ABAH BIOFLUX
Animal Biology & Animal Husbandry
International Journal of the Bioflux Society

Changes of ovarian fluid compositions and sperm quality parameters in koi (ornamental *Cyprinus carpio*) during spawning season

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Abstract. The purpose of the present study was to analyze changes of ovarian fluid, seminal plasma compositions, also sperm production characteristics (spermatocrit, sperm density and sperm volume) and sperm motility traits (sperm movement duration and percentage of motile spermatozoa) during spawning season (February to May) in koi, *Cyprinus carpio* (Linnaeus, 1758). Ovarian fluid and semen samples were collected from 10 female and 15 male fish in each month respectively. The results suggested that percentage of motile spermatozoa, sperm movement duration and sperm volume had not significantly changes (P>0.05), but spermatocrit and sperm density shown significant changes (P>0.05) among February, March, April and may. On the other hand, no significant differences were recorded in ovarian fluid compositions. Analysis seminal plasma compositions shown that Sodium, glucose and total protein significantly changed (P<0.05) between four term, and other seminal plasma compositions had not changes (P>0.05) during spawning season. The present study described that evaluation of these parameters will help in development of the basic knowledge about reproduction biology in koi.
Key Words: koi, spermatological parameters, biochemical compositions, spawning season.

چکیده: هدف از مطالعه حاضر، بررسی تغییرات مایع تخمدان، ترکیب پلاسمای سمینال، همچنین خصوصیات تولید اسپرم (اسپرماتوکریت، تراکم اسپرم و حجم اسپرم دهی) و ویژگیهای حرکتی اسپرم (مدت زمان حرکت و درصد تحرک اسپرم) ماهی کوی *Cyprinus carpio* (Linnaeus, 1758) در فصل تخم ریزی (اسفند تا خرداد ماه) بود. نمونه های مایع تخمدانی و سمن در هر ماه از 10 مولد ماده و 15 مولد نر جمع آوری شدند. نتایج نشان داد که درصد تحرک اسپرم ، دوره تحرک اسپرم و حجم اسپرم دهی اختلاف معنی داری نداشت (P>0/05)، اما درصد اسپرماتوکریت و تراکم اسپرم اختلاف معنی داری را در میان ماه های اسفند، فروردین، اردیبهشت و خرداد نشان داد (P<0/05). از سوی دیگر، اختلافی در ترکیبات مایع تخمدانی مشاهده نشد. آنالیز ترکیبات پلاسمای سمینال نشان داد که میزان سدیم ، گلوکز و پروتئین کل دارای اختلاف معنی داری (P<0/05) بین 4 ماه بوده و دیگر ترکیبات پلاسمای سمینال تغییرات معنی داری در طول فصل تخم ریزی نداشتند (P>0/05). مطالعه حاضر نشان می دهد که ارزیابی این پارامترها می تواند در توسعه دانش بیولوژی تولید مثل در ماهی کوی کمک کننده باشد.
کلمات کلیدی: کوی، پارامترهای اسپرم شناختی، ترکیبات بیوشیمیایی، فصل تخم ریزی

Introduction. The koi (*C. carpio*) is a member of the carp Family Cyprinidae and is one of the most widely recognized of all fishes, at least in countries where ornamental species are maintained in homes. Koi are extremely hardy, so they make excellent aquarium species as well as good laboratory species. This species has high potential in terms of breeding and cultivation due to resistance and adaptability to condition of farm. It was one of the earliest fish to be domesticated, and is one of the most commonly kept aquarium fish. The spawning mats are placed on the bottom of the pond when the broodfish approach spawning condition. Most koi breed in captivity, particularly in pond settings. Breeding usually happens after a significant temperature change, often in spring. Ovarian fluid is a maternally derived liquid that surrounds the egg mass inside the female fish and is expelled during spawning. The ovarian fluid surrounding the eggs during spawning may influence varying aspects of fertilization, such as sperm motility characteristics (Lahnsteiner 2002) or sperm-egg recognition (Amanze & Iyengar 1990). Investigating the properties of ovarian fluid in fish has been studied, and evidence suggests there is scope for further research. Seminal fluid produced by the efferent duct provides an ionic environment that maintains the viability of spermatozoa after their release from the testis into the sperm duct (Stoss 1983; Billard 1986). Season can significantly affect the composition of the seminal plasma (Ciereszko 2008). In several teleost species, the sperm quality parameters change during the spawning season in carp (*Cyprinus carpio*), tilapia (*Oreochromis mossambicus*) (Kruger et al 1984), rainbow trout (*Onchorhynchus mykiss*) (Munkittrick & Moccia 1987) and common barbel (*Barbus*

barbus) (Alavi et al 2008). The changes could be in terms of spermatozoa concentration, sperm volume, and seminal plasma composition pH or sperm motility parameters such as percentage of sperm motility and sperm velocity (Billard et al 1995; Alavi et al 2008). In cyprinids, spermatozoa are immotile in the seminal plasma because of high osmolality (Morisawa et al 1983). Numerous factors can affect the quality of semen and seminal plasma composition. This factor includes season, temperature, nutrition, stress, hormonal stimulation, milt contamination and short- term storage (Ciereszko 2008). Composition of seminal plasma are included inorganic constituent (Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+}) involved in the process of inhibition or activation of sperm motility (Morisawa 1985) and also organic compounds such as (glucose, cholesterol, protein) are found in seminal plasma (Lahnsteiner et al 1994). Compared to the evaluation ovarian fluid of salmonids fish, less attention has been paid to that of the other fish. However, comprehensive information is little on many aspects of ovarian fluid quality in cyprinids. In this study, we determined pH, ionic (K^+, Na^+, Cl^-, Ca^{2+}, Mg^{2+}) and biochemical (total protein, glucose, cholesterol) composition of the seminal plasma, ovarian fluid, also sperm production characteristics (spermatocrit, sperm density, sperm volume) and sperm motility parameters (duration of sperm motility and percentage of motility) during spawning season in the koi.

Material and Method. Female and male koi were obtained from a reared hatchery at Gonbad, Iran. To stimulate fish for spawning injected intraperitoneally: *0.5 mL* kg^{-1} O*vaprim* (sGnRHa+dompridon). Milt samples were collected during the 2010 spawning season (February, March, April and May) from 60 males. Fish were dried to avoid activation of sperm by water, urine and blood, and then milt was collected by applying gentle abdominal pressure. Ovarian fluid was also collected from mature two-year-old female koi during the 2010 spawning season (n=40).

For the analysis and evaluation of sperm 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 semen diluted placed on a glass microscope slide and motility was recorded using a camera (Panasonic wv.cp240 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. Percentages of motile spermatozoa after activation (%) were measured. Only forward moving sperm were judged motile, those simply vibrating or turning on their axes was considered immotile (Aas et al 1991). Spermatocrit was defined as the ratio the total volume of white package material to the total volume of semen ×100 (Rurangwa et al 2004). Microhaematocrit capillary tubes (75mm lengths and 1.1-1.2mm diameter) were filed with semen and end of each tube was sealed with clay. The capillary tubes were centrifuged at 3000 for 8 min in centrifuge (Sigma, 13 USA). Spermatozoa concentration was calculated with haemocytometer, with this aim a droplet of diluted semen with 0.3% NaCl solution was placed on a Thomas haemocytometer slide (depth 0.1mm) with a cover slip and counted using light microscopy. After a few minutes (to allow sperm sedimentation), the number of spermatozoa was counted at×200 magnification and expressed as spermatozoa $\times 10^9$ per ml). Sperm volume was measured in graduated tubes and expressed as µL. All experiments were performed in triplicate at room temperature (20-22°C).

For the determination of ovarian fluid and seminal plasma compositions, the ovarian fluid was pipetted gently out of the egg batch and into screw–cap tubes with minimal head space to minimize air equilibration. Ovarian fluids were centrifuged at 3000 rpm for 8 min. Sperm samples were centrifuged at 3000 rpm g per 8 min at 4°C and then seminal plasma was collected. Plasma was centrifuged twice to avoid possible contamination with spermatozoa. The pH of ovarian fluids and seminal plasma were immediately determined using a laboratory pH meter (pH meter, Iran 762) and samples were frozen at -20^{0C} until analysis. Two mineral (Ca^{+2} and Mg^{+2}) and three biochemical parameters (total protein, glucose and Cholesterol) of the ovarian fluid and seminal plasma were measured spectrophotometric method (S2000-UV/VIS England). The

concentration of Na^+ and K^+ were determined with flame photometer (Jenway PFP, England) (standard kits from parsazmoon, Tehran, Iran).

A data analysis was done with ANOVA (Duncan test) using SPSS version 16. Before Analysis data by ANOVA Duncan test were used for normality of data distribution and homogeneity of variance. A one-way analysis of variance(ANOVA) was carried out to determine variation of on sperm movement duration, seminal plasma pH, $Na^{+,}$ K^+, Ca^{2+} and Mg^{2+}, Spermatocrit, total protein, glucose, cholesterol, sperm density, sperm movement duration and sperm volume during spawning season. The Duncan test was used for comparisons between means at a 0.05 significant level. All mean values represent mean ± standard deviation (S.D.).

Results and Discussion. Ovarian fluid compositions are presented in Table 1. As the seen the table, among pH and ionic (Na^+, K^+, Cl^-, Ca^{+2} and Mg^{+2}) and biochemical (total protein, glucose) composition of ovarian fluid were not observed significantly changes (P> 0.05) during spawning period. Sperm production characteristics (spermatocrit and sperm density) were observed increased in beginning to the middle part of the spawning period and then gradually decreased towards the end of the reproductive period, peaked during the initial part of the spawning period, and differences significant were observed (P<0.05) in breeding season (Figs 3-4).

On the other hand, no significantly changes recorded (P>0.05) for sperm movement duration, percentage of motile spermatozoa and semen volume during spawning season. The seminal plasma compositions are shown in Table 2. According to results, significantly changes in values of sodium, glucose and total protein during the spawning season were detected (P<0.05): a decrease from the beginning to the middle part of the spawning period and then an increase towards the end of the reproductive period was detected, but Na^+ concentration gradually increased from the beginning to the end of the reproductive period, peaked during the medium part of the (April) of the spawning period. On the other hand, no differences significant were obtained (P>0.05) from values of the pH and K^+, Cl^-, Ca^{2+} and Mg^{2+} ion concentrations of seminal plasma. The higher content of Ca^{2+} and Mg^{2+} ions were recorded during the middle part of the spawning period (March: 0.71 ± 0.21 and 1.63 ± 0.39 respectively) compared to the beginning (February: 0.64 ± 0.90 and 1.40 ± 0.44 respectively) or the end (May: 0.64 ± 0.32 and 1.44 ± 0.32 respectively). Also K^+ ion concentrations gradually decrease from the beginning toward the middle and then increase the end part of the spawning period was detected.

Table 1

Concentrations principal inorganic ions and biochemical compositions of the ovarian fluids of koi

Parameters	February	March	April	May	Statistics
Na^+ (mmol L^{-1})	128.40 ± 3.5	130 ± 1.8	125 ± 4.3	134 ± 2.7	NS
K^+ (mmol L^{-1})	2.26 ± 0.13	2.54 ± 0.32	2.44 ± 0.35	2.39 ± 0.41	NS
Cl^- (mmol L^{-1})	137.2 ± 5.1	134.6 ± 6.2	134.5 ± 4.2	132.2 ± 3.5	NS
Ca^{+2} (mmol L^{-1})	0.49 ± 0.18	0.51 ± 0.42	0.53 ± 0.37	0.50 ± 0.26	NS
Mg^{+2} (mmol L^{-1})	0.66 ± 0.14	0.63 ± 0.22	0.64 ± 0.23	0.63 ± 0.29	NS
pH	8.1 ± 0.12	8. 3± 0.10	8.3 ± 0.11	8.2 ± 0.10	NS
glucose (mg l^{-1})	3.3 ± 0.19	3.4 ± 0.68	3.6 ± 0.49	3.7 ± 0.34	NS
total protein (gdl^{-1})	3.5 ± 0.76	3.28 ± 0.52	3.24 ± 0.47	3.36 ± 0.41	NS

Values without the same superscripts are not significantly different.

Discussion. There is seasonal variation in quality of male gamete in some carp. Changes in the quality of semen during spawning season have been reported in teleosts (Billard et al 1977). In this study, the maximum and minimum spermatocrit and sperm density were recorded in February and May. A similar result has been reported for spermatocrit in rainbow trout (*Onchorhynchus mykiss*) (Munkittrick & Moccia 1987). In contrast to our research, Suquet et al (2005) reported sperm density and spermatocrit were not changed in Atlantic cod (*Gadus morhua*) during spawning season. In general, sperm production characteristics (spermatocrit and sperm density) are used to evaluation sperm quality (Rurangwa et al 2004).

Table 2

Changes in seminal plasma quality during the spawning season

Parameters	February	March	April	May	*Statistics*
Na^{+} (mmol L^{-1})	116.73±14.16 ^{d}	131.42±11.53 ^{cd}	156.12±18.21 ^{a}	155.68±34.29 ^{ab}	*P*< 0.05
K^{+} (mmol L^{-1})	22.61±2.02	24.46±6.44	23.27±1.09	28.38±6.36	NS
Cl^{-} (mmol L^{-1})	139.45±14.41	142.31±16.22	145.52±7.63	147.42±17.08	NS
Ca^{+2} (mmol L^{-1})	0.70±0.11	0.69±0.18	0.68±0.09	0.66±0.28	NS
Mg^{+2} (mmol L^{-1})	1.57±0.36	1.60±0.28	1.52±0.34	1.51±0.17	NS
pH	8.8±0.12	8.7±0.10	8.7±0.11	8.6±0.09	NS
Glucose (mg l^{-1})	0.21±0.028 ^{a}	0.16±0.006 ^{bd}	0.14±0.22 ^{cd}	0.13±0.05 ^{bc}	*P*< 0.05
Total protein (gdl^{-1})	0.08±0.003 ^{a}	0.05±0.001 ^{ab}	0.04±0.003 ^{bc}	0.03±0.002 ^{d}	*P*< 0.05
Cholesterol (mg l^{-1})	0.026±0.23	0.059±0.42	0.023±0.003	0.018±0.002	NS

Different letters correspond to significantly different results.

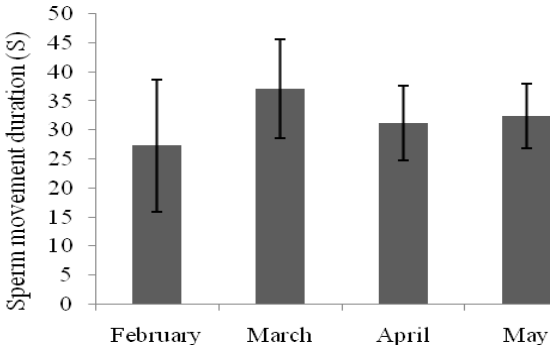

Fig. 1. Changes in sperm movement duration during the spawning period (n=60 males).

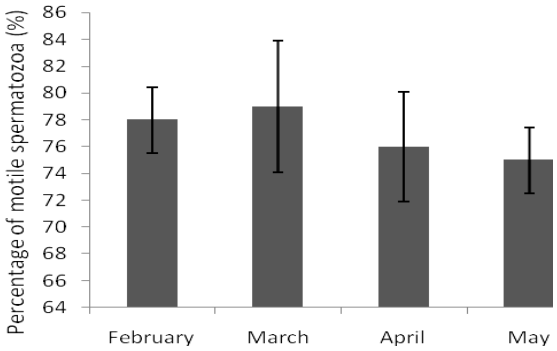

Fig. 2. Changes in percentage of motile spermatozoa during the spawning period (n=60 males).

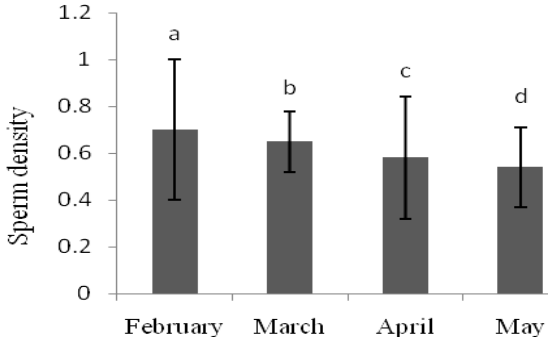

Fig. 3. Changes in sperm density of koi during spawning season (different letters correspond to significantly different results (*P*< 0.05) (n=60 males).

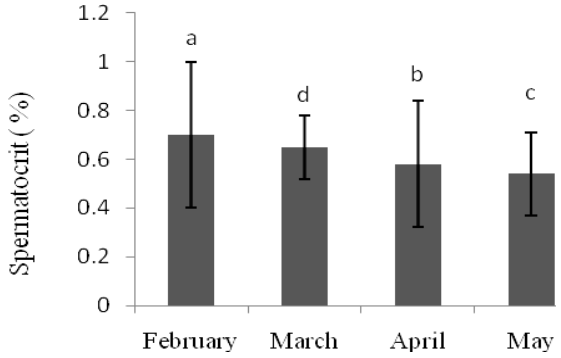

Fig. 4. Changes in spermatocrit of koi during spawning season (different letters correspond to significantly different results (P< 0.05) (n=60 males).

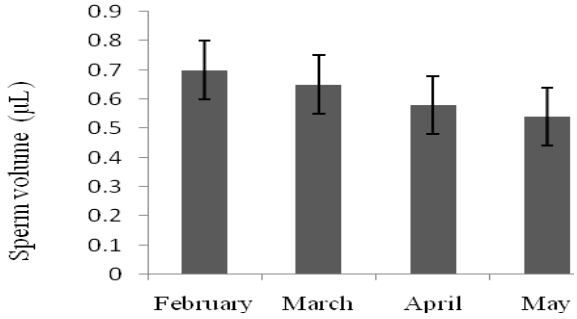

Fig. 5. Changes in sperm volume of koi during the spawning season (n=60 males).

In teleost fish, sperm motility is a one of biomarkers for assessment of sperm quality (Lahnsteiner et al 1998). Also, semen with an increased percentage of motile spermatozoa would have a better chance to successfully fertilize larger numbers of eggs. The present study demonstrated that sperm movement duration and percentage of motile spermatozoa were not changed during spawning period. In agreement with our results, Suquet et al (1994) observed percentage of motile spermatozoa during the reproductive season of cod (*Gadus morhua*) were not difference significant (P>0.05). Also, similar result about sperm movement duration has been reported in rainbow trout (*Onchorhynchus mykiss*), brook trout (*Salvilenus alpinus*) and Atlantic salmon (*Salmo salar*) (Benau & Terner 1980). In this study, sperm volume was not changed during spawning season, but other workers reported that seasonal changes in semen volume may due to the age of spawner, the maintenance circumstance of the spawner (Buyukhatipoglu & Holtze 1984) and inducing agents (Billard & Marcel 1980). The present study analyzed ovarian fluid compositions in females of koi. Because, there is little information is available about ovarian fluid compositions in cyprinids (especially ornamental fish) and more study have been done on females of salmonids family. We compared our results with other results obtained from salmonids fish. In this research observed that pH, ionic (Na^+, K^+, Cl^-, Ca^{+2} and Mg^{+2}) and biochemical (total protein, glucose) composition of ovarian fluid were not changed during spawning period, whereas Lahnsteiner et al (1995) reported ovarian fluid composition (Na^+, K^+, Ca^{+2} and glucose) of four species includes rainbow trout (*Onchorhynchus mykiss*), brook trout (*Salvilenus alpinus*), lake trout (*Salmo trutta lacustris*) and danube compounds (Na^+, K^+, Cl^-, Ca^{2+} and Mg^{2+}) and low concentrations of organic substances such as sugars, cholesterol and protein (Rurangwa et al 2004). The ionic composition of seminal plasma may vary through during the reproductive season (Alavi & Cosson 2006).

The results of this experiment in koi show that Na^+ ion concentration present a significant difference (P<0.05) during spawning season. In agreement with our results, Suquet et al (2005) reported that during breeding season of Atlantic cod (*Gadus morhua*)

Na^+ ion concentration was significant change (P<0.05). Several studies have shown that the extent of inhabitation of sperm motility by K^+ ion change during spawning season. The seasonal variation of K^+ ion concentration in seminal plasma of cyprinids has been reported and disagree to results were obtained in the present experiment. Limit information is available on the organic composition of the carp semen. Munkittrick & Moccia (1987) reported a decline in rainbow trout seminal plasma concentration of sodium, potassium and chloride ions during the spawning season. These differences may due to the differences in secretary activity of the spermatic duct epithelium of individuals since the formation of the seminal plasma in fish (inorganic as well as organic compounds) is a secretion process of the spermatic duct epithelium (Marshal 1986; Marshal et al 1989; Lahnsteiner et al 1994). The presence of glucose in seminal plasma is necessary for high energy demand of the testes during spermatogenesis or lipid synthesis of spermatozoa (Soengas et al 1993). The protein content is highly variation throughout the year (Billard & Cosson 1990). In this study, significant changes of organic composition of seminal plasma (glucose and total protein) were detected. Protein changes are importance because of the protective role they play for spermatozoa in many fish species (Rouxel et al 2008). In addition, White & Macleod (1963) indicated that protein has a protective role. There is little information about lipid and cholesterol and their role in seminal plasma. Billard et al (1995) expressed that lipid and cholesterol has a protective effect against environment change (especially water temperature) when semen is released.

Conclusions. The information obtained from the present study can lead to more efficient gamete management and used to select high quality mature males for fertilizing eggs in a commercial ornamental fish aquaculture.

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Received: 18 September 2010. Accepted: 25 October 2010. Published online: 27 October 2010.
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How to cite this article:
Taati M. M., Jafaryan H., Mehrad B., Golpour A., 2010 Changes of ovarian fluid compositions and sperm quality parameters in koi (ornamental *Cyprinus carpio*) during spawning season. ABAH Bioflux **2**(2):83-90.