Influence of broodstock age on sperm quality traits in *Rutilus frisii* and its effect on fertilization success

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Abstract

In this study, age-dependent changes of sperm quality parameters in *Rutilus frisii* were investigated. Also, fertilization capacity of breeders was tested using two age groups of males (3 and 4 year old breeders) and one female group (i.e. 4 year old breeders). For fertilization trials, the breeders were crossed randomly among age classes. Between the two age groups, spermatological parameters did not show vary significant differences. Some composition of the seminal plasma such as osmolality showed significant difference and was higher in older fish. Higher fertilization rate was observed when ova were fertilized with 4 years old semen samples. Relationships between sperm motility characteristics (percentage and duration of sperm motility) and chemical properties of seminal plasma were investigated. In this regard, were found between the duration of sperm motility and Na^+, and Cl^-, respectively. Also, the percentage of motile spermatozoa had significantly positive relationships with the concentrations of Ca, Mg and pH of semen. On the other hand, osmolality of the seminal plasma was positively correlated with protein. Our results shows that selection of broodstock based on age can be used as a simple procedure to achieve higher results in fertilization process; also understanding of such correlations can be useful to evaluation of sperm quality and make media (extender) for dilution of semen and improving sperm motility parameters of kutum.

Keywords: Sperm traits, Fertilization success, Biochemical parameters, *Rutilus frisii*

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Introduction

Rutilus frisii, belongs to cyprinids, which is the most famous, valuable, commercial and economic teleost in the Caspian Sea (Razavi Sayad, 1995). It is only found in the Caspian Sea and its main habitat is to south of the Caspian Sea, especially in Iran's coasts (Razavi Sayad, 1995). Artificial reproduction and culture of this fish in land pools and renewal of its stocks in the Caspian Sea started since 1982 (Emadi, 1995). Sperm quality is a measure of the ability of sperm to successfully fertilise an egg. Physico-chemical parameters are directly correlated with the fertilization capacity of semen and could potentially be used as a measure of sperm quality. Sperm quality can be influenced by factors such as size of individuals (Aas *et al*., 1991). Often, older, more experienced males produce higher semen volume with higher sperm density and higher fertilization capacity as compared to the younger, less mature fish (Aas *et al*., 1991). In fish farming industry, sperm quality mainly depends on the maintenance condition and intensity of use of the same male and also on the condition of hypophysial injection. Furthermore, the knowledge about the trend in sperm quality is very important for developing semen technology of carp species in selective breeding programs as only the high quality breeders meet the necessary requirements. Most common parameters employed to study sperm biology are duration of motility, percentage of spermatozoa exhibiting forward motility, semen volume, sperm density and spermatocrit. Estimation of motility is of great importance, as semen must be fully motile to achieve egg penetration. Both percentage of spermatozoa and duration of motility are regarded to play a major role in achieving higher fertilization success. The quality of sperm usually refers to the motility which is a prerequisite factor determining the semen fertilizing ability (Lahnsteiner *et al.*, 1997). Good quality eggs are defined as those which exhibit low levels of mortality at fertilization, eying, hatching and first feeding and those which produce the fastest growing and healthiest fry (Benau and Turner, 1980; Aas *et al*., 1991;). Larval quality is also affected by sperm and egg quality (insemination ratio, spermatocrit etc.), which is taken from available broodfish. So, high quality egg or semen means high quality larval fish. The aim of the present work was to verify whether the age of male kutum can affect the most important spermatological indices and subsequently fertilization capacity.

Materials and methods

Broodstocks

The experiments were performed on kutum broodfish at Shahid Ansari Cyprinid Fish Complex, Rasht, Iran. Kutums were captured from the Sefid Rood River inlets to the Caspian Sea during spawning migration (water temperature 9-12ºC). For this purpose 10 mature females and 20 males were randomly selected from the ponds. The average total weight and total length for three and four year males were

510±48.5 g, 38.7±1.41 cm and 996±155.2 g, 48.4±2.13 cm, respectively. Also, average of total weight and total length for four year females was 1180±43.5 g, 51.2±1.41. Males and females were naturally stripped. Care was taken to avoid contamination of the semen with water, mucus, blood cells, faeces or urine. Semen of each male was collected and the sperm batches were transported to the laboratory under cold conditions (4 ºC) until used for analysis and fertilization.

Sperm motility analysis

An activating solution of 0.3% NaCl was used for estimating motility. For the evaluation of motility, about 1 µL of semen was placed on a test tube and 1000 µL of activation solution was added and thoroughly mixed with the tip of a pipette, about 10 µL of the diluted semen was placed on a glass microscope slide and motility was recorded using a camera (Nikon 50i Japan) mounted on a phase contrast microscope (Leica USA). Each motility determination was performed in triplicate for each semen sample. The duration of sperm motility was measured immediately after initiation of sperm activation until 100% spermatozoa were immotile and expressed as sperm movement duration. The percentages of motile spermatozoa were defined as the percentage of progressively motile spermatozoa within each activated sample. Progressively motile spermatozoa were defined as actively swimming in a forward motion. Only forward moving sperm was judged motile and sperm cells that vibrated in place were not considered to be motile. Observations were made within two hours of semen collection. Semen was drawn into glass microhaematocrit capillary tubes (75 mm length, 1-1–1-2 mm internal diameter) until 60–80% of the tube volume was occupied by semen. One end of the tube was then sealed with clay and the tubes were centrifuged for 8 min at 3,000 g (Eppendrof-5415D Germany). Spermatocrit was defined as the ratio of the total volume of white package material to the total volume of semen ×100 (Rurangwa *et al*., 2004). Measurements were taken in triplicate for each sample, and the average of the three measurements was used for the results.

Seminal plasma indicies

Sperms were centrifuged to obtain seminal plasma at 3000 g for 10 min (Eppendorf AG, Hamburg, Germany) and the supernatant (seminal plasma) was collected. Plasma was centrifuged twice to avoid possible contamination with spermatozoa and then samples were frozen at -20°C until analysis. Immediately after plasma collection, the osmolality and pH were measured with an osmometer (Melting Point Osmometer Nr 961003, Roebling Company, Berlin, Germany) and (pH meter, Iran 762), respectively. The minerals (Ca^{+2}, Mg^{+2} and Cl^{-}) were measured spectrophotometrically (S2000-UV/VIS England). The concentrations of Na^{+} and K^{+} were

determined with flame photometer (Jenway PFP, England) (Standard kits from Parsazmoon, Tehran, Iran). The alkaline phosphatase (ACP) and biochemical parameters (total protein, glucose, cholesterol, triglyceride and urea) were measured by auto-analyzer (Caretium-XI-921, Germany) using enzymatic procedures with a diagnostic kit (Pars Azmoon Co, Tehran, Iran).

Fertilization capacity evaluation

Fresh ovulated eggs were obtained from females and pooled just prior assessment. To control variation among the qualities of egg, eggs from each broodstocks were pooled separately in order to minimize variations in gamete quality. For fertilization trials semen samples of each age were used for eggs fertilization . Fertilization took place in dry plastic dishes. Afterward, the pooled semen samples were added equally to dishes containing pooled eggs and then mixed. The fertilization solution (3 g of urea, 4 g of NaCl in 1 L distilled water) was used according to the dry fertilization technique. Following fertilization, the eggs were stirred for 1 h and then eggs rinsed with hatchery water and placed into the incubator. Fertilization rate was determined as the percent of the eyed eggs about 6 h after the fertilization. Hatching occurred between 1–2 days at water temperature 20-24.5°C. The following equations were used to calculate fertilization capacity.

Fertilization rate: number of fertilized egg/ total eggs× 100 (Bromage and Cumalantunga, 1998)

Hatching rate = (number of healthy fertilized eggs / number of fertilized eggs) ×100 (Hanjavanit *et al*., 2008)

Data analysis

Since the data on sperm quality parameters were normally distributed (Shapiro-Wilk test), Student-pair tests were used to test statistical significance. All statistical analyses were performed using the statistical program SPSS 10.0. Data were presented as mean±SD.

Results

Sperm quality properties of two age groups are given in Table 1. There were no significant diffrences in spermatological parameters. Seminal plasma composition such as osmolality and potassium significantly changed between the two age classes and was higher in older fish (Table 2). The highest fertilization rate was observed in 4 year old males (Fig. 1). Similar trend was also observed for hatching rate, but values were not statistically different between 3 and 4 year old individuals (Fig. 2). Larvae length did not show significant difference between ages (Fig. 3). Significant negative and positive relationships were detected for the duration of motility vs. Ca^{+2} and Cl^{-} of semen, respectively (Figs. 4, 5). A negative relationship was recorded for the percentage of motile spermatozoa vs. Ca^{+2}, Mg and pH of semen (Figs. 6, 7 and 8). There were no relationships between metabolites composition of seminal plasma (glucose, total protein and cholesterol) and duration of sperm motility and percentage of motile

spermatozoa. On the other hand, osmolality in seminal plasma was positively correlated with protein (Fig. 9).

Table 1: Spermatological parameters of 3 years old of *Rutilus frisii.*

Variables	3-years old	4-years old	Mean ± SD
Sperm of duration of motility (sec)	45[a]	85[a]	63.4 ± 9.4
Percentage of motile spermatozoa (%)	80[a]	85[a]	81.7 ± 5.3
Sperm density ($mL \times 10^{-9}$)	16.3[a]	18.[a]	17.9 ± 2.5
Spermatocrit (%)	35[a]	56[a]	45 ± 5.9

Similar letters in same parameters show no significant differences ($p>0.05$).

Table 2: Some seminal plasma composition between two age groups in *Rutilus frisii.*

	Age	
Parameters	**2 years old**	**3 years old**
Sodium ($mmol^{-1}$)	96 ± 5.7[a]	96.6 ± 6.4[a]
Potassium ($mmol^{-1}$)	32 ± 1.6[b]	34.5 ± 2.4[a]
Chloride ($mmol^{-1}$)	100.1 ± 7.9[a]	107.3 ± 7.6[a]
Calsium ($mmol^{-1}$)	4.2 ± 1.4[a]	3.5 ± 1.02[a]
Magnesium ($mmol^{-1}$)	2.2 ± 0.62[a]	2.2 ± 0.47[a]
Glucose ($mgdL^{-1}$)	3.1 ± 1.2[a]	2.3 ± 0.68[a]
Protein (gdL^{-})	2.9 ± 1.3[a]	2.9 ± 1.2[a]
Cholesterol ($mgdL^{-1}$)	18.3 ± 5.8[a]	22.4 ± 8.4[a]
Osmolality ($mOsmol\ kg^{-1}$)	329.6 ± 11.9[b]	343.4 ± 10.9[a]
Triglyceride ($mgdL^{-1}$)	26.3 ± 8.2[a]	23.4 ± 7.5[a]
Urea ($mgdL^{-1}$)	18.7 ± 3.3[a]	20 ± 3.5[a]
Alkaline phosphatase (IU/L)	23.5 ± 16.2[a]	30.6 ± 17.1[a]
pH	7.7 ± 0.51[a]	7.6 ± 0.26[a]

Values with the different alphabetic letters are significantly different ($p<0.05$).

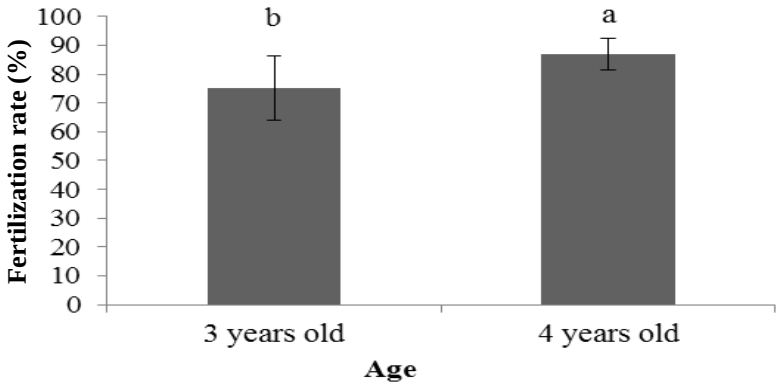

Figure 1: Fertilization rate of 3 and 4 years old males (Mean±SD). Different letters indicate significant differences ($p<0.05$).

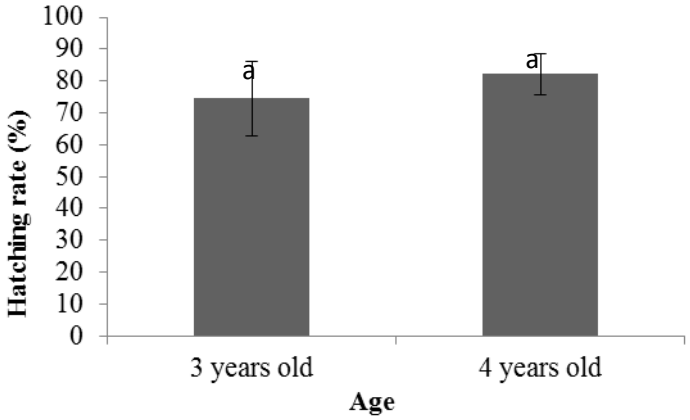

Figure 2: Hatching rate of 3 and 4 years old males (Mean±SD). The similar letters show no significant difference (p>0.05).

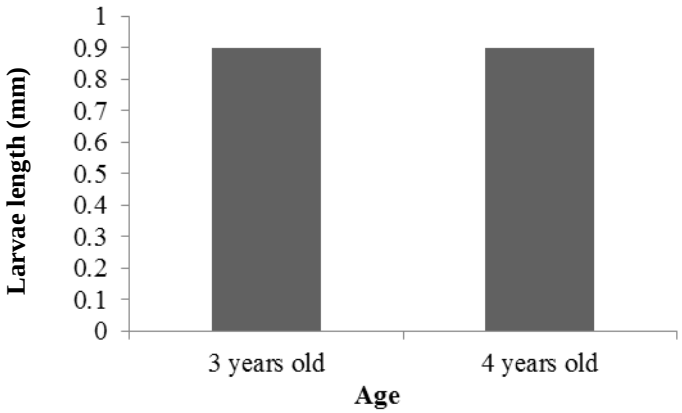

Figure 3: Larvae length of 3 and 4 years old males.

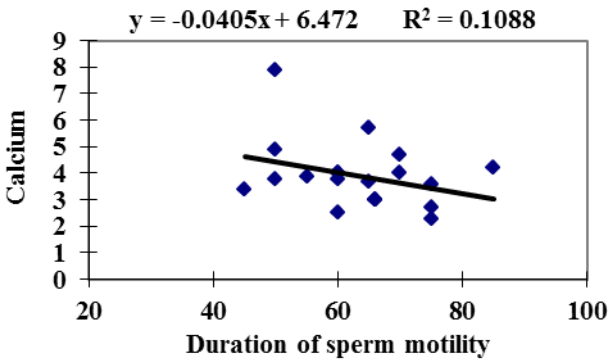

Figure 4: Negative relationships between duration of sperm motility and Ca^{+2} in *Rutilus frisii* (independent variable: duration of sperm motility, dependent variable: Ca^{+2}).

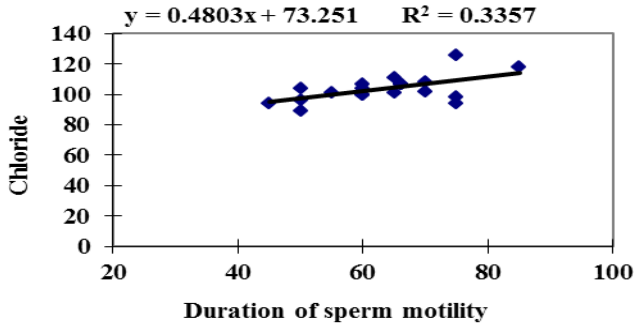

Figure 5: Positive relationships between duration of sperm motility and Ca+2 in *Rutilus frisii* (independent variable: duration of sperm motility, dependent variable: Cl-).

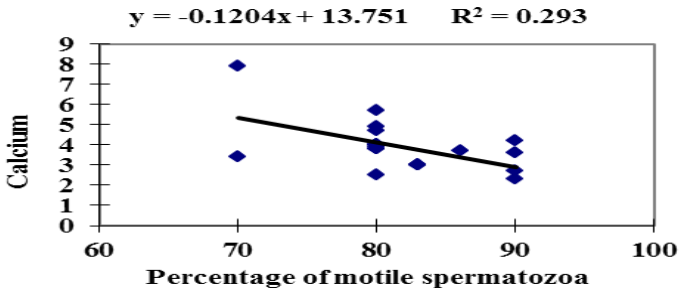

Figure 6: Relationship between percentage of motile spermatozoa and Calsium of seminal plasma in *Rutilus frisii.*

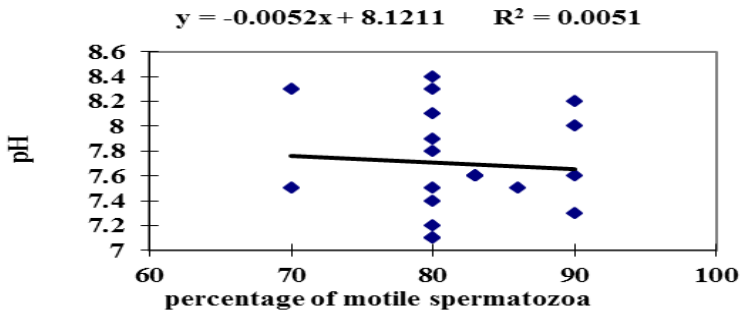

Figure 7: Relationship between percentage of motile spermatozoa and Magnesiumof seminal plasma in *Rutilus frisii.*

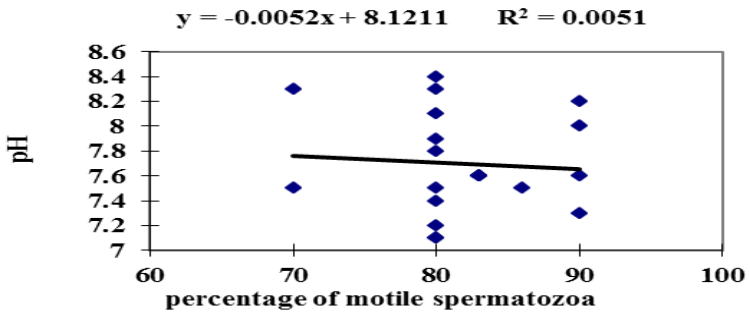

Figure 8: Relationship between percentage of motile spermatozoa and pH of seminal plasma in *Rutilus frisii.*

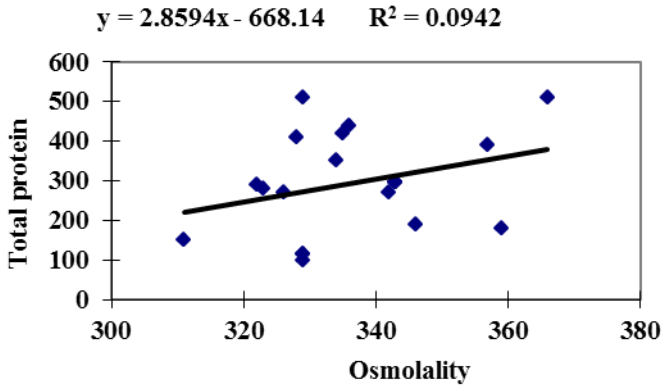

Figure 9: Relationship between the osmolality and total protein in *Rutilus frisii* (independent variable: osmolality, dependent variable: total protein.

Discussion

Viable sperm is an essential component in success of fish farming industry and the success of reproductive process is dependent on a supply of high quality gametes (Daye and Glebe, 1984). Sperm quality also is an important factor that increases the efficiency of artificial fertilization (Billard *et al*., 1995; Bromage, 1995). Sperm production characteristics and quality might be affected by both fish size and physiological status. Also, feeding condition, age, environmental factors or dilution ratio may change sperm quality (Kjorsvik *et al*., 1990; Lee and Donaldson, 2001). Relationships found in this experiment between fish age and sperm quality indicies indicates that physical condition of mature fish has no influence on sperm quality. In addition, sperm quality may be affected by genetics, diet, and environmental stress (water quality and fish density) or disease. Khodzher (1981) reported no change in the duration of sperm motility of Baikal Omul in the age range of 6-14 years. In our study, spermatological parameters were not affected by age of the broodstocks. The percentages of motile sperm were not similar among the three groups of stripped bass *Morone saxatilis* (Vuthiphandchai and Zohar, 1999) and smallmouth yellow fish *Barbus aeneus* (Vlok and Vuren, 1988). Similar results in terms of spermatocrit were observed in sockeye salmon, *Oncorhynchus nerka* and Atlantic salmon, *Salmo salar* where the spermatocrit values were higher in 3 year old males than in other age classes (Daye and Glebe, 1984; Hoysak and Liley, 2001). Some authors confirmed that with increase in male broodstocks age, spermatocrit value would decrease (Liley *et al*., 2002), which is contrary to our findings. The differences might be due to feeding conditions, husbandry procedures, age, environmental factors, spawning time or dilution ratio. Several authors have shown that fertilization capacity is directly correlated with sperm traits in rainbow trout (Munkittrick and Moccia, 1987). Our results confirmed Bozkurt (2006) study who found a correlation between sperm

quality parameters and fertilization success for the rainbow trout using subjective methods for motility determination. The ionic and organic composition of the seminal plasma can indicate fish fertilization capacity (Ciereszko *et al*., 2000). Depending on ionic concentration, most of these ions are involved in regulating sperm motility through contributing to the intracellular ionic composition by regulating osmolality (Billard and Cosson, 1992). The present study describes that only osmolality of seminal plasma was different between two age classes and was higher in 4 year old males (p<0.05). Also, several studies have confirmed that composition of seminal plasma has a great influence on the biological quality of the semen and these factors are directly related to the fertilization success (Rurangwa *et al*., 2004; Alavi and Cosson, 2005). Fish seminal plasma has a unique composition regarding the presence of the organic and inorganic components which support the viability of spermatozoa (Morisawa *et al*., 1983; Lahnsteiner *et al*., 1993; Lahnsteiner *et al*., 2004). In this regard, interactions of ions present in the seminal fluid with the sperm membrane do influence the membrane potential (Ciereszko *et al*., 2000) and represent a mechanism of inhibition of spermatozoa in the seminal plasma or sperm duct (Boitano and Omoto, 1991), allowing the maintenance of the potential of motility before release to the surrounding medium (Morisawa and Morisawa., 1988). In the present experiment, a positive significant relationship was observed between the duration of motility and Cl^- of semen. Also a negative relationship was found between the percentage of motile spermatozoa and Ca^{+2}, Mg and pH of semen. In agreement with our results, similar relationships were found in other fish. For example, the percentage of motile spermatozoa vs. pH in rainbow trout (Lahnsteiner *et al*., 1998), Chinook salmon, *Oncorhynchus tshawytscha* (Ingermann et al., 2002) , Mg^{2+} in *Alburnus alburnus* (Lahnsteiner *et al*., 1995) and also Grass Carp (Khara, 2014) have been reported. With regard to these correlations, studies suggest that seminal plasma characteristics could influence on the potential motility of kutum spermatozoa before sperm ejaculation.

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