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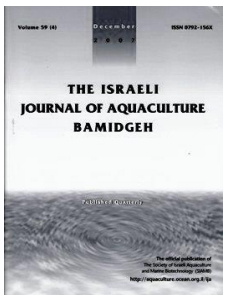

Effects of Seminal Plasma Properties on Percentage and Duration of Shabut (*Barbus grypus* Heckel,1843) Sperm Motility

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Abstract

The aim of this study was to demonstrate the effects of seminal plasma properties (Ca^{2+}, Na^{+}, K^{+} and Mg^{2+}, glucose, urea, triglyceride, total protein concentrations, pH and osmolality) on progressive sperm motility percentage and duration in *Barbus grypus*. Osmolality (mOsmol/kg) ranged from 81-263, while ionic contents (mM) varied between 20-79 Na^{+}, 1.2-58.4 K^{+}, 0.50-1.05 Ca^{2+}, and 0.51-1.44 Mg^{2+} respectively. Spermatozoa density was 15.12±1.11 $x10^{9}$/mL, semen volume 3.87±0.41 mL while total protein, urea triglyceride and glucose were 0.60±0.17 mg/mL, 4.08± 0.08 mg/dL, 8.42±0.62 mg/dL, 5.17±0.11 mg/dL respectively. The data obtained allows comparison of variations in semen parameters and enables us to determine how these parameters relate to motility characteristics. The groups formed by hierarchical cluster analysis (CA) based on the semen properties corresponded to sperm motility characteristics. According to principal component analysis (PCA) results, osmolality, protein concentration, Na^{+} and K^{+} were strongly correlated with motility characteristics and were responsible for total variation in data compared to other parameters. Multivariate statistical methods as well as ionic ratios were used to analyze the data.

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Introduction

Fish seminal plasma is composed of minerals, enzymes, and metabolites such as triglycerides, glycerol, fatty acids, glucose, free amino acids, and proteins (Cabrita et al. 2009). These constituents of seminal plasma support sperm cells and provide an optimal environment. Changes in the composition of seminal plasma might impair its protective functions and may also harm sperm quality which affects the total period of sperm motility and percentage of motile sperm observed visually (Stoss 1983; Cosson 2004; Ciereszko 2008). In other words, the presence or absence of the mineral constituents influences osmolality, pH, and consequently, percentage and duration of sperm motility (Morisawa et al. 1983).

Osmolality levels affect motility without consideration of temperature, pH, or composition of activating medium in many fish species (Ciereszko 2008). Moreover, the interactions and influence of some ions such as K^+ and Ca^{2+} are the key to understanding sperm motility for many fish groups (Alavi and Cosson 2006). Even though it is known that spermatozoa motility is affected positively after incubation in K^+-rich media, spermatozoa of Cyprinid species are less sensitive to K^+ than the Salmonidae and Acipenceridae species (Alavi and Cosson 2006; Redondo-Muller et al. 1991). The influx of extracellular Ca^{2+} into sperm cells has a unique capacity to activate sperm motility in *Cyprinus carpio* sperm (Krasznai et al. 2000). Additionally, Na^+ has been described as having a secondary role in the activation and regulation of sperm motility (Ciereszko 2008). Intracellular alkalinization induced activation of sperm motility as a result of activation of the Na^+/H^+ exchanger (Marian et al. 1993). Some metabolites such as triglycerides, fatty acids, and glucose, are indicative of energy metabolism, while other constituents such as lipids and proteins may also affect sperm motility (Lahnsteiner et al. 1993).

Some studies related to sperm motility and/or characteristics of seminal plasma have been carried out on some Cyprinid species such as *C. carpio* (Redondo-Muller et al. 1991; Morisawa et al. 1983), *Tinca tinca* (Linhart et al. 2003), *Alburnus alburnus* (Lahnsteiner et al. 1996), *Barbus barbus* (Alavi et al. 2009), *Ctenopharyngodon idella* (Bozkurt et al. 2008), *Hypophthalmichthys molitirix* (Rahman et al. 2011), *Abramis brama* (Glogowski et al. 1999), *Aristichthys nobilis* (Khara et al. 2013), *Carassius auratus* (Morisawa et al. 1983) and *Barbus sharpeyi* (Alavi et al. 2010). *Barbus grypus* is abundant in the Tigris-Euphrates basin, encompassing the drainage basin of the Tigris and Euphrates rivers and their tributaries (Coad 1996). Its spawning period is from late April-May to mid June-early August. It is considered an omnivorous species (Sahinoz et al. 2007; Kahkesh et al. 2011). Although some parameters related to motility characteristics of *B. grypus* sperm are known (Ogretmen et al. 2014), there is no knowledge of the biological charachteristics such as seminal plasma composition, osmolality, pH, sperm volume, and density, of its semen.

The aim of this study was to reveal the effects of seminal plasma properties (Ca^{2+}, Na^+, K^+ and Mg^{2+}, glucose, Urea, Triglyceride, total protein concentrations, pH and osmolality) on percentage and duration of progressive sperm motility. This was done using Hierarchical Cluster Analysis (CA) in an effort to classify samples based on measured seminal plasma characteristics, and then to determine which of these components significantly affected the total variation among the samples with Principal Component Analysis (PCA).

Materials and Methods

Broodstock management. This study was conducted in June 2012 at the aquaculture department of General Directorate State Hydraulic Works, Fish Production Station Atatürk Dam Lake, Urfa, Turkey. Six year old *B. grypus* males used in this study were produced in 2006 to enhance fish stocks of dam lakes in the Euphrates basin. They were held in sand-gravel ponds under a natural photoperiod regime. Twelve mature *B. grypus* (average weight 960.26±197 g) mean total length 50.3±7.92 cm) were selected randomly from a pond and transferred to a hatchery and maintained in a 10,000 L concrete pond. Fish were fasted 48 h prior to sperm collection. Water conditions were

24.0±0.3°C, 8.9±0.2 mg/L oxygen, pH 8.3, salinity 0.1% during the spawning season of June 2012.

Sperm collection and motility analysis. Males were anesthetized with 2-phenoxyethanol (500 µl/L) and injected with a single injection of 1 mg/kg of carp pituitary extract (CPE). After 24 h the males were anesthetized again and their sperm was collected by manual abdominal stripping. The sperm was stored in glass tubes and only samples that were uncontaminated with water, blood, urine, or feces were used. Sperm samples were stored in ice until analysis. An activating solution of 0.3% NaCl was used to measure motility. Motility was evaluated with a light microscope equipped with a phase-contrast attachment (Nikon Ci-S) at 400X magnification. About 1 µl of semen was placed on a glass microscope slide and 50 µl of activation solution was added. Sperm motility was measured immediately after sperm activation. Sperm motility was tested visually after activation as a percentage of progressively motile spermatozoa, and total duration of motility (in seconds). The sperm motility percentages were estimated as the percentage of cells that exhibited progressive forward movement (Horváth et al. 2003), and the duration of motility was determined as the time when forward movement stopped and circular movement began. Spermatozoa density was determined according to the hemocytometric method (Ciereszko and Dabrowski1993) and was expressed as $x10^9$/mL.

Measures of seminal plasma composition ionic content, metabolite composition, pH and osmolality. Seminal plasma was collected after centrifugation at 1370 g for 10 min at room temperature and stored in Eppendorf (Sigma-Aldrich, Germany) vials at -80 °C until analysis. Seminal plasma was centrifuged twice to avoid possible contamination with spermatozoa. Major cation and metabolite levels such as Na^+, K^+, Ca^{2+}, Mg^{2+}, glucose, protein, cholesterol, triglyceride and urea levels, were determined with original Abbott-Aeroset auto analyser kits (Chicago, IL, USA). Osmolality was measured with a cryoscopic osmometer (Gonotech, Osmomat 30, Germany) and is expressed as mOsmol/kg. pH was measured with a micro pH meter (WTW, pH 320) within 30 min of sampling. All measurements were replicated three times.

Data Analysis. The data are presented as minimum and maximum values, and mean and standard error of mean (SEM). Pearson Correlation coefficients were used to show the paired associations between measured variables. The relationship between the measured parameters and sperm motility characteristics are presented as linear regression equations. Statistical significance is determined at $p < 0.01$ and $p < 0.05$. The data of the defined semen variables were analyzed by hierarchical cluster analysis (CA) to identify similarities among the semen samples based on the measured variables from individual fish semen. With CA a complete linkage method was used and the variation between samples was calculated using square Euclidean distances. The dendrogram similarity scale ranged from zero (greatest similarity) to 25 (least similarity).

Variability due to defined variables among the semen samples is explained by means of Principal Component Analysis (PCA). To equalize the effect of variables measured on different scales, all data were normalized or standardized for PCA and CA respectively prior to analysis. All statistical analyses were carried out using SPSS version 19.0.

Results

Spermatological parameters and seminal plasma composition of the semen samples. The minimum, maximum, means and SEM of the chemical composition levels, osmolality, and pH in the seminal plasma, and some spermatological parameters of *B. grypus* are shown in Table 1.

Table 1. The chemical composition levels, osmolality, and pH, in the seminal plasma and some spermatological parameters (volume, density, motility percentage and duration) of Barbus grypus (N=12).

	Min	*Max*	*Mean*	*SEM*
Protein (mg/ml)	0.06	1.91	0.6	0.17
Urea (mg/dL)	4	5	4.08	0.08
Triglyceride (mg/dL)	7	14	8.42	0.62
Glucose (mg/dL)	5	6	5.17	0.11
Ca^{2+} (mM)	0.5	1.05	0.64	0.05
Na^{+} (mM)	20	79	33.32	6.07
K^{+} (mM)	1.2	58.4	16.9	5.46
Mg^{2+} (mM)	0.51	1.44	1.07	0.08
pH	6.9	8.5	7.56	0.18
Osmolality (mOsm/kg)	81	263	141.42	19.2
Semen Volume (ml)	2.1	6.2	3.87	0.41
Sperm Density ($x10^9$/mL)	9.85	22.56	15.12	1.11
Sperm Motility (%)	57.5	95	79.58	3.34
Sperm Motility duration (s)	33	72	48.67	4.18

The linear relationships and the pairwise associations between measured variables and motility characteristics. The regression coefficients (r) and the coefficients of determination (R^2) between the semen parameters and sperm motility characteristics are also presented in Table 2. Both the percentage of motile cell and duration of sperm motility decreased when osmolality of seminal plasma increased. Relatively, sperm motility characteristics at pH levels around 7.0 were lower than at pH levels of 8 and 8.5. Enhanced protein, Ca^{2+}, Na^{+} and K^{+} concentrations in the seminal plasma improved sperm motility. Sperm motility characteristics were more or less constant in relation to Mg^{2+} concentrations. Semen volume and density were strongly related to sperm motility characteristics.

Table 2. Slope, intercept, and R^2 of the regression line for the scattering comparison and Pearson correlation coefficients (r) between progressive motility percentage and duration and the other parameters.

	Dependent variables							
	progressive motility percentage				*progressive motility duration*			
	Regression coefficients		*R^2*	*r*	*Regression coefficients*		*R^2*	*r*
Independent variables	*Slope*	*Intercept*			*Slope*	*Intercept*		
Protein	10.99	72.95	0.31	0.561	20.11	36.53	0.67	0.819**
Urea	3.18	66.59	0.01	0.079	11.27	2.64	0.05	0.225
Triglyceride	1.86	63.95	0.12	0.345	4.81	8.22	0.51	0.714**
Glucose	9.5	30.5	0.1	0.32	23.8	74.3	0.41	0.640*
Ca^{2+}	24.57	63.9	0.14	0.371	58.78	11.15	0.5	0.709**
Na^{+}	0.25	71.24	0.21	0.455	0.56	31.84	0.54	0.733**
K^{+}	0.29	74.68	0.23	0.474	0.65	37.75	0.71	0.843**
Mg^{2+}	9.044	69.86	0.04	0.212	10.32	37.58	0.03	0.193
pH	12.97	-18.48	0.47	0.682*	13.84	-55.97	0.34	0.581*
Osmolality	0.01	65.51	0.33	0.572	0.19	21.54	0.77	0.881**
Semen Volume	4.77	61.13	0.34	0.579*	9.22	13.01	0.8	0.895**
Sperm Density	1.4	58.4	0.22	0.464	3.46	3.53	0.84	0.914**

Similarities among the semen samples based on measured variables in relation to sperm motility. In hierarchical cluster analysis, samples are grouped based on similarities. The similarities between the semen samples are presented in a dendrogram (Fig. 1). The dendrogram similarity scales that are generated by the SPSS program range

from zero (greatest similarity) to 25 (least similarity) as linkage distances. According to the cluster analysis (CA), two well-defined clusters are visible at linkage distances of 25. The CA dendogram based on measured variables of seminal plasma also characterized sperm motility .

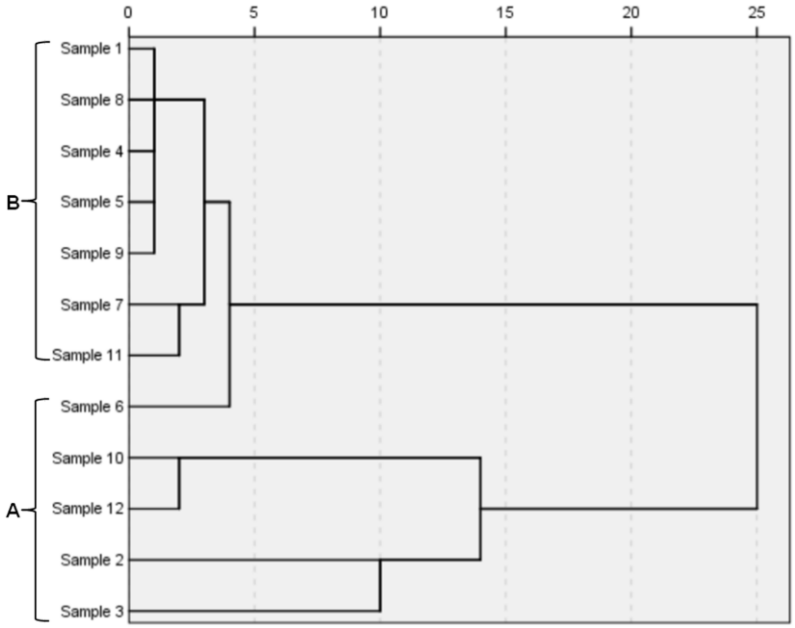

Fig. 1 Dendrogram of the Euclidean distance between the samples from each individual Barbus grypus based on the semen parameters (seminal plasma protein concentration, Urea, Triglyceride, Glucose, Ca2+, Na+, K+, Mg2+ ,pH, Osmolality, Volume, Density). Group A had motility duration >55 s and percentage >80%, Group B had motility duration <55 s and percentage <80% (except for sample 9 (85%) and 11 (95%)).

Source of variability among the semen samples. Principal component analysis (PCA) was applied to the standardised values of the measured variables of semen. According to PCA results, PC1 explained 87.5 % of the total variance in the data while PC2 explained 12.1%. In other words, first and second components together revealed 99.6 % of the cumulative variation. The score plot of the samples for PC1 and PC2 shown in Fig. 2 are an indication of the distribution of the samples. All samples with more than 50 s motility duration, and 80 % motility, are separate from the other samples and displayed within encircled areas.

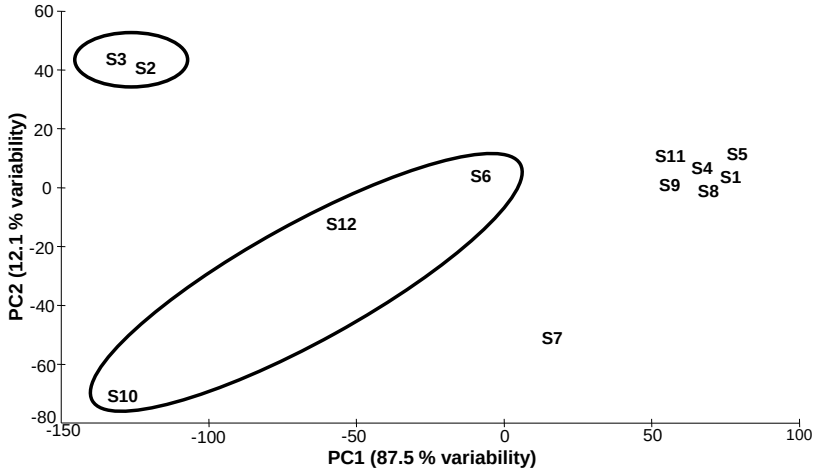

Fig. 2 Principal component analysis (PCA) scores plot for semen samples (S) based on the variables seminal plasma protein concentration, Urea , Triglyceride, Glucose, Ca2+, Na+, K+, Mg2+ ,pH, Osmolality, Volume, Density. First component (PC1) explained 85.5% of total variation; second component (PC2) explained 12.1 % of total variation.

Discussion

Sperm motility determines the fertilizing ability of semen (Cosson et al. 1991; Lahnsteiner et al. 1997). There is a correlation between seminal plasma biochemistry and sperm motility in fish (Linhart et al. 2003; Alavi et al. 2004; Bozkurt et al. 2008; Alavi et al 2009; Li et al. 2011). Seminal plasma with its unique composition reflects the functions of the reproductive system (Ciereszko et al. 2000) and offers considerable

knowledge of semen motility characteristics (Alavi et al. 2004; Li et al. 2009). Basic knowledge of seminal plasma composition is important and necessary in aquaculture especially for new aquaculture species such as *B. grypus*. Our study reveals the optimum spermatological parameters for *B. grypus* culture and supports new information on the relationship between seminal plasma composition and motility characteristics.

The variation in concentration levels of the measured ions, Ca^{2+}, Na^{+}, K^{+} and Mg^{2+} and others may have been the result of the stage of sexual maturity of the six-year old *B. grypus* males used in the study. *B. grypus* males begin to mature sexually after five or six-years under conditions of the Ataturk Dam Lake, Turkey. This data enables a comparison of variations in semen parameters and to understand sperm motility.

The CA helped to identify relatively homogeneous groups of samples based on semen quality. According to these the dendrogram separated the samples into two groups, groups A and B, (Fig. 1). Sperm motility (percentage and duration) was also shown in these groups. In Group A, motility duration was >55 s and percentage of motile sperm was >80%. In Group B, motility duration was <55 s and percentage of motile sperm was <80%.

The PCA showed variables responsible for variation between the semen samples. The plot of the first two components supported the CA results and differentiated samples 2 and 3; from samples 6, 10 and 12, and from the other samples (Fig. 2). According to the PCA results, the first component was dominated by osmolality which was responsible for the largest variance, and protein concentration responsible for the second largest variance. Osmolality, protein concentration, Na^{+} and K^{+} respectively were responsible for the total variation in the data. These variables affected the differences between the samples though sample 7 was separated from the others based on its principal component plot (Fig. 2). This sample had a relative optimum protein concentration (0.93 mg /mL), but a low osmolality value (103 mOsm/kg), that moved it to group B instead of group A (Fig. 1). Motility decreased when osmolality and pH were lower than 150 mOsmol/kg and 7.5 respectively. Osmolality was higher than 200 mOsmol/kg and significantly enhanced sperm motility; highest osmolality value was 263 mOsmol/kg in this study. Regression equations indicated that enhanced osmolality levels positively affect motility duration (Table 2). Group A described in Fig. 1 had osmolalities higher than 150 mOsmol/kg and pH levels between 7.5-8.5. The optimum pH and osmolality values were similar to other Cyprinid species. In other Cyprinids, osmolality levels were between 258 and 346 mOsmol/kg in *C. carpio* (Redondo-Muller et al. 1991; Kruger et al. 1984), 230±82 mOsmol/kg in *T. tinca* (Linhart et al. 2003), 254-267 mOsmol/kg in *A. alburnus* (Lahnsteiner et al. 1996) 249-294 mOsmol/kg in *B. barbus* (Alavi et al. 14) for mature individuals and 274.5±9 mOsmol/kg in *B. sharpeyi* (Alavi et al. 2010).

Sperm volume was 2.1-6.1 mL in *B. grypus* and relatively lower in *C. idella* (Bozkurt et al. 2008), and *C. carpio* (Bozkurt et al. 2009). This could be because the males used in the study were approximately 1 kg and in their second year of maturation. In cyprinids, sperm concentrations varied at different levels depending on the species, [2.82±0.08 $x10^{9}$ mL *H. molitirix* (Rahman et al. 2011), 11.68± 4.33 $x10^{9}$ /mL for *A. brama* (Glogowski et al. 1999), 15.43±0.72 $x10^{9}$ /mL for *C. idella* (Bozkurt et al. 2008), 28.80± 1.23 $x10^{9}$ /mL for *C. Carpio* (Bozkurt et al. 2009), 14.6 ±2.2 $x10^{9}$ /mL for *B. sharpeyi* (Alavi et al. 2010)]. For *B. grypus*, maximum sperm concentrations were $x10^{9}$ /mL, average as $x10^{9}$/mL. In this study, the samples 2, 3, 10, 12, had a higher motility percentage and duration, greater than 4.8 mL sperm volume, and 19.81±1.00 $x10^{9}$ mL sperm concentration.

There was more variation in seminal plasma metabolites and protein concentrations than in urea, triglyceride, and glucose levels. In group A (Fig. 1), all triglyceride concentrations were greater than 7 mg/dL while triglyceride levels in group B which had less motility percentage and duration values, were less than 7 mg/dL. Protein concentrations and the other metabolites were relatively higher than other investigated Cyprinid species (Alavi et al. 2010; Bozkurt et al. 2008; Bozkurt et al. 2009).

K^{+}, Na^{+} and Ca^{2+} levels in seminal plasma have been measured as 46.01±1.52, 1000.44±2.37, 3.01±0.09 mM for *H. molitirix* (Rahman et al. 2011); 47±10.17, 97±3.9,

3.6±2.47 mM for *Aristichthys nobilis* (Khara et al. 2013); 1.9 ±0.6, 18.4±1.3, 0.6 ±0.2 mM for *T. tinca* (Linhart et al. 2003); 105.1±2.24, 67.12±1.06, 1.96±0.17 mM for *C. carpio* (Bozkurt et al. 2009), 28.8 ±0.9, 70.0±3.4, 2.1±0.1 mM for *B. Sharpeyi* (Alavi et al. 2010) respectively. In this study, average K^+, Na^+ and Ca^{2+} concentrations were 16.90±5.46, 33.32±6.07, 0.64±0.05 mM respectively for *B. grypus*. Mg^{2+} concentration was 1.07±0.08 mM for *B. grypus*, and 5.7±3.44 mM for *A. nobilis* (Khara et al. 2013), 0.5±0.1 mM for *T. tinca* (Linhart et al. 2003), and 0.9±0.1 mM *B. Sharpeyi* (Alavi et al. 2010). In this study, Na^+ values were less than 20 mM in group B, and more than 20 mM in group A. K^+ concentration was 27.5 mM in sample 12 with 72 s, while 45.1 mM in sample 2 with 59 s. As in other Cyprinids (Alavi et al. 2009), not only excess K^+ but also lack of K^+ had an inhibitory effect on motility duration. Ionic ratios of seminal plasma (quantity of ions) were related to sperm motility characteristics. The optimum ratios are Na^+/K^+ (1/1), Na^+/ Ca^{2+} (40), Ca^{2+}/Mg^{2+} (2) in Cyrinid species such as *Carassius auratus* and *C. Carpio* (Morisawa et al. 1983). Na^+/K^+ ratio affects sperm motility duration; Na^+/Ca^{2+} and Ca^{2+}/Mg^{2+} ratios are related to percentage of motile spermin *Rutilus rutilus caspicus* during spawning (Golpour et al. 2011). In our study, the ionic ratios of seminal plasma related to groups A and B were described with CA (Fig. 1). Samples in group B had lower motility levels with ratios of $Na^+/K^+ > 2$, $Na^+/ Ca^{2+} < 12$, and $Ca^{2+}/Mg^{2+} <2$, than group A .

This study shows that chemical composition levels, osmolality and pH of seminal plasma, sperm volume, and density affect sperm motility and duration. It also offers new information on semen composition of *B. grypus* and the relationship between this and motility thus providing a basis for further studies on reproduction, not only for *B. grypus* but also for other Cypinid species. Based on semen properties the CA and PCA techniques demonstrated the effect of the studied variables with comparable and comprehensible data on ionic ratios of seminal plasma that should be used.

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