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# Effects of Stripping Frequency on Semen Quality of Endangered Caspian Brown Trout, *Salmo trutta caspius*

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**Abstract: Problem statement:** Because of dramatic declines in stocks of endangered Caspian brown trout males, *Salmo trutta caspius* in Caspian Sea, each male brooder is stripped indispensably more than once during the spawning season in other to artificial insemination in hatchery. The aim of the present study was to assay the changes of indicators of semen quality (sperm motility, sperm production, semen volume and chemical composition of seminal fluid) during these sequential strippings. **Approach:** The 11 tagged males were stripped four times every 12-14 days with beginning of spermiation period (2 December 2008) towards its end (10 January 2008). One-way Analysis Of Variance (ANOVA) was employed to analyze differences between means of semen parameters. Also, the relationships between semen parameters were tested using the bivariate correlation coefficients of Pearson. **Results:** The semen volume, sperm density, osmolality and the concentrations of $Na^+$, $Cl^-$, $K^+$, $Ca^{2+}$, $Mg^{2+}$ and total protein gradually decreased whereas the values of glucose and triglyceride had no significant changes during sequential strippings. Also, the values of semen pH, the percentage (5s post-activation) and duration of motility were statistically stable until third stripping but a decrease was recorded for these parameters in the fourth stripping. As well as, significant positive correlations were found for sperm density vs. $K^+$, $Cl^-$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$, total protein, spermatocrit; the percentage of motile spermatozoa Vs $Ca^{2+}$, $Mg^{2+}$, $K^+$, $Cl^-$, $Na^+$, total protein and also the duration of motility Vs $K^+$, $Cl^-$, total protein and pH. **Conclusion:** The semen quality of Caspian brown trout males decrease in successive strippings during spawning season. Also, the knowledge on values and correlations between the sperm motility characteristics and the composition of seminal fluid could be useful to formulation of a species-specific extender solution for cryopreservation of semen of Caspian brown trout.

**Key words:** Semen quality, spawning season, sequential strippings

## INTRODUCTION

The availability of semen with desirable quality is one of the critical factors necessary to increase the efficiency of artificial fertilization of fish species. Several studies have described semen characteristics which can influence quality. For example: Sperm density, sperm motility and the composition of seminal fluid[34]. Seminal fluid has a unique composition regarding the presence of the organic and inorganic components which support the viability of spermatozoa. For example: Minerals (Potassium, Sodium, Magnesium, Calcium and Chloride), pH, osmolality, proteins, glucose and triglyceride[12,18,19,23,29]. As well as the composition of seminal fluid, sperm motility and sperm density determine the fertilization capability of spermatozoa and often are used to estimate semen quality[2,17,24].

Few studies have shown that the fish semen characteristics could be affected by stripping frequency[9,13,32,35,37]. The Caspian brown trout, *Salmo trutta caspius* is a critically endangered anadromous species that has been considered for a biological conservation program in the southern part of the Caspian Sea[16].

In recent years, because of dramatic declines in the capture of male brooders and subsequent insufficient availability of semen, the males are stripped more than once during the spawning season. In the present study, the qualitative parameters of semen i.e. chemical composition of seminal fluid (Triglyceride, glucose, total protein, $Na^+$, $Cl^-$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, pH, osmolality), sperm production (semen volume, sperm density, spermatocrit,) and sperm motility were measured over four successive strippings during the spawning season.

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The aim of the present investigation was to assay the ability of Caspian brown trout to produce of semen with desirable quality during the sequential strippings.

Specific objectives of this study were to (1) estimate the number of needed males in the hatchery on the basis of their ability in the production of semen during the spawning season and the number of available mature females; (2) determine the possible semen components which may be related to sperm motility. This may be useful for formulation of a species-specific extender solution for cryopreservation of semen of Caspian brown trout.

## MATERIAL AND METHODS

The experiment was carried out at the Kalardasht Salmonids Reproduction Center (KSRC), Iran, during the spawning season of Caspian brown trout. Males were captured from the Sardabrood and Tonekabon Rivers during their up stream reproductive migration and then transferred to the broodstock pond at the KSRC. In KSRC, Males were hand-stripped every week to detect the onset of spermiation. The 11 tagged males (TL: 59.3±4.7 cm, TW: 1503.6±113.3 g) were selected with occurrence of first spermiation and stripped repeatedly every 12-14 days from 2 December 2008 to 10 January 2008 (altogether four times during the spawning season). Before stripping, the fish were anaesthetized in 100 ppm of MS222 (tricaine methane sulfonate) and then semen samples were collected by massage from the anterior portion towards the genital papilla. Care was taken to avoid contamination of the semen with water, mucus, blood cells, faeces and urine. Immediately after semen collection, the osmotic pressure and pH of semen were measured with an osmometer (Melting Point Osmometer Nr 961003, Roebling Company, Berlin, Germany) and a semi-microelectrode pH Meter (SM102 pH Meter) respectively.

A two-step dilution was used for motility activation according to the method suggested by Billard and Cosson (1992)[5] for salmonid fish. Firstly, the semen was prediluted in saline solution (composed of 80 mM NaCl, 40 mM KC1, l mM $CaC1_2$ and 20 mM Tris-HCl 1 $L^{-1}$ of distilled water (final pH = 9)) at a ratio of 1/100 and secondly, the prediluted semen was subjected to a second dilution in a physiological saline solution at a ratio of 1/20 and immediately 1 µL of solution was placed on the microscope stage and motility was analyzed by a semi-quantitative method[34]. In this method, the motility was recorded by a videocamera coupled with the optical lens of a microscope. The video recordings were reviewed and the motility was expressed as the percentage and duration of motility. Only forward-moving sperm were judged motile, those simply vibrating or turning on their axes was considered immotile[1].

The semen volume was measured by scaled vials. Also, two different methods were used to determine the sperm production. One method was spermatocrit and another sperm density. Spermatocrit was determined by centrifuging of semen for 10 min at 5000 rpm in a haematocrit centrifuge (D-78532, Tuttlingen, Zentrifugen, Germany) according to Piironen[32] and sperm density by haemocytometer counting chamber according to Caille *et al*.[11].

To analyze the chemical components in seminal fluid, the semen was separated from the seminal fluid by centrifugation (Heraeus, Sepatech, Labofuge 200, Germany, 5000 rpm for 10 min). Magnesium, chloride and calcium were measured colorimetrically using an Auto-analyser Technican (RA 1000, Technicon-Swords, Dublin, Ireland). Potassium and Sodium were determined with a flamephotometer (CORNING 480, Corning, Medfield, MA, USA). Also, glucose, triglyceride and total protein of seminal fluid were measured spectrophotometrically (Cintra 40 UV-Visible Spectrometer, GBC) (standard analysis kits from Ziestchem, Tehran, Iran).

**Statistical analysis:** All parameters were expressed as means and standard deviations. One-way Analysis Of Variance (ANOVA) was employed to analyze differences between means. Because percentage data (the percentage of motile spermatozoa and spermatocrit) did not have a normal distribution, proportional data were converted by angular transformation (arcsin $\sqrt{p}$) prior to analysis by ANOVA. The sperm motility characteristics (percentage of motile spermatozoa and duration of motility) and sperm density were tested on correlations with the parameters of the seminal fluid using the bivariate correlation coefficients of Pearson. Also, this test was used to analyze the correlations between semen volume, sperm density and spermatocrit.

## RESULTS

The values of semen volume, spermatocrit and sperm density continuously declined significantly over the four stripping times (Table 1, $p<0.05$). The percentages of motile spermatozoa at 5s post-activation was significantly lower in the fourth stripping than in the first, second and third stripping (Table 1, $p<0.05$). Also, the percentages of motility decreased with time

after activation in each semen sample, reaching 0 % in all samples within 45s post-activation. The duration of motility was significantly stable until third but it decreased in the fourth stripping (Table 1, $p<0.05$). The osmolality and the concentrations of $Na^+$, $Cl^-$, $K^+$, $Ca^{2+}$, $Mg^{2+}$ decreased in successive strippings (Table 2, $p<0.05$). The values of glucose and triglyceride had no significant changes over four times stripping, but the concentration of total protein decreased continuously (Table 2, $p<0.05$). The values of semen pH were stable until the third stripping but a sudden decrease was recorded in the fourth stripping (Table 2, $p<0.05$).

Also, positive correlations were found for semen volume Vs sperm density (Bivariate coefficient: 0.851, $p<0.01$), spermatocrit Vs sperm density (Bivariate coefficient: 0.979, $p<0.01$), semen volume Vs spermatocrit (Bivariate coefficient: 0.820, $p<0.01$), sperm density vs. $K^+$, $Cl^-$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$, total protein of seminal fluid (Table 3, $p<0.01$), the percentage of motile spermatozoa vs. $Ca^{2+}$, $Mg^{2+}$, $K^+$, $Cl^-$, $Na^+$, total protein of seminal fluid (Table 3, $p<0.01$), also the duration of motility Vs $Cl^-$, $K^+$, total protein and pH of seminal fluid (Table 3, $p<0.01$). As well as, the other possible correlations between the seminal fluid parameters have been shown in Table 4.

Table 1: Mean (with standard deviation) for duration and percentage of motility, spermatocrit, sperm density and semen volume during sequential stripping from Caspian brown trout during the spawning season (n = 42 semen samples, 3 males yielded no semen in fourth stripping)

| | Stripping data | | | |
|---|---|---|---|---|
| Traits | 2 December (2008) | 16 December (2008) | 29 December (2008) | 10 January (2008) |
| Percentage of motility (5 sec after post-activation) | 66.8±8.4$^a$ | 65.9±8.3$^a$ | 60.4±5.7$^a$ | 52.8±6.2$^b$ |
| Duration of motility (sec) | 44.0±1.7$^a$ | 43.0±2.8$^a$ | 42.0±2.2$^a$ | 39.0±4.4$^b$ |
| Sperm density ($\times10^9$sperm $mL^{-1}$ semen) | 4.4±0.6$^a$ | 3.6±0.5$^b$ | 2.0±0.5$^c$ | 1.1±0.5$^d$ |
| Spermatocrit (%) | 45.6±5.7$^a$ | 37.3±4.4$^b$ | 22.5±6$^c$ | 20.0±3$^c$ |
| Semen volume (mL) | 19.2±1.8$^a$ | 18.0±1.6$^a$ | 11.7±2.1$^b$ | 6.4±4.2$^c$ |

The values with the same letter above are not significantly different

Table 2: Mean (with standard deviation) for chemical parameters of seminal fluid during sequential strippings from Caspian brown trout during the spawning season (n = 42 semen samples, 3 males yielded no semen in fourth stripping)

| | Stripping data | | | |
|---|---|---|---|---|
| Traits | 2 December (2008) | 16 December (2008) | 29 December (2008) | 10 January (2008) |
| $Na^+$ (mM $L^{-1}$) | 143.20±9.2$^a$ | 131.7±6.3$^b$ | 127.6±4.5$^{ab}$ | 122.60±3$^a$ |
| $Cl^-$ (mM $L^{-1}$) | 147.00±8.6$^a$ | 133.7±6$^b$ | 124.3±4.2$^c$ | 120.60±8.4$^c$ |
| $K^+$ (mM $L^{-1}$) | 38.50±7.3$^a$ | 36.2±7$^a$ | 28.7±3.8$^b$ | 23.20±3.2$^c$ |
| $Ca^{2+}$ (mM $L^{-1}$) | 1.90±0.2$^a$ | 1.4±0.3$^b$ | 1.3±0.2$^b$ | 0.90±0.3$^c$ |
| $Mg^{2+}$ (mM $L^{-1}$) | 1.30±0.2$^a$ | 1.0±0.2$^b$ | 0.9±0.1$^{bc}$ | 0.90±0.2$^c$ |
| Triglyceride (mM $L^{-1}$) | 0.30±0.2$^a$ | 0.3±0.1$^a$ | 0.3±0.15$^a$ | 0.40±0.1$^a$ |
| Glucose (mM $L^{-1}$) | 1.90±0.9$^a$ | 2.0±1.1$^a$ | 1.7±0.5$^a$ | 2.00±0.9$^a$ |
| Total protein (mg $mL^{-1}$) | 0.60±0.1$^a$ | 0.3±0.1$^b$ | 0.2±0.1$^c$ | 0.10±0.1$^d$ |
| Osmolality (mOsmol $Kg^{-1}$) | 207.00±14.6$^a$ | 188.2±8.7$^b$ | 180.0±8.5$^{bc}$ | 172.70±6.8$^c$ |
| pH | 8.03±0.13$^a$ | 8.0±0.10$^a$ | 8±0.13$^a$ | 7.77±0.31$^b$ |

The values with the same letter above are not significantly different

Table 3: The correlations between the chemical of seminal fluid, sperm density, the percentage of motile spermatozoa and duration of motility during sequential strippings from Caspian brown trout during the spawning season (n = 42 semen samples, 3 males yielded no semen in fourth stripping)

| | $Na^+$ | $Cl^-$ | $K^+$ | $Ca^{2+}$ | $Mg^{2+}$ | Glucose | Triglyceride | Total protein | Osmolality | pH |
|---|---|---|---|---|---|---|---|---|---|---|
| Sperm density | 0.731** | 0.733** | 0.750** | 0.861** | 0.749** | ◦ | ◦ | 0.857** | 0.684** | 0.412** |
| Motility (%) | 0.506** | 0.593** | 0.884** | 0.487** | ◦ | ◦ | ◦ | 0.440** | 0.586** | ◦ |
| Duration of motility | ◦ | 0.380** | 0.360** | ◦ | ◦ | ◦ | ◦ | 0.382** | 0.351** | 0.690** |

Statistically significant correlations are indicated as follows: **: $p<0.01$ and non-significant as ◦. Data: Bivariate coefficient

Table 4: Correlations between the chemical parameters of seminal fluid during sequential strippings from Caspian brown trout during the spawning season (n = 42 semen samples, 3 males yielded no semen in fourth stripping)

| | $Na^+$ | $Cl^-$ | $K^+$ | $Ca^{2+}$ | $Mg^{2+}$ | Glucose | Triglyceride | Total protein | Osmolality | pH |
|---|---|---|---|---|---|---|---|---|---|---|
| $Na^+$ | - | 0.806** | 0.624** | 0.711** | 0.584** | ◦ | ◦ | 0.713** | 0.843** | ◦ |
| $Cl^-$ | ** | - | 0.672** | 0.629** | 0.559** | ◦ | ◦ | 0.727** | 0.922** | ◦ |
| $K^+$ | ** | ** | - | 0.597** | 0.533** | ◦ | ◦ | 0.567** | 0.668** | ◦ |
| $Ca^{2+}$ | ** | ** | ** | - | 0.771** | ◦ | ◦ | 0.820** | 0.648** | 0.367** |
| $Mg^{2+}$ | ** | ** | ** | ** | - | ◦ | ◦ | 0.690** | 0.597** | 0.352** |
| Glucose | ◦ | ◦ | ◦ | ◦ | ◦ | - | ◦ | ◦ | ◦ | ◦ |
| Triglyceride | ◦ | ◦ | ◦ | ◦ | ◦ | ◦ | - | ◦ | ◦ | ◦ |
| Total protein | ** | ** | ** | ** | ** | ◦ | ◦ | - | 0.694** | 0.359* |
| Osmolality | ** | ** | ** | ** | ** | ◦ | ◦ | ** | - | ◦ |
| pH | ◦ | ◦ | ◦ | * | * | ◦ | ◦ | * | ◦ | - |

Statistically significant relationships are indicated as follows: **: $p<0.01$, *: $p<0.05$ and non-significant as ◦: Data: Bivariate coefficient

## DISCUSSION

According to our results, the values of semen volume, sperm density and spermatocrit declined by increasing of stripping frequency as previously reported for Atlantic salmon, *salmo salar*[1], but unlike that seen over weekly stripping f rom landlocked salmon, *Salmo salar M. sebago girard*[32] and biweekly stripping from rainbow trout[35].

The decrease of spermatocrit during sequential strippings may be due to the three possible reasons including the decreasing of spermiation rate, higher hydration of testis and the higher secretion of seminal fluid. The hydration could be due to the hypotonicity of freshwater environment, possibly causing the dilution of semen and leading to a higher semen volume and subsequently lower sperm density and spermatocrit (a negative relationship) as this subject is supported by the observations of Morisawa *et al*.[28]. But, with regard to the positive correlations between semen volume, spermatocrit and sperm density, it is likely that the decrease of spermatocit over four successive strippings is more a result of a decrease in the spermiation rate than an increase of seminal fluid secretion and hydration of testis. Because of the close positive correlation between sperm density and spermatocit, it is comprehended that the variations in the spermatocrit of semen samples is due to the changes of spermatozoa number not spermatozoa size as this correlation was previously reported by Hatef *et al*.[14] in Caspian brown trout. The only report found on seasonal change in spermatozoa size is by Billard *et al*.[7], referring to unpublished data which suggests that spermatozoa size may vary during the year in seabass, *Dicentrarchus labrax* and halibut, *Hippoglossus hippoglossus*.

On the whole, the specific pattern of sperm production during sequential stripping may be resulting from the effects of possible physiological and environmental variants on spermiation process during the spawning season. The assessment of the ability of males in the production of semen during the spawning season could be useful to estimate the number of needed males in the hatchery with regard to the number of available mature females.

In present study, significant positive correlations were found between sperm density and the concentrations of $Na^+$, $Cl^-$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, total protein. Similar correlations were reported in landlock salmon for sperm density Vs $Na^+$, $K^+$,$Ca^{2+}$, $Mg^{2+}$[32] and sperm density vs. total protein in Siberian sturgeon, *Acipenser baeri*[33]. the close correlations between the concentration of $Na^+$,$Cl^-$,$K^+$,$Ca^{2+}$, $Mg^{2+}$ and sperm density reveals their regulation during spermiation of Caspian brown trout. Likely, such regulation may be controlled by pituitary hormones as Sanchez-Rodriquez *et al*.[35] have reported the gonadotropin control of the sodium levels in the seminal fluid of rainbow trout. Lahnsteiner *et al*.[23] reported that some proteins of seminal fluid were shown to have a key role in the motility of spermatozoa. It is possible that in dense semen, more concentrations of these types of proteins are required for the stimulation of sperm motility than in semen with a low spermatozoa density.

In the present research, the values of ions and total protein of seminal fluid showed a decreasing trend during sequential strippings. This decreasing trend may be a reflex of the change of secretary activity in the fish spermatic duct during the spawning season since the formation of the seminal fluid in fish (inorganic as well as organic compounds) is a secretion process of the spermatic duct epithelium[18,20,25]. Several studies have recorded the correlations between mineral content of seminal fluid and osmolality[2] as these correlations were found in Caspian brown trout. Thus, the decrease of osmolality during sequential strippings may be related to the declining of mineral content of seminal fluid over the four stripping times during the spawning season. In this regard, probably the $Na^+$ and $Cl^-$ are the main electrolytes that play a major role in maintaining the osmolality of the seminal fluid[28], as the highly correlation was recorded between osmolality and the concentrations of $Na^+$ and $Cl^-$ of seminal fluid of Caspian brown trout.

Irrespective of this reasoning, other factors including contamination of semen by urine during stripping[38] and hydration of semen during spermiation period[28] may affect on the semen osmolality of fish.

In all semen samples of Caspian brown trout, percentages of motility decreased with time and reached 0 % 45s post-activation. This could be resulting from the gradual decreasing of energetic resources of sperm especially ATP concentration as this problem has been suggested for other teleost fish[11,30]. Also, significant correlations were observed between the percentage of motile spermatozoa and $Na^+$, $Cl^-$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, total protein and osmolality of seminal fluid, also between the duration of motility and $K^+$, $Cl^-$, total protein and pH of seminal fluid. In agreement with our results, similar correlations were found in other fish. for example, the percentage of motile spermatozoa Vs $Na^+$, $K^+$, pH, osmolality in rainbow trout[23], percentage of motile spermatozoa vs. pH in Chinook salmon, *Oncorhynchus tshawytscha*[15], percentage of

motile spermatozoa Vs $Na^+$,$Mg^{2+}$ $Cl^-$ and the duration of motility Vs $Na^+$ in Persian sturgeon, *Acipenser persicus*[3], percentage of motile spermatozoa Vs $Na^+$, $K^+$, total protein, pH, osmolality in *Alburnus alburnus*[21], although the negative relationships were recorded between the percentage of motility and the concentrations of $Ca^{2+}$, $Mg^{2+}$, $Na^+$, $K^+$ in European eel[31].

Generally, interactions of ions present in the seminal fluid with the sperm membrane do influence the membrane potential[12] and represent a mechanism of inhibition of spermatozoa in the seminal fluid or sperm duct[8], allowing the maintenance of the potential of motility before release to the surrounding medium[29]. According to past data, it was revealed that the low $K^+$ levels of surrounding medium is key factor for activation of salmonid fish spermatozoa[4,29], also the increase of semen pH may be responsible for acquisition of motility of spermatozoa in the rainbow trout *Oncorhynchus mykiss* and chum salmon, *Oncorhynchus keta*[7,26,27] during passage of spermatozoa from the testis to the spermatic duct i.e., before sperm ejaculation to spawnig medium (water).

With regard to this point, the highly significant correlations between percentage of motile spermatozoa and $K^+$ and also the duration of motility and pH suggest that these parameters may be the most important seminal fluid characteristic influencing on the potential of motility of Caspian brown trout spermatozoa in the seminal fluid. Thus, the $K^+$ and pH of semen may be two main indicators of semen quality of Caspian brown trout. The positive correlation between the percentage of motility and total protein of seminal fluid could be related to the key role of some proteins in the motility of sperm cells[23] or their influences on motility by buffering the seminal plasma, as proteins reveal also a positive correlation to pH in Caspian brown trout[21].

The lipids and monosaccharides such as triglycerides and glucose serve as energy resources for spermatozoa energy metabolism[19]. Thus, Low triglycerides and glucose levels could therefore be indicative of inadequate energy resources, reduced motility rate and fertilization capacity. In this study, there were no correlations between sperm motility and the concentrations of triglycerides and glucose. Thus, this problem may be due to the existence of a threshold level for these components in related to sperm motility so that probably the influence of these parameters on sperm motility is revealed in very low concentrations.

The cryopreservation of Caspian brown semen is a strategy for permanent access to spermatozoa in this species[36]. On the other hand, the dilution of semen during this process is necessary for better efficiency of crystallization. But, during the dilution, the majority of sperm cells lose their motility with time due to simultaneous activation in diluent solution. Because of chemical properties of seminal fluid, Fish spermatozoa are immotile in seminal fluid. Thus, formulation of diluent solution (extender solution) mimic to specific properties of seminal fluid is necessary. Therefore, the determination of the values of chemical parameters of semen and their relationships with sperm motility characteristics could be useful in order to formulation of a species-specific extender solution (diluent) for cryopreservation of Caspian brown trout semen.

## CONCLUSION

In present research, the understanding of the changes in the sperm production over sequential stripping during the spawning season could be useful for estimation of the number of needed males in the hatchery with regard to the number of available mature females. Also, the values and correlations between the sperm motility characteristics and the composition of seminal fluid could be useful in order to formulation of a species-specific extender solution (diluent) mimic to seminal fluid for cryopreservation of Caspian brown trout semen. in this regard, the pH and $K^+$ of seminal fluid may be the main indicators of semen quality of Caspian brown trout due to the their close correlations with sperm motility characteristics.

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