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Effects of different levels of coelomic fluid on spermatozoa mobility trait in the Persian sturgeon (*Acipenser persicus*)

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Abstract. Effects of coelomic fluid on spermatozoa mobility trait, in sturgeons, have not been known well. This research aims to determine the content of inorganic (magnesium, sodium, potassium and calcium) and organic ions (cholesterol and total protein) in coelomic fluid and to investigate the effects of different levels of coelomic fluid on some spermatozoa mobility traits (duration of sperm mobility and the percentage of motile sperms) in Persian sturgeon brood stocks. Hence milts have been sampled from several male brood stocks and have been mixed by coelomic fluid in different ratios (1 to 20, 15, 10, 7.5, 5, 2.5, 1, 0.5, 0.33, 0.25, 0.20 and 0), then mobility traits have been measured by stereomicroscope. Results of this research show that, if coelomic fluid is added to sperm more than 2.5 times, all sperms of Persian sturgeon brood stocks will perish and if coelimic fluid is added to sperm less than 2.5 times, the duration of sperm mobility and the percentage of motile sperms will increase significantly, comparing to controled group (which does not have coelimic fluid) ($P<0.05$). Besides, the maximum duration of sperm mobility and percentage of motile sperms was respectively observed among those treated groups in which the ratio of coelomic fluid to sperm equaled to 1:0.33 and 1:0.5 ($P<0.05$).
Key Words: Coelomic fluid; Spermatozoa mobility trait; Persian sturgeon.

چکیده. اثرات مايع سلوميک بر ويژگي هاي حرکتي اسپرم در ماهيان خاوياري به خوبي شناخته نشده است. هدف از اين تحقيق تعيين مقادير يونهاي غير آلي (منيزيم، سديم، پتاسيم و کلسيم) و آلي (کلسترول و پروتئين کل) مايع تخمداني و بررسي اثرات مقادير مختلف مايع سلوميک روي برخي پارامترهاي حرکتي اسپرم (طول دوره تحرک اسپرم و درصد اسپرم هاي متحرک) مولدين قره برون مي باشد. براي انجام اين کار 1 نمونه ميلت از مولدين نر با نسبت هاي مختلف مايع سلوميک (20، 15، 10، 7/5، 5، 2/5، 1، 0/5، 0/33، 0/25، 0/2 و 0) با هم مخلوط گرديده و سپس پارامترهاي حرکتي توسط دستگاه استرئوميکروسکوب اندازه گيري گرديد. نتايج اين تحقيق نشان داد که افزودن بيش از 2/5 برابر مايع سلوميک به اسپرم سبب مرگ کامل اسپرم ماهي قره برون شده و در اثر اضافه کردن مايع سلوميک در مقادير کمتر 2/5 برابر اسپرم، سبب افزايش معني دار طول دوره تحرک و درصد اسپرم هاي متحرک مولدين قره برون نسبت به گروه شاهد که فاقد مايع سلوميک مي باشد، مي شود. همچنين در بين گروه هاي آزمايشي **تيماري** که نسبت مايع سلوميک به اسپرم برابر 1:0/33 و 1:0/5 بود به ترتيب حداکثر طول دوره تحرک و درصد اسپرم هاي متحرک ديده شد ($P<0/05$).
کلمات کليدی: مايع سلوميک، پارامترهاي حرکتي، قره برون

Introduction. Sturgeon is one of the most valuable seafood species which has been known since antiquity. This species, due to a very long historical record, is called living fossil. Major amount of sturgeon of the world (92-90%) is extracted from the Caspian Sea (Deetlaff et al 1993). Persian sturgeon (*Acipenser persicus*) (Borodin, 1897) is an anadromous fish which belongs to the Acipenseridae family and spreads widely in the southern part of Iranian coastal waters of the Caspian Sea. In 1997, this species, for the first time, has been introduced as an independent species (Birstein et al 1997).

In fishes with external fertilization during the spawning season, male and female fishes of those with external fertilization, release their gametes into the water, at the same time. Sperm mobility is the main factor in fertilization success (Rudolfsen et al 2008). The fish spermatozoa mobility starts after sperm transfer to the aqueous environment (in the natural reproduction) or in culture media (Stoss 1983; Cosson et al 1999). Water, the agent against aquatic environment, can cause physiological changes in gametes which will lead to activation of sperm mobility and unfertilized eggs (Jamieson 1991). Therefore in hypertonic water, the swelling and mortality of sperm cells reduce sperm mobility duration (Cosson 2004). When the eggs were in contact with fresh water they changed quickly to non-fertilized eggs, which leaded to the closure micro pills and ultimately osmotic swelling is preventing conception (Billard & Cosson 1992).

In addition to factors mentioned above, at the start of sperm mobility, the speed of sperm swimming and the duration of sperm swimming should be directly affected by the concentration of ions (K^+, Na^+, Ca^{2+}, Mg^{2+}), the rate of osmotic, pH (Stoss 1983; Wojtczak et al 2007), viscosity (Lauga 2007), rate of dilution and other components of external medium (Yoshida & Nomura 1972; Stoss 1983; Billard et al 1995b; Cosson et al 1999; Ingermann et al 2003).

Several studies have investigated the seminal fluid composition (Alavi & Cosson 2006) but information about the composition of coelomic fluid (ovarian or peritoneal) is narrow (Hirano et al 1978; Lahnsteiner et al 1995; Wojtczak et al 2007).

Coelomic fluide is formed by filtered blood plasma and secreted actively from ovary epithelia (Hirano et al 1978). This liquid has pH between 8.4 to 8.8 and contains sodium, potassium and calcium ions, glucose, fructose, cholesterol, phospholipids, proteins and free amino acids (Lahnsteiner et al 1995). In sturgeon, this liquid inhibits the spermatozoa mobility and hereupon separats the duration of eggs fertilization (Deetlaff et al 1993). Billard (1983) used coelimic fluid in concentration of 1% and 0.1% in the eggs of rainbow trout - *Oncorhynchus mykiss* (Walbaum, 1792), and found that the duration of sperm motion and percentage of fertilized egg increased. Turner & Montgomerie (2002) have also studied on the effects of coelomic fluid on fish spermatozoa movement of Arctic Char - *Salvelinus alpinus* (Linnaeus, 1758). According to this study, coelomic fluid has significant effect on the period, the percentage and speed of spermatozoa of this species. Unlike above results, Elofsson et al (2003), have investigated some effects of saline solution with coelomic fluid on spermatozoa motion of fifteen spines - *Spinachia spinachia* (Linnaeus, 1758). In this species, coelomic fluid with saline solution had no effect on the spermatozoa motion. However, the mechanism of action on the coelomic fluid on spermatozoa motion is unknown (Litvak & Triple 1998).

In fresh water (Billard 1983) and saltwater fishes (Suquet et al 1992), dilution of sperm is the principal factor in the induction of sperm mobility (Stoss 1983, Billard & Cosson 1992) and in the ability to maintain the productivity of sperm. Since the duration of sperm mobility is short and quality of sperm motion varies during the movement phase, the dilution rate has become a key rule. Hence the rate of diluted sperm determines the sperm energy and extra diluted may lead to mortality of sperms (Billard & Cosson 1992; Billard et al 1995a). High dilution (1:1000 or 1:2000) is necessary to start synchronization of all spermatozoa mobility (Billard et al 1995b). Numerous studies showed that in Acipenseridae, compared with teleost fishes, sperms are active in low dilution rates (Gallis et al 1991; Toth et al 1997; Alavi et al 2002). Several studies have imported that the best sperm mobility in sturgeons is produced in lower dilution rate (1:50) (Gallis et al 1991; Alavi et al 2002; Alavi & Cosson 2005).

Considering the above points, coelomic fluid properties that come out of gametes have an important role in the fertilization process. Therefore, this study was performed to compare the effects of different ratios of coelomic fluid on sperm motion (duration of sperm movement and percentage of motile sperms).

Material and Method. A milt from persain sturgeon brood stock (combination of five male sperms) and a coelomic fluid of female brood stock were collected in Shahid Marjani breeding and growth sturgeons' center, Gorgan, Iran, in March, 2008. Brood stocks were kept apart from each other in Kourenski pounds with 14-17 °C and 8.2 mg O_2. Calculation of the time of hormone injection was done on the basis of germinal vesicle (GV) and sexual maturity index (Deetlaff et al 1993), according to the method of Van Eenennam et al (2001), depending on the water temperature and the fish weight (Billard 2000). Approximately 24 hours after first injection, spawning of female brood stock was participated. Then female fish was killed by a head strike. After that it was dried and cleaned with a towel and blood sample was taken with cutting branches. The abdomen area was incised, the eggs were extracted, placed in the cotton filter and coelomic fluid purified and collected. Milts were also extracted with special syringes that inserted in to the gonopores of alive males. After that, coelomic fluid and milts were put separately into 1.5 mL micro tube, with the least upper atmosphere air space to minimize their air exchanges. Then a flask containing ice and samples was transferred to the Central

Laboratory of Agricultural Sciences and Natural Resources at University of Gorgan for future analysis. However temperature of milt and coelomic fluid were at 4°C during analysis to save their mobility.

Coelomic fluid and milt extracted from brood stocks were mixed together with different ratios (1 to 20, 15, 10, 7.5, 5, 2.5, 1, 0.5, 0.33, 0.25, 0.2 and 0) (for example in the ratio of 1:5, 1 mL of milt with 5 mL of ovarian fluid were mixed and 1:0 was controled group). After adding distilled water (50 times of sperm volume, to stimulate sperm mobility) to this solution, it was put on the stereomicroscope device (microscope equipped with a CCD camera attached to computer, Panasonic WV-CP240, Japan) (Cosson et al 2000) with magnification number 10 to measure the duration of sperm movement and percentage of motile sperms (time started in less than 7 seconds) and a digital camera recorded the sperm movement by high resolution. The shelf life of sperm for each prototype was measured by stopwatch. The stopwatch started to work once milt was activated by water and stopped when sperm movement stopped (Leach & Montgomery 2000, Turner & Montgomery 2002). Then Adobe premier software (Version 6) was used to get pictures every 10, 20, 30, 40 seconds after the activation of sperms. These pictures changed to 30 forms (slides) and we randomly selected four forms (i.e. Form 1, 4, 7 and 10). Positions of 10 spermatozoa in these randomly selected pictures and also the percentage of motile sperms were calculated.

It should be noted that the diluted ratio of milt and coelomic fluid with water was 1:50, observed around 50 sperm in each point viewed under the microscope (in addition to being suggested by the Gallis et al 1991, Alavi et al 2002, Alavi & Cosson 2005, we gained this ratio by trial and error). All treatments were done thrice and to avoid experimental error, all measurements were observed by a viewer (Cosson et al 2000).

Spermatocrit of milt is significantly different among males and among species and in different seasons (Patkin 1999). Spermatocrit and sperm density in seminal fluid are generally used to evaluate sperm quality (Rurangwa et al 2004). For measuring the spermatocrit, some tubes containing semen were centrifuged in the 3000 round of centrifugal machine for 8 minutes (Eppendorf AG 22311 Hamburg, centrifuge 5415D) then hematocrit reader was used to determine the percentage of sperm to seminal fluid (percentage of white space to the total milt volume) (Fitzpatrick et al 2005).

Furthermore, sperm density was measured by haemacytometry standard method with diluted sperm in ratio of 1:2000 by water, using a microscope with 10 magnification phase contrast black background and it was written units per $\times 10^9$ mL semen.

In order to studying the biochemical index of sperm and coelomic fluid between indices, samples were located into 1.5 mL micro tubes and tubes were centrifuged in the 13,000 round for 5 minutes and then they were transferred to new vials. Vials of coelomic fluid and sperm were stored at -20 °C for future biochemical compounds study. Since cell membrane can polarize the ions in seminal fluid and stimulate sperm mobility (Morisawa et al 1999) we measured sodium and potassium ions concentration by flam photometer (Jenway pfp England) and the concentration of calcium, magnesium ions, glucose, total protein and cholesterol by the spectrophotometer (S200-UV.VIS England) in seminal and coelomic fluid, using quantitative biochemical kits of serum or plasma parameters (Turker et al 2004).

Statistical analysis of data was done thrice, by One-Way ANOVA with Duncan test at the level of 95 % using SPSS 16. Statistically significance was set at the level of $P<0.05$ with ± standard deviation (SD).

Results and Discussion. Fork length and total weight of the female brood stock before extraction eggs were 171 cm and 34 kg respectively. The fertilization rate of the first and second generation were 63% and 49% respectively.

Average of spermatocrite and density of sperm in male brood stocks were 11.33 ± 15 and $10^9 \times (1.75 \pm 0.86)$ respectively. The Biochemical analysis of coelomic fluid and sperm have been presented in Table 1 and the mean and variance of fish sperm motion parameters influenced by different ratios of coelomic fluid have been shown in Tables 2 and 3.

Table 1

Biochemical analysis of coelomic fluid and sperms (mono valence and bivalence)

Samples	*Glucose mg.dL*	*Cholesterol mg.dL*	*Total protein g.dL*	*Magnesium mg.dL*	*Calcium mg.dL*	*Sodium mM.L*	*Potasium mM.L*
Coelomic fluid	22.89	19.35	0.25	0.64	5.92	147.08	1.91
Sperm	6.52	8.31	0.15	0.93	9.21	73.65	3.57

As shown in Table 2, adding different ratios of coelomic fluid to sperm will cause a significant change in duration of movement and percentage of motile sperms ($P<0.05$).

Table 2

Compared means of sperm mobility traits parameters in Persian sturgeon influenced by different ratio of sperm to coelomic fluid

Ratio of sperm to coelomic fluid	1:20 1:15 1:10 1:7.5	1:5	1:2.5	1:1	1:0.5	1:0.33	1:0.25	1:0.2	1:0
Duration of sperm movement	0 0 0 0	0	96 ± 15.55^{d}	366 ± 33.61^{ab}	344.3 ± 34.58^{b}	381.25 ± 71.45^{a}	363.3 ± 56.09^{ab}	378.58 ± 148.82^{ab}	285 ± 87.80^{c}
Percentage of motile sperms	0 0 0 0	0	69 ± 1.41^{c}	87.87 ± 6.85^{ab}	88.37 ± 2.82^{a}	86.37 ± 7.72^{ab}	85.87 ± 8.11^{ab}	87.33 ± 6.97^{ab}	82.7 ± 8.71^{b}

Different letters (a, b and c) indicate significant differences between groups ($P<0.05$).

Results showed that in Persian sturgeon if the coelomic fluid in sperm increases more than 2.5 times, complete mortality of sperm will occure. On the other hand, adding coelomic fluid, with a ratio less than 2.5 times, to the sperm leads to significant increases in the duration of movement and percentage of motile sperms compared to the controled group that does not have coelomic fluid. Furthermore, among the treatments in the experimental groups, those groups in which the ratios of coelomic fluid to sperm were 1:0.33 and 1:0.5 respectively show significant higer duration of movement and percentage of motile sperms ($P<0.05$) (Tables 2 and 3). Concludingly those sperms could be closer to eggs micro pills in such environment (Iwamatsu et al 2003).

Table 3

Analysis of variances of sperm mobility traits parameters in Persian sturgeon influenced by different ratios of sperm to coelomic fluid

Dependent variable	*SV*	*df*	*Sum of squares*	*Means square*	*F computed*	*p value*
Duration of sperm movement	Between groups	6	187848.667	31308.11	91.125	0.000
	Within groups	14	4810.000	334.571	-	-
	Total	20	192658.667	-	-	-
Percentage of motile sperms	Between groups	6	824.952	137.492	13.948	0.000
	Within groups	14	138.000	9.857	-	-
	Total	20	962.952	-	-	-

The Increase in sperm mobility and the possibility of continuous existence of sperm by coelomic fluid can mainly be attributed to coelomic fluid ionic balance (Scott & Baynes 1980). Liley et al (2001) also state that the beneficial role of coelomic fluid in sperm mobility cab be effective in male fertility, because in such environment, males release their milt closer to eggs.

According to our study results, Rosengrave et al (2008) found that activated sperm in coelomic fluid, compared to activated sperm in fresh water, causes prolonged sperm mobility. Furthermore, in several species of fish, this liquid has been identified to increase swimming speed of the motile sperm and long-time direct movement of spermatozoa, compared to the other groups activated in fresh water, salt water or buffer solution (arctic charr - Turner & Montgomery 2002, Urbach et al 2005; Atlantic cod *Gadus morhua* (Linnaeus, 1758) - Litvak & Triple 1988; rainbow trout - Rosengrave et al 2008). Turner & Montgomery (2002) also showed in a research that in Arctic Charr, the increase in the concentration of coelomic fluid up to 50 % causes a significant increase in duration of movement and the percentage of motile sperm. 5% concentration did not show any positive or negative effect on these factors. Furthermore Hugunin et al (2008) expressed that coelomic fluid may have no effect on eggs, but instead it prevents sperm mobility or connect the sperms with eggs. Besides, they found that if coelomic fluid is washed completly by buffer solution, embryo viability in rainbow trout, compared to other groups, will be higher.

Coelomic fluid is affective on some factors containing mammals for example on spermatozoa absorption (Oliviera et al 1999), the acceleration of the capitation (spermatozoa development process that carried in females reproductive system) (Ravnik et al 1990), the stimulation acrosome activity (Suarez et al 1986), the acceleration of spermatozoa (Oliviera et al 1999) and longevity of spermatozoa (Zhu et al 1994).

There is little information about the effects of the bivalent cation on sperm mobility in semen sturgeons. Cations are available to have antagonistic effects on sperm mobility to avoid the effects of potassium ion (Alavi & Cosson 2006). Contrary to carp fish (Perchec et al 1993), sperm mobility of sturgeons is very sensitive to very low concentrations of potassium (Cosson et al 1999). It has also been proved that in sturgeons, potassium can control sperm activity in very low concentrations (Cosson et al 1999).

The duration of sperm movement and the percentage of motile sperms can decrease by an excessive increase of Na^+ (Morisawa et al 1983). Alavi et al (2005) have demonstrated that sperm concentration shows more biological sensitivity to the concentration of sodium when it comes to 50 mM or more. Toth et al (1997) also reported that the concentration of sodium in 40 mM or more has inhibitory effect on the activation of sperm mobility in Lake sturgeon - *Acipenser fulvescens* (Rafinesque, 1817). In Siberian sturgeon - *Acipenser baeri* (Brandt, 1869), Na^+ had no effect on sperm mobility when it was in the amplitude of seminal plasma concentration (20 mM) (Gallis et al 1991). In our study, due to high concentration of sodium ions (147.08 mmol) in coelomic fluid (Table 1) and considering that spermatozoa in salt water fish are active in the hyper osmotic environment, it can be concluded that such high levels of sodium in coelomic fluid are due to increased osmotic pressure and the mobility of spermatozoa of Persian sturgeon.

Alavi et al (2004) showed that the spermatozoa of *A. persicus* have high sensitivity to calcium concentration. Calcium, like sodium, could reduce inhibitory effects of potassium on the activation of spermatozoa in Sturgeons (Billard et al 1999; Alavi et al 2002; Linhart et al 2002).

There is little information about the effects of magnesium ions on sperm mobility in teleost fish and sturgeons. Linhart et al (2002) reported that magnesium can rapidly improve the movement of spermatozoa and the percentage of motile sperms in the *Polyodon spathula* (Walbaum, 1792). A research on intracellular mechanisms of sperm mobility in teleost fishes has proved key role of magnesium in starting the activation of sperm mobility, especially in membrane of sperm (Cosson et al 1999). Alavi & Cosson (2005) found that in *A. persicus* which has 10 mmol magnesium, highest duration of sperm movement and percentage of motile sperms are observed.

Significant negative relationships are found between duration of sperm movement and the amount of calcium and magnesium ions in coelomic fluid of Chinook salmon - *Oncorhynchus tshawytscha* (Walbaum, 1792) brood stocks. Besides, there is a significant dependency between calcium and magnesium ions in coelomic fluid and the lowest percentage of mobility in coelomic fluid was found in coelomic fluid that had the least amount of magnesium (Rosengrave et al 2009).

Cholesterol may also has protection affect against environmental changes (especially temperature). A research has shown that in trout there is a clear correlation between cholesterol and the duration of sperm movement (Secer et al 2004).

According to what was mentioned about the negative effects of excessive density of some ions in coelomic fluid (including potassium) on sperm mobility, these negative factors can cause immobilization treatments containing higher amounts of coelomic fluid, were been attributed to excessive density ions mentioned. On the other hand, some components of coelomic fluid influences may increase in ATP metabolism and energy production (Perchec et al 1995; Lahnsteiner et al 1997).

Conclusion. Our research was similar to previous studies (Litvak & Trippel 1988; Yanagimachi et al 1992; Turner & Montgomery 2002) and showed that adding coelomic fluid to the sperms of *A. persicus* directly affects on spermatozoa mobility traits and either complete elimination or the increase of the coelomic fluid more than 2.5 times, will cause significant decrease in the sperm mobility and therefore the possibility of the fertilization of sperms ($P<0.05$) reduces. On the other hand, adding the coelomic fluid to sperm, less than 2.5 times, will cause significant increase in the duration of sperm movement and percentage of motile sperms, compared to the controled group which did not have coelomic fluid and which was activated by water ($P<0.05$). Furthermore, among the treatments in this experiment, those groups in which the ratio of sperm to coelomic fluid was 1:0.33 and 1:0.5, respectively had the maximum duration of sperm movement and maximum percentage of motile sperms ($P<0.05$).

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