson and Lewander, 1973), which seems to indicate that response to food deprivation might be dependent on the experimental conditions. Effects of fasting on immune parameters have been evaluated in a few fish species (Sitjà-Bobadilla and Pérez-Sánchez, 1999, Shoemaker et al., 2003). It has been reported to decrease the expression of genes encoding a number of secreted immune related proteins, including serum amyloid A, complement factor B

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and serotransferrin in the Atlantic salmon (Martin et al., 2010). Since the assessment of blood parameters during periods of fasting and refeeding could be a valuable tool in evaluating the health status of fish, the present study examined the effects of short-term fasting and subsequent re-feeding on the alteration of hematological and non-specific immune indices in *Mesopotamichthys sharpeyi(*Günther, 1874) fingerlings.

2. Materials and methods

2.1. Fish and rearing condition

640 *Mesopotamichthys sharpeyi* (Günther, 1874) fingerlings with mean weight of 6.13±0.40 g were used in the experiment. Before the beginning of the experimental trial, the fish were disinfected with brackish water, and in order to acclimatize to laboratory conditions were maintained for 2 weeks in 300 L tanks. During this period fish were fed with a commercial pelleted diet for carp (21 Beyza Co, Shiraz, Iran) containing 41.04% crude protein, 5.83% crude fat and 11.45% ash, two times a day (9:00 am and 17:00 pm). After the acclimatization period, fish were weighed individually and distributed randomly to nine 300 L fiberglass tanks (40 fish/per tank) that were equipped with aeration system. The experimental condition were maintained at optimum with temperature 25.6 ± 0.9 C, pH= 8.05±0.31 and dissolved oxygen= 7.61±0.51 under natural photoperiod

2.2. Experimental design

To investigate the effects of fasting and refeeding on blood hematological and immune parameters, three treatments of 4, 8 and 16 days fasting were considered. All the experimental treatments were in triplicates. After fasting, all experimental groups were fed for 32 days. Total duration of the experiment was 48 days. The experimental design was such that end of fasting and the beginning of the feeding period of all treatments coincided. All experiments were conducted indoors.

2.3. Sampling

Fish were deprived of food one day before sampling procedure. 30 fish from each treatment (10 fish from each replicate) were randomly sampled and anesthetized with 2-phenoxy-ethanol (%2). The blood samples were taken from caudal vein at the beginning of the trial (as initial sample) and at the end of the fasting and re-feeding periods for each treatment. Because of the small fish size, blood samples of 3-4 fish were pooled (Urbinate et al., 2004). Blood samples were divided into two parts. One of which was added to heparinized tube and another part for immune parameters assay was centrifuged immediately at room temperature, serum was separated and stored at -20 ºC until analysis.

2.4. Analysis

Red blood cells (RBCs) and WBCs s were counted immediately with a Neubauer hemocytometer after dilution with Natt & Herrick's solution (Houston, 1990). For hematocrit (Hct) determination capillary tubes were filled with blood and spun in a hematocrit centrifuge at 12,000 g for 5 min and hematocrit values were read as percentage (Karimi et al., 2013). For hemoglobin (Hb) assay Drabkin's solution was added to blood and then solution was centrifuged (3500 g for 6 min) to remove interferents, afterwards blood hemoglobin concentration (Hb) was determined with a spectrophotometer (Model RA 1000, Technicon Corporation, USA) at 540 nm using the method of Blaxhall and Daisley (1973). The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated (Houston 1990) according to the following formulas:

MCV (fl) = 10 × (PCV per RBC)
MCH (pg) = 10 × (Hb per RBC)
MCHV (%) = 100 × (Hb per PCV)

The serum lysozyme activity was conducted in accordance with the method of Sankaran & Gurnani (1972) which is based on the ability of lysozyme to destroy the peptidoglycan layer of Gram-positive bacteria (*Micrococcus lysodeikticus*). *Micrococcus lysodeikticus* lyophilized cells (Sigma, USA) were suspended in phosphate buffer (pH 7.2) at a concentration of 0.25 mg/ml and used as a substrate solution. 200 µl of serum, diluted with an equal volume of phosphate buffered saline (PBS), were added to 1.3 ml of the substrate solution at 25 ºC and measured immediately at 450 nm. After 30 minutes incubation in a humidified environment at 25 ºC, the optical density was again measured. A lyophilized hen egg-white lysozyme (Sigma, USA) was used to develop the standard curve.

Total immunoglobulin level of serum was assayed by lowery micro method modified by Siwicki & Anderson (1993). This method requires precipitating immunoglobulins out of the serum with polyethylene glycol (10000 kDa).

2.5. Statistical analyses

Statistical analysis was performed using SPSS software (version 16.0, Chicago, IL) at the significance level of 5%. The results are presented

as means ± standard error. Data were tested for normality by Kolmogorov–Smirnov test and analysis of variance (ANOVA) was employed to reveal significant differences in measured variables between experimental groups in each sampling time. When a difference was detected ($P<0.05$), Tukey's multiple comparison test was used to discriminate differences between the treatments. Differences between fed and food deprived groups at each treatment were analyzed by independent *t*-test.

3. Results

At the end of the fasting period, no significant differences were observed in the number of WBCs between the initial levels and the different fasting treatments ($P>0.05$), whereas after re-feeding the number of white WBCs was significantly higher in the starved groups compared to initial level ($P<0.05$). In addition, comparison of changes in the number of WBCs after re-feeding showed a significant increase ($P<0.05$) in the number of WBCs (Fig. 1).

The changes in the number of RBCs, hemoglobin concentration and hematocrit during fasting and re-feeding are shown in Table 1. The number of RBCs, hemoglobin concentration (Hb) and hematocrit (Hct) decreased after 16 days of fasting significantly ($P<0.05$). Maximum number of RBCs was recorded in the initial samples ($2.06\pm0.07\times10^3/mm^3$) and the lowest number ($1.48\pm0.13\times10^3/mm^3$) was observed on the 16th day of fasting. RBCs and Hct did not recover after re-feeding periods, while no significant differences were found between Hb concentration in the initial samples and the other groups after re-feeding periods ($P>0.05$). The comparison of changes in the number of RBCs in each treatment showed a significant decline only in the 8-days starved group ($P<0.05$), whereas changes in Hb levels in each sampling time were not statistically significant ($P>0.05$). The comparison of Hct at the end of fasting and re-feeding represented significant differences in 4 and 8 days starved groups ($P<0.05$), although in 16-days fasting group no significant differences were recorded between the end of fasting and re-feeding times ($P>0.05$).

There were no significant changes in MCV during all periods of fasting and re-feeding. Moreover, comparison of variations of this index in each treatment showed the lack of significant differences in different sampling time ($P>0.05$) (Table 1).

As shown in Table 1, food deprivation caused a significant reduction in MCH and MCHC indices in the food-deprived groups of 4 and 8 days in comparison with the initial samples ($P<0.05$). However, at the end of the refeeding period no significant difference was observed for both the indices ($P>0.05$). The comparison of MCH changes in each treatment indicates that there was a significant difference between the end of fasting and the end of re-feeding only in 16-days fasting group ($P<0.05$).

Table 1. Hematological parameters of *Mesopotamichthys sharpeyi* (Günther, 1874) fingerlings after fasting and refeeding in experimental groups. Different small letters indicate significant differences between the base group and the other experimental groups at the end of starvation periods and the end of Re-feeding. Symbol (*) represents significant differences in each treatments. Data are presented as mean±SE

Index	Control	4 days starvation		8 days starvation		16 days starvation	
		End of starvation	End of refeeding	End of starvation	End of refeeding	End of starvation	End of refeeding
RBC($\times10^3/mm^3$)	2.06 ±0.07^{a}	1.84±0.05^{ac}	1.50±0.27^{ac}	1.96±0.08a*	1.30±0.17^{ac}	1.48±0.13^{bc}	**1.14±0.15^{bc}**
Hb(g/dL)	12.26±0.17^{a}	11.53±0.14^{a}	11.56±1.80^{a}	11.80±0.23^{a}	11.20±1.47^{a}	9.60±0.61^{b}	**10.80±0.96^{a}**
Hct(%)	28.90±0.65^{a}	27.43±0.46^{a*}	20.25±1.41^{bc}	29.10±1.05^{a*}	19.26±2.17^{bc}	20.73±2.38^{b}	**17.50±2.38^{bc}**
MCV(fL)	16.46 ±0.25^{a}	16.28±0.29^{a}	16.38±0.73^{a}	16.12±0.46^{a}	14.69±0.49^{a}	15.20 0.28 a	**15.25±0.21^{a}**
MCH(pg)	89.16 ±5.19^{a}	68.30±1.24^{bc}	78.83±7.24^{a}	65.46±3.51^{bc}	86.93±7.22^{a}	71.46±5.94^{ac*}	**95.23±4.63^{a}**
MCHC(g/dL)	56.34±3.49^{a}	42.12±0.64^{bc*}	57.83±1.82^{a}	40.60±1.15^{b*}	59.18±3.35^{a}	47.16±4.05^{ac*}	**62.46±2.86^{a}**

Serum lysozyme activity and the total immunoglobuli did not show significant changes among the intial and experimental treatments (P>0.05) throughout the experiment (Fig. 2). Moreover, comparison of variations of both the indices, indicated that there were no significant differences between the end of fasting and the end of re-feeding in all groups (P>0.05).

4. Discussion

According to the results of this study, fasting caused no significant changes in the number of WBCs (P>0.05), while the number of these cells after refeeding period significantly increased (P<0.05). Similarly, in gilthead sea bream (*Sparus aurata*) no significant differences in total WBCs were observed following fasting (Sala-Rabanal et al., 2003). Smirnova (1965) in burbot fish (*Lota lota*), Kawatsa (1966) in Rainbow trout (*O. mykiss*) and Johansson-Sjobeck et al (1975) in European eel (*Anguilla anguilla*), showed that WBC count is reduced during periods of fasting. Moreover, a decline in the number of WBCs has been reported under food deprivation in juvenile Siberian sturgeon (*Acipenser baerii*) and Beluga sturgeon (*Huso huso*) (Morshedi et al., 2011). Reduction in WBC count was attributed to damaged capacity of the immune system during fasting periods (Smirnova, 1965, Johansson-Sjobeck et al. 1975).

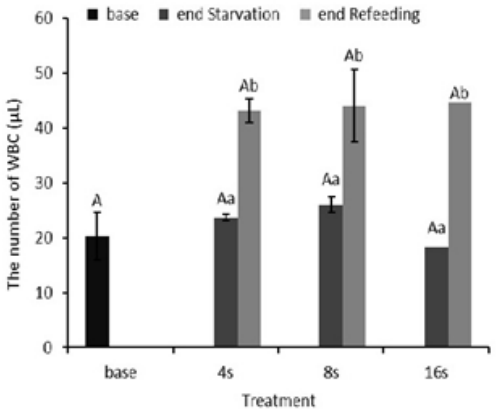

Fig. 1. *Mesopotamichthys sharpeyi* (Günther, 1874) Blood WBCs fingerlings after fasting and refeeding in experimental groups. Similar capital letters represents significant differences between the base sample and other experimental groups at the end of starvation and after re-feeding (P < 0.05). Small letters indicate changes in the activities of these enzymes in each treatment

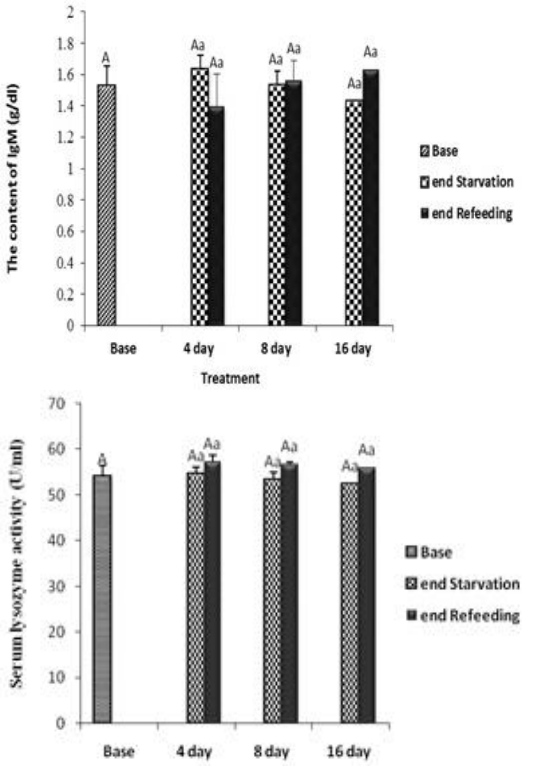

Fig. 2. Effects of starvation and Re-feeding on Ig M (a) and lysozyme activity (b) in *Mesopotamichthys sharpeyi* (Günther, 1874). Similar capital letters represent significant differences between the base sample and other experimental groups at the end of starvation and after re-feeding (P < 0.05). Small letters indicate changes in the activities of these enzymes in each treatment

Starved *M. sharpeyi* showed a remarkable decrease in the RBCs after 16-days of food deprivation (P<0.05). Similar results were reported by Rios et al (2005) in Tiger Fish (*Hoplias malabaricus*) after 240 days of fasting. In addition, Rios et al (2011) reported a similar decrease in *Prochilodus Lineatus* after 5 weeks of food restriction. Also, a significant reduction were reported in the number RBCs in Marble goby (*Oxyeleotris Marmorata*) after 4 weeks of fasting (Sridee and Boonanuntanasarn, 2012). In channel catfish (*Ictaluros panctacus*) no significant difference in RBCs was observed at 2 and 4 week periods of food deprivation (Shoemaker et al., 2003). Similar results were observed in Siberian sturgeon and Beluga sturgeon (Morshedi et al., 2011).

There are conflicting results about effect of fasting on the hemoglobin and hematocrit values. Some studies showed an increase in the hematocrit content in response to fasting periods of 90, 145 and 47 days in the Japanese eel (*Anguilla japonica*), the borbut and European eel, respectively (Sano 1962, Smirnova 1965, Johansson-Sjobeck et al., 1975). The increase in the hematocrit was also

reported in starved Beluga sturgeon by Morshedi et al (2011), whereas a decrease in the hematocrit and haemoglobin values were observed in food-deprived carp (*Cyprinus carpio*) and rainbow trout, respectively (Kawatsa, 1966). Reduction in the hematocrit content in Pike (*Esox lucius*), *Hoplias Malabaricus* and Siberian sturgeon (Kristoffersson and Broberg, 1971, Rios et al., 2005, Morshedi et al., 2011). On the other hand, Larsson and Lewander (1973) stated that fasting did not affect the hematocrit and haemoglobin content in the starved European eel for 150 days. Furthermore, studies conducted on European seabass (*Dicentrarchus labrax*) and black spot sea bream (*Pagellus bogaraveo*) showed that fasting does not affect the hematocrit and haemoglobin content (Caruso et al., 2011). Similar results on starved gilthead seabream and red porgy (*Pagrus pagrus*) had been reported (Sala-Rabanal et al., 2003, Caruso et al., 2012).

In the present study, MCV index during periods of fasting and re-feeding remained unchanged. Similar findings have been obtained in starved *Hoplias malabaricus* (Rios et al., 2005). Similarly, no significant change was reported in the MCV of juvenile Siberian sturgeon and Beluga sturgeon (Morshedi et al., 2011) during fasting periods, while Sridee and Boonanuntanasarn (2012) found an increase in the MCV index in starved *Oxyeleotris marmorata*.

At the end of the fasting period both MCH and MCHC indices showed a significant decrease in treatments 4 and 8 days fasting that was in accordance with decrease of RBCs and Hct. Decrease in MCHC has also been reported in *Oxyeleotris Marmorata* (Sridee and Boonanuntanasarn, 2012) under fasting condition . Inconsistent with the present results, a remarkable increase in MCH and MCHC indices was reported in *Malabaricus Hoplias* after 180 days of food deprivation that is caused by a decrease in RBC volume (Rios et al., 2005). Nevertheless, no significant change had been reported in the MCH index of starved Siberian sturgeon and Beluga sturgeon (Morshedi et al., 2011).

Fish, like other vertebrates have an efficient immune system which protects them from substances or agents recognised to be foreign to the organism (Eliss, 1999). There is little information about relation of fasting and refeeding and immune parameters. No change in lysozyme and IgM was recorded after fasting and refeeding. Caruso et al., (2011) found similar findings in blood lysozyme as it was not affected after 31 days fasting in *Dicentrarchus labrax* but in *Pagellus bogaraveo* its value was lower in starved fish. It was also affected in blood and kidney of European eel after 58 days of fasting (Caruso et al, 2011). Based on the performed research, the effects of experimental fasting periods are dependent on internal and external factors. Hence, the choice of season, photoperiod, temperature and age of the fish is among the factors that have a considerable impact on the test results (Navarro and Gutierrez, 1995). This issue could explain the differences in reports of effects of food deprivation in fish and the contradiction in the finding of the present study with previous studies. Also, increase in lysozyme activity as a part of immune system in response to stress has been reported (Demers and Bayne, 1997), so it could be concluded that in the present study the fasting duration did not provoke stress responses to cause an increase in lysozyme activity.

The assessment of serum values of IgM could be used not only for the diagnosis of disease, but provide valuable information which could be used for taking preventive and managenment measures in aquaculture (Cuesta et al., 2004). IgM values were not affected by experimental fasting and refeeding. There are no reports in fish species, but in Mongolian gerbil similar results have been reported (Xu et al., 2011). Increase (Effros et al. 1991, Jolly 2004, Zysling et al. 2009) or decrease (Demas and Nelson 1998, Bilbo and Nelson 2004, Liang et al. 2004, Martin et al. 2008) in IgM value after fasting was reported in other species. WBC counts increased after refeeding in all groups but no significant changes were recorded in experimental group, which indicates lack of a direct association between IgM production and WBC.

In conclusion, it can be stated that despite the relatively high resistance of cold-blooded animals, including fish, most of the examined hematologic parameters in this study showed a significant decrease, but after re-feeding, all parameters returned to their normal values, except RBC and Hct. On the other hand the immune response was not affected by fasting and also its duration. Since the evaluation of these parameters alone cannot present a clear picture of the physiological status of the fish, analysis of other physiological parameters is recommended in future studies in this species.

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