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RESEARCH PAPER

A Preliminary Study on Sperm Morphology, Motility and Composition of Seminal Plasma of Shirbot, *Barbus grypus*

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Abstract

In the present study, we investigated some qualitative parameters of milt in an endemic cyprinid fish, Shirbot, *Barbus grypus*. The parameters including: sperm motility, sperm density and the composition of seminal plasma (calcium, sodium and potassium, phosphorous, cholesterol, glucose. In this regard, 13 males ready to spawning (TW: 2846 ± 324 g, TL: 65.8 ± 4.8 cm) were provided from the South Aquaculture Research Center. Then, fish were injected during 12 days intraperitoneally in one stage with 2 mg/kg.bw pituitary extract and after 10-12 h, the spermiation occurred and sperm collection was carried out. According to results, the mean values of assayed parameters were: sperm motility duration: 108±13.6 s; sperm density: 18.8±2.44; seminal plasma pH: 7.87±0.2; % spermatocrit: 76.07±9.57; glucose: 9.41±1.29 mg/dl; cholesterol: 25.71±2.99 mg/dl; total protein: 1.27±0.29 mg/dl; Na^{+}: 93.73±7.78 mmol/l; K^{+}: 44.3±4.5 mmol/l; Ca^{++}: 0.86±0.06 ; Mg^{++}: 1.43±0.18 mmol/l; total sperm length: 4.17±0.34 µm; sperm head length: 1.2±0.2 µm; sperm flagellum length: 3.01±0.21 µm. Also, significant correlation was found between sperm motility duration and pH of seminal plasma and also spermatocrit and sperm density.

Keywords: Endemic fish, sperm, seminal plasma, *Barbus grypus.*

Introduction

The endemic fish species are ecologically important and they considered as gene banks of an ecosystem. Knowledge on all biological aspects of these species especially reproduction properties may help to their appropriate management and conservation in the nature. In this regards, many studies have investigated the reproduction properties of fishes especially gamete quality, the studies which followed the aquacutural and conservational goals .Sperm motility and sperm density determine the fertilization capability of spermatozoa and are considered as the qualitative parameters of fish spermatozoa (Suquet *et al*., 1982; Billard *et al*., 1993; Linhart *et al*., 1994; Krol *et al*., 2006). Seminal plasma has a special composition composed of organic and inorganic (ions) components which support the viability of spermatozoa (Piironen and Hyvarinen, 1983; Lahnsteiner *et al*., 1993 (a,b); Ciereszko *et al*., 2000). K^{+} is a key ion controlling sperm motility in Salmonidae and Acipenseridae in combination with osmotic pressure (Alavi *et al*., 2006). In cyprinidae, the sperm motility is under control of seminal plasma osmolality and the motility is prevented when the osmotic pressure is high (Alavi *et al*., 2006). Many studies have shown that the composition of seminal plasma affects the sperm motility of fishes. In this regard, interesting results were found in fish species, for example, the positive correlation between % sperm motility and Na^{+}, K^{+}, pH, osmolality in rainbow trout, *Onchorhynchus mykiss* (Lahnsteiner *et al*. 1998), % sperm motility and pH in Chinook salmon, *Oncorhynchus tshawytscha* (Ingermann *et al*., 2002), % motility vs. Na^{+}, K^{+}, total protein, pH and osmolality in *Alburnus alburnus* (Lahnsteiner *et al*. 1996) and also negative correlations between the % motility and the concentrations of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} in European eel, *Anguilla anguilla* (Perez *et al*. 2003). A recent study by Öğretmen *et al*. (2014) indicated that motility of shabut, *Barbus grypus* spermatozoa is in under influence of the composition of activation solution especially K^{+} and Ca^{++} concentrations. In this regard, the concentrations more than 20 mM K^{+} and 10 mM Ca^{++} significantly decreased the sperm motility of shabut.

The Shirbot, *Barbus grypus* is one of the endemic and freshwater fish of Iran. This species is nutritionally omnivorous and ecologically euryhaline and eurytherme and widely distributed in Iran, Turkey, Syria and Iraq (Nikpei, 1996; Abdoli, 2000). Shirbot is favorable among indigenous residents of south western provinces of Iran, especially Khuzestan Province (Banaee and Naderi, 2014). The growth rate of this species is high and sometimes reaches up to 20 Kg. Thus, Shirbot can be a good candidate for aquaculture in the southern regions of Iran. Understanding the reproduction properties of Shirbot can help to their management and conservation in the nature and also to artificial reproduction in hatchery condition. Thus, in

the present study we investigated some reproductive parameters including sperm motility, sperm density and the composition of seminal plasma (calcium, sodium and potassium, phosphorous, cholesterol, glucose).

Materials and Methods

The experiment was carried out at the South Aquaculture Research Center, Ahvaz, Iran during May to April. Altogether, 13 males ready to spawning (TW: 2846 ± 324 g, TL: 65.8 ± 4.8 cm) were captured from nature (Karun river) and then transported to the South Aquaculture Research Center. After 3 days acclimation to pond condition, male fish were injected during 12 days intraperitoneally in one stage with 2 mg/kg.bw pituitary extract. Before injection, the fish were anaesthetized using 100 pp of MS222 (tricaine methane sulphonate). After injection, males were checked every 6 h interval up to spermiation. During the course of the experiment, the water condition (temperature: 24-25 °C, dissolved oxygen: 8-8.5 mg/l and pH: 7.2-7.3) of each pond was checked and was in normal range. After 10-12 h, the spermiation occurred and sperm collection was carried out by hand-stripping. In this regard, the milt collection was carried out by massage from the anterior portion of the belly (testis region) towards the genital papilla. Special care was taken to avoid the contamination of semen by water, mucus, blood cells, faeces or urine. Immediately after milt collection, the pH of milt samples was measured with a pH meter. Then an aliquot of milt samples were considered for motility and the sperm density analysis and remaining centrifuged (13000 g for 10 min) to separate seminal plasma. To evaluate the duration of sperm motility, a 100 µl freshwater placed on a glass slide under light microscopy (Nikon eclipse 50i) equipped to monitor and then 10 µl fresh sperm was added using a microsampler. Then, the duration of sperm motility was recorded by a time recorder (Secer 2004). Only forward-moving spermatozoa were classified as motile, while sperm cells simply vibrating or turning on their axes (circular movement) were considered as immotile (Aas *et al*., 1991).

The spermatocrit is defined as the ratio of white packed material volume to the total volume of semen × 100 (Rurangwa *et al*., 2004). Microhaematocrit capillary tubes (75 mm in length and 1.1–1.2 mm in diameter) were used for spermatocrit measurement. Microhaematocrit capillary tubes filled with milt were centrifuged at 5000 rpm for 10 min in a D-78532 centrifuge (Tuttlingen, Zentrifugen, Germany) and then spermatocrit was calculated on the basis of the ratio of spermatozoa volume (white part) to total volume of milt × 100. To assay sperm density, at first milt samples were diluted 1:1000 times by pipetting10 µl semen in 990 µl of water (Ciereszko and Dabrowski, 1993). Then, a haemocytometer counting chamber was used to determine the spermatozoa density. A droplet of the diluted milt was placed on a haemocytometer slide (depth 0.1 mm) with a coverslip and counted using light microscopy equipped to monitor according to Alavi *et al*. (2006). The light microscope equipped to monitor (400 X) with accuracy of 0.01 µm was used to investigate the morphological properties of spermatozoa including total length, head length and flagellum length.

The seminal plasma concentrations of calcium and phosphorous were assayed photometrically using SELECTRA XL automated chemistry analyzer (ELITech group, vital scientific, Chicago, IL (USA) according to… the protein, cholesterol and glucose were measured spectrophotometrically (RA 1000 Technicon) using standard analysis kits (standard analysis kits from Parsazmoon, Tehran, Iran). Also, the concentration of sodium and potassium were assayed by an automatic electrolyte analyzer (Convergys ISE NG, GmbH, Germany).

Statistical Analysis

The SPSS software was used to analyze data. All correlations were tested using the bivariate correlation coefficients of Pearson. Then, linear and non-linear regression models were investigated using regression fits.

Results

According to our results, the range of all assayed parameters (n=13) were as follow: sperm density: 15-23 × 10^9 ; spermatocrit: 60-90 %; duration of sperm motility: 90-132 s; milt volume: 2.9-4.5 ml; seminal plasma pH: 7.65-8.27; glucose: 8.2-12.2 mg/dl; cholesterol: 21-29.3 mg/dl; total protein: 0.97-1.9 ; Na^+: 84.45-103 mM/l; K^+: 36.8-49.3 mM/l; Ca^{++}: ; Mg^{++}:1.2-1.55 mM/l; total sperm length: 3.48-4.66 µm; sperm head length: 0.88-1.33 µm; sperm flagellum length: 12.6-13.3 µm. Also, the mean ± SD all parameters have been presented in Table 1. Also, significant correlation was found between sperm motility duration and

Table 1. Milt parameters of Shirbot, *Barbus grypus*

Milt parameters	Mean±SD
sperm density (× 10^9)	18.8±2.44
Spermatocrit (%)	76.07±9.54
duration of sperm motility (s)	108±13.6
milt volume (ml)	3.95±0.54
seminal plasma pH	7.87±0.2
Glucose (mg/dl)	9.41±1.29
Cholesterol (mg/dl)	25.71±2.99
total protein (mg/dl)	1.27±0.29
Na^+ (mmol/l)	93.73±7.78
K^+ (mmol/l)	44.3±4.5
Ca^{++} (mmol/l)	0.86±0.06
Mg^{++} (mmol/l)	1.43±0.18
total sperm length (µm)	4.17±0.34
sperm head length (µm)	1.2±0.2
sperm flagellum length (µm)	13.01±1.21

seminal plasma pH (Figure 1); sperm motility duration and glucose concentration (Figure 2) and also spermatocrit and sperm density (Figure 3).

Discussion

Knowledge on biological aspects of fish especially reproductive properties is essential to their management and conservation in the nature. In this regard, attentions to endemic fish species are very important since these species are of the most important part of local ecosystem. These species help to sustain the parts of ecosystem. In the present study, some reproductive properties of an endemic fish species, Shirbot, *Barbus grypus*. In our study, the values of K^+ and Ca^{++} were at the rage of those assayed in other cyprinidae (K^+: 20-78.87 mmol/l; Ca^{++}: 0.7-10.6 mmol/l) (Alavi and Cosson 2006). However, the Ca^{++} concentration was lower than many cyprinid fishes (Alavi and Cosson 2006). The values of Na^+ and Mg^{++} in Shirbot were higher than other cyprinid fish (Alavi and Cosson 2006) (Na^+: 51.3-71.2 mmol/l; Mg^{++}: 0.02-0.3 mmol/l). The interactions of ions present in the seminal plasma with the sperm membrane affects the membrane potential (Ciereszko *et al*., 2000) and represent a mechanism of inhibition of

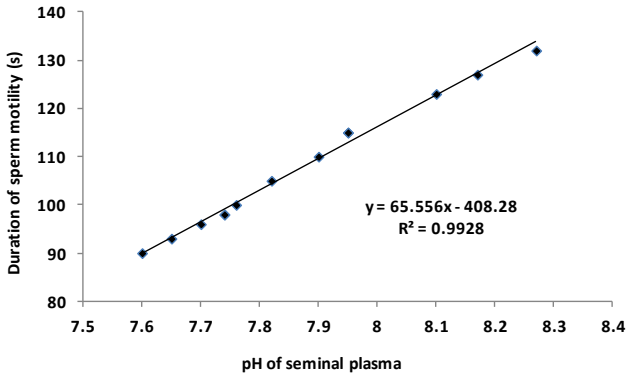

Figure 1. The relationships between seminal plasma pH and duration of sperm motility in Shirbot, *Barbus grypus*.

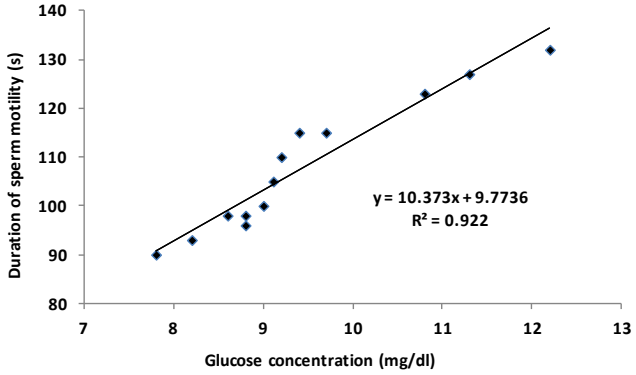

Figure 2. The relationships between glucose concentration and duration of sperm motility in Shirbot, *Barbus grypus.*

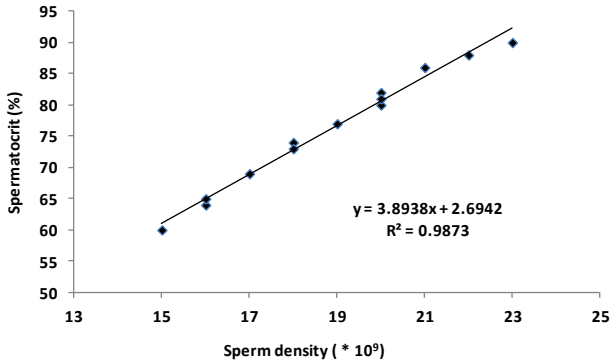

Figure 3. The relationships between sperm density and spermatocrit in Shirbot, *Barbus grypus.*

spermatozoa in the seminal plasma or sperm duct (Boitano and Omoto, 1991). The inhibition of spermatozoa motility in seminal plasma saves the potential of the motility and allows the initiation of motility when spermatozoa are ejaculated to the water (Krasznai *et al*., 2000). According to our literature review, Na^+ and Cl^- have been considered as main electrolytes involving in maintenance of the osmolality of seminal plasma (Morisawa *et al*., 1979). It seems that the Shirbot needs more Na^+ and Mg^{++} in seminal plasma to maintain the optimum osmolality for spermatozoa. The values of glucose, total protein and cholesterol were higher in shirbot than in other cyprinid fish (Verma *et al*., 2009). Also, in a recent study on shirbot, it was found that shirbot spermatozoa are activated in activation solution with K^+ and Ca^{++} concentrations of 5-80 mM and 5-60 mM respectively (Öğretmen *et al*., 2014). Thus, it seems that the osmolality of seminal plasma is main controlling factor of sperm motility in seminal plasma of shirbot than ions especially K^+ since we recorded ranges of 20-78.87 mmol/l and 0.7-10.6 mmol/l for K^+ and Ca^{++} respectively.

Commonly, monosaccharides such as glucose serve as energy sources for sperm motility in fish (Stoss, 1983; Lahnesteiner *et al*., 1993b), thus different concentrations of glucose in fish seminal plasma could be related to differences in spermatozoa energy metabolism among fish species. Also, some studies have confirmed the key role of some proteins in the motility of fish spermatozoa through buffering the seminal plasma (Lahnestiner *et al*., 2004; Lahnestiner *et al*., 1996). Thus, it seems that the more level of protein is needed for viability of shirbot spermatozoa compared to other cyprinid fishes. There is insufficient information about the role of cholesterol in seminal plasma, in spite of its identification in the seminal plasma of freshwater fish (Billard *et al*., 1995). Lipids and cholesterol might have a protective effect against environmental changes (especially in temperature) that occur when fish semen is released (Bozkurt *et al*., 2008).In our study, the values of sperm production (sperm density and spermatocrit were at the rage of other cyprinid fishes (Verma *et al*., 2009; Bozkurt and Öğretmen F. 2012). The positive relationship between sperm density and spermatocrit of Shirbot suggests that the evaluation of sperm quantity by spermatocrit determination is better than the spermatozoa counting method from facility and time-saving aspects. In our study, positive relationship was found between pH of seminal plasma and the duration of sperm motility. According to previous studies, during the passage of spermatozoa from the testis to the spermatic duct an increase in external pH may be responsible for the acquisition of motility in some salmonid fish (Morisawa and Morisawa, 1986, 1988; Billard *et al*., 1995) and therefore the seminal fluid pH may also effect the final maturation of spermatozoa (Lahnesteiner *et al*., 1998). Thus, the significant correlation between the duration of motility and semen pH in shirbot may be related to this problem. In addition, it seems that the motility of shirbot spermatozoa in higher in seminal plasma with pHs more than 8. Similar result has been reported for same species in a previous study, where the highest motility was found in pH of 9 (Öğretmen *et al*., 2014). In the present study, some morphological parameters of shirbot spermatozoa were investigated. The morphological properties of sperm may be related with systematics and phylogenetic features and therefore interesting under taxonomical aspects (Jamieson, 1991; Lahnsteiner and Patzner, 2008).

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