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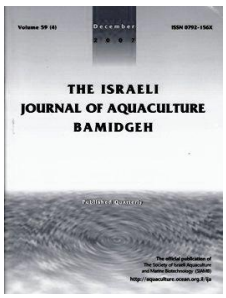

Quantitative Characteristics and Short-term Storage of *Salmo coruhensis* Sperm

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Abstract

This study determined spermatologic characteristics of semen from *Salmo coruhensis* and analyzed an extender for its short-term storage. Sperm was collected from 15 males in the spawning season by abdominal massage. Volume, motility, duration of motility, density, spermatocrit, and pH of the sperm were determined, as well as the correlation between these characteristics and mean body weight and total length. The sperm volume was 1.6±0.64 ml, motility was 90±5.17%, duration of motility was 129.5±38.78 s, density was 13.0±4.93 × 10^9/ml, spermatocrit was 41.5±6.63, and pH was 7.4±0.16. Percent motile sperm negatively correlated with fish weight and total length and positively correlated with motility duration (p<0.05). Sperm stored in an extender containing glucose remained motile for 24 days, while sperm stored in extenders containing calcium chloride or magnesium chloride remained motile for only 14 and 18 days, respectively.

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Introduction

Quality gametes from broodstocks in aquaculture are important for obtaining quality offspring. Analysis of sperm characteristics provides essential data for improving gamete handling as well as short-term and long-term storage of sperm to be used in artificial fertilization (Billard et al., 1995; Linhart et al., 2004). A lack of knowledge regarding sperm limits the efficient and economic management of male broodstock while information on sperm can help to select better semen and improve protocols for cryopreservation and artificial propagation.

Commonly-evaluated sperm parameters include volume, spermatozoa concentration, number of spermatozoa in the sperm, percentage of motile sperm, sperm pH, and fertilization success (Billard et al., 1993, 1995; Bloom and Ottobre, 2001; Tekin et al., 2003). Sperm quality, seminal plasma characteristics, and the preservation of sperm have been studied in pike perch (Bokor et al., 2008) and mirror carp (Bozkurt et al., 2009). In *Oncorhynchus mykiss*, milt quality (Aral et al., 2007) and the effect of ascorbic acid supplementation on sperm quality (Canyurt and Akhan, 2008) have been studied while sperm has been cryopreserved in a glucose-based extender (Bozkurt et al., 2005). The relationships between body condition, physiological parameters, and biochemical parameters have been studied in *Salmo trutta fario* sperm (Bozkurt et al., 2006a), as has the fertilizing ability of short-term preserved spermatozoa of *S. trutta abanticus* (Hatipoğlu and Akçay, 2010) and the relationship between seminal plasma composition and sperm quality in *S. trutta magrostigma* (Bozkurt et al., 2011).

The native range of brown trout includes Europe, western Asia, and northern Africa (Behnke, 2002). *Salmo coruhensis* is a newly identified species from the lower and middle parts of streams and rivers that drain into the southern and southeastern Black Sea (Turan et al., 2009). The objectives of this study were to determine basic quality parameters of sperm of wild *S. coruhensis*, to examine the relationships between body size and sperm characteristics, and to develop a suitable extender for short-term storage of *S. coruhensis* semen for use in hatcheries during the spawning season.

Materials and Methods

Broodstock and collection of sperm. The experiment was carried out at the Iyidere Fisheries Research Center (IFRC) in Rize University, Rize, Turkey. Brooders were held in raceways under a natural photoperiod regime and fed a commercial trout diet at 2% of their body weight per day. During the spawning season, the average water temperature was 12.9±0.58°C (8.0-18.5°C). Fifteen mature *S. coruhensis* males (206.7±58.49 g, 26.9±2.6 cm) were randomly selected from this broodstock and used as semen donors. The fish were not fed 48 h prior to sperm collection, then anesthetized in 30 ppm benzocaine. Their abdomens and urogenital papillas were dried before stripping. The semen was collected into individual 10-ml graded glass tubes by gentle abdominal massage from the anterior portion of the testis towards the genital papilla. Each male was stripped once and the total volume of milt was measured. The semen samples were held on crushed ice (4ºC) until analysis, undertaken within 4 h. Special care was taken to avoid contamination of semen with urine, mucus, or blood cells.

Sperm density and spermatocrit. Sperm density was determined according to the hemacytometric method (Şahin et al., 2012). Semen was diluted 100 times by pipetting 10 µl semen into a solution of 990 µl 0.7% NaCl. One droplet of diluted semen was placed on a hemocytometer slide (depth 0.1 mm) with a coverslip and allowed to settle for 3-5 min, sperm cells were counted in 16 chamber cells using light microscopy (×400), and spermatozoa density was expressed as no. × 10^9/ml. Spermatocrit (%) was defined as 100(volume of white packed material:total semen volume), as per Rurangwa et al. (2004). Heparinized microhematocrit capillary tubes (75 × 1.1-1.2 mm) were filled with semen, one end was sealed with clay, and the tubes were centrifuged at 10,000 rpm for 10 min.

Sperm motility, duration of motility, and pH. The motility of fresh spermatozoa from each male was determined immediately after collection. The percent spermatozoa exhibiting rapid, vigorous, forward movement was determined subjectively under a

microscope (×400) by diluting the semen with an activation solution (0.3% NaCl) at a ratio of 1:100 (1 µl sperm:99 µl activation solution). The duration of the spermatozoa movement was assessed with a sensitive chronometer (1/100) that was simultaneously activated with the addition of the activation solution into the samples. Sperm motility was observed in triplicate. All experiments were performed at 17-20°C. To avoid subjective error, all measurements were carried out by the same investigator and under the same conditions. pH was measured by indicator papers (Merck 6.4-8).

Sperm extenders. Three extenders containing calcium chloride, magnesium chloride, or glucose (Liu et al., 2006) were evaluated (Table 1). The semen and extenders were kept at 4ºC prior to dilution. The semen and extender were mixed at a ratio of 1:3 (semen:extender) and placed into 2-ml plastic vials. After rapid shaking, the vials were put into a refrigerator at 4°C. No oxygen and antibiotics were added to the stored semen. Samples of stored semen were analyzed for sperm motility at 2-day intervals until motility stopped.

Table 1. Composition of sperm extenders for *Salmo coruhensis* (g/l).

Extender	*NaCl*	*KCl*	*$CaCl_2$*	*$MgCl_2$*	*$NaHCO_3$*	*Glucose*
Calcium chloride	8.75	0.20	0.20	-	0.30	-
Magnesium chloride	8.75	0.20	-	0.20	0.40	-
Glucose	7.25	0.40	-	-	0.80	2.0

Statistical analysis. The average of three measurements was used in all statistical analyses. Data are expressed as means±SD. Motility data were normalized through arcsine transformation. Pearson correlation analysis was used to estimate spermatologic parameters. Differences between parameters were analyzed by one-way analysis of variance (ANOVA). Significant means were subjected to a multiple comparison test (Tukey HSD) for post-hoc comparisons at a level of $\alpha = 0.05$. Analyses were carried out using SPSS 15.0 for Windows Statistical Software Package.

Results

Spermatologic parameters were rather variable (Table 2). Sperm weight and length significantly correlated with each other (Table 3). Percent motility significantly correlated with weight, total length, and motility duration. Sperm stored in the extender containing glucose remained motile for 24 days, while sperm stored in the extenders containing calcium chloride or magnesium chloride remained motile for only 14 and 18 days, respectively (Table 4).

Table 2. Characteristics of *Salmo coruhensis* sperm (n = 15).

Characteristic	*Mean±SD*	*Minimum*	*Maximum*
Weight (g)	206.7±58.49	126.0	328.0
Total length (cm)	26.9±2.66	23.0	32.0
Volume (ml)	1.6±0.64	0.4	2.5
Motility (%)	90±5.17	30	100
Movement duration (s)	129.5±38.78	64	217
Density ($\times 10^9$/ml)	13.0±4.93	4.8	21.5
Spermatocrit (%)	41.5±6.63	31.0	52.0
pH	7.4±0.16	7.2	7.7

Table 3. Correlations between length, weight, and spermatologic characteristics of *Salmo coruhensis*.

	Weight	*Length*	*Volume*	*Motility*	*Duration*	*Density*	*Spermatocrit*
Length	0.974*	-	-	-	-	-	-
Volume	-0.334	-0.289	-	-	-	-	-
Motility	-0.654**	-0.531**	-0.181	-	-	-	-
Duration	-0.041	0.002	-0.191	0.587**	-	-	-
Density	0.233	0.252	0.003	-0.101	0.095	-	-
Spermatocrit	-0.447	-0.489	0.325	0.213	0.071	0.353	-
pH	-0.153	-0.144	-0.023	0.144	0.038	0.258	0.190

* $p<0.01$, ** $p<0.05$

Table 4. Percent motile *Salmo coruhensis* sperm stored in different extenders.

Day	Extender			Undiluted semen
	Calcium chloride	Magnesium chloride	Glucose	
2	69.4±5.3[a]	78.9±6.5[b]	92.8±4.4[c]	84.4±5.3[b]
4	57.8±6.7[a]	72.2±8.3[b]	87.8±5.7[c]	81.7±7.1[c]
6	48.9±6.0[a]	60.0±7.1[b]	85.0±6.1[c]	77.2±6.2[c]
8	42.2±8.3[a]	58.9±7.8[b]	72.8±6.2[c]	52.2±8.3[b]
10	20.6±7.3[a]	40.0±8.7[b]	68.9±9.3[c]	46.7±7.1[b]
12	12.2±4.4[a]	21.1±6.5[b]	65.6±6.8[c]	23.3±4.3[b]
14	8.3±2.5[a]	18.3±6.1[b]	60.6±9.5[c]	16.7±5.0[b]
16	0	12.5±4.6[a]	57.5±4.6[b]	12.5±4.6[a]
18	-	7.2±2.6[a]	52.2±6.7[b]	8.3±2.5[a]
20	-	0	48.3±2.6	0
22	-	-	20.8±3.8	-
24	-	-	15.8±3.8	-

Values in a row with different superscripts significantly differ $p<0.05$.

Discussion

The volume of *S. coruhensis* sperm (1.6±0.64 ml) is low compared to other freshwater species: *O. mykiss* (18.17±2.74 ml, Bozkurt et al., 2005), *S. trutta fario* (3.9±1.48 ml, Bozkurt et al., 2006a), *S. trutta macrostigma* (13.93±0.84 ml, Bozkurt et al., 2011b), *S. trutta abanticus* (7.4±0.3 ml, Hatipoğlu and Akçay, 2010), and *Cyprinus carpio* (13.9±11.4 ml, Bozkurt et al., 2005; 2.75 ml, Bozkurt, 2006), but high compared to *Carasobarbus luteus* (0.75±0.05 ml, Aral et al., 2004), *O. mykiss* (1.22±0.22 ml, Aral et al., 2007), and *Liza abu* (45.76±3.55 µl, Şahinöz et al., 2008). Sperm density is lower than in *C. carpio* (Bozkurt et al., 2005; Bozkurt, 2006) and *S. trutta abanticus* (Hatipoğlu and Akçay, 2010) but higher than in *O. mykiss* (Bozkurt et al., 2005; Aral et al., 2007), *S. trutta caspius* (Hatef et al., 2007), and *S. trutta macrostigma* (Bozkurt et al., 2011b).

After freshwater activation, 90±5.17% of spermatozoa were motile, higher than in *O. mykiss* (72.29±10.79%, Bozkurt et al., 2005; 73.25±5.15%, Aral et al., 2007; 78.25±3.63%, Canyurt and Akhan, 2008), *S. trutta abanticus* (75.2±3.24%; Bozkurt et al., 2006b), *S. trutta magrostigma* (80.37±2.36%, Bozkurt et al., 2011b), and *S. trutta fario* (81.0±10.74%, Bozkurt et al., 2006a). Spermatozoa that were immotile in the seminal fluid were rapidly activated in contact with fresh water and remained motile for 64-217 s, similar to values reported by Büyükhatipoğlu and Holtz (1984), Babiak et al. (1999), and Tekin et al. (2003), but higher than reported by Bozkurt et al. (2005) and Tuset et al. (2008): 78-174 and 22-33 s, respectively.

The duration and motility of sperm can vary according to season (Benau and Terner, 1980) or biochemical composition and osmolality of the seminal plasma (Alavi et al., 2009). The most reliable indicator of sperm quality is spermatozoa motility and this indicator is used to select sperm for insemination. Using subjective estimation methods to determine motility, there is a correlation between motility rate and fertilization capacity in rainbow trout semen (Ciereszko and Dabrowski, 1994). Long motility duration of fish spermatozoa can enhance the fertilization success of individual males.

Sperm quality is usually evaluated by volume, motility, and motility duration. The present study shows that *S. coruhensis* produce sperm with a very low density compared to other salmonids. Differences in sperm production can be related to age and weight of males, sampling period, and method (Suquet et al., 1994, 1998), rearing conditions, nutrition, breeding seasonality, method of spawning induction, ecology, spawning behavior of broodfish (Piironen and Hyvarinen, 1983; Rurangwa et al., 2004), feeding conditions and regime, environmental factors, or spawning time (Bozkurt et al., 2006b). The correlations between body weight-length and spermatologic parameters were insignificant, as in *S. trutta fario* (Bozkurt et al., 2006a), suggesting that small insignificant correlations between fish size and sperm quality are not related to the size of mature fish but are probably affected by genetic and environmental factors.

Appropriate diluents are important for the cold preservation of fish sperm (DeGraaf et al., 2004; Bozkurt et al., 2011ab). In this study, three extenders were tested for cold storage of *S. coruhensis* sperm. The sperm were best preserved in the extender containing glucose, diluted at a ratio of 1:3 (semen:extender). Further work is needed on *S. coruhensis* sperm and egg quality to fully understand the reproductive potential of this species and to develop broodstock management protocols. Our results can be used to select high quality mature males for fertilizing eggs in a commercial aquaculture operation and provide a basis for future evaluation and control of reproduction in *S. coruhensis.*

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