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Ionic composition and osmolality of paddlefish (*Polyodon spathula*, Acipenseriformes) seminal fluid

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Abstract. Changes in ionic composition as Na^+, K^+, Ca^{2+} and Mg^{2+}, osmolality in seminal fluid, percentage of motile spermatozoa and velocity were investigated in response to CPP and different dosage of LHRHa. The lowest velocity of sperm was observed after use CPP treatment. The velocity of spermatozoa, significant main effect of the treatment ($P < 0.0001$) and the time of sperm collection ($P < 0.0104$) were evaluated. The osmolality of seminal fluid was different between experimental groups of LHRHa (48.0–62.7 $mOsmol.kg^{-1}$) and CPP (33.0–46.3 $mOsmol.kg^{-1}$) treatments. The osmolality was significantly higher on the first day and one-half, then declined on day three, ranging from 33.0 to 62.7 $mOsmol.kg^{-1}$. Analysis of variance showed significant main effects of the treatment ($P < 0.0001$) and the time of sperm collection ($P < 0.0002$) on the osmolality of seminal fluid. The level of Na^+ and K^+ ion was different between experimental groups of LHRHa and CPP treatment. The highest concentration of 11.11 $mmol.l^{-1}$ was observed at Na^+ ion. Then the concentrations declined on the level 1.56, 0.52 and 0.36 $mmol.l^{-1}$ for K^+, Ca^{2+} and Mg^{2+} ions, respectively. There were highly positive correlations between osmolality of seminal fluid and dosage of LHRHa treatment ($r = 0.84$), velocity of spermatozoa and osmolality of seminal fluid ($r = 0.57$) and osmolality of seminal fluid and Na^+ concentration at seminal fluid ($r = 0.70$). Injection with LHRHa increased quality of sperm as velocity of sperm, level of Na^+, K^+ and osmolality at seminal fluid compared to CPP treatments.

Key words: Aquaculture, Ionic contents, Motility of sperm, Paddlefish *Polyodon spathula*, Reproduction, Spermiation

Introduction

A lot of chemical investigations of seminal fluid were reviewed or recently published on teleostean fish species as salmonids (Baynes et al. 1981; Lahnsteiner et al. 1998; Glogowski et al. 2000), cyprinids (Gosh 1985) and other species as African catfish (*Clarias macrocephalus*, Tan-Fermin et al. 1999), but very rarely on chondrostean fish species as lake sturgeon (*Acipenser fulvescens*, Toth et al. 1994). In Atlanic salmon (*Salmo salar*, Hwang and Idler 1969) and grass carp (*Ctenopharyngodon idela*, Gosh 1985), ionic ratios of Na^+/K^+ and Mg^{2+}/Ca^{2+} are clearly different between sperm and seminal fluid. Ionic concentration in seminal fluid of rainbow trout (*Oncorhynchus mykiss*) changes in dependence on the time of spawning (Munkitrick and Moccia 1987) and in some species it can reach such low osmotic level that spermatozoa could probably be spontaneously activated without water (Cosson et al. 1999). Further, ionic concentration in seminal fluid of freshwater teleosts is higher than that of chondrostean species, both freshwater and marine (Cosson et al. 1999; Mims 1991; Toth et al. 1997). Osmolality of seminal fluid in freshwater teleosts ranges from 232 to 400 $mOsmol.kg^{-1}$ (Billard et al. 1986; Linhart et al. 1999; Tan-Fermin et al. 1999; Suquet et al. 1994) compared to 38–100 $mOsmol.kg^{-1}$ in Siberian sturgeon (*Acipenser baeri*, Gallis et al. 1991; Williot et al. 2000).

The quantity of sperm is limited during the reproductive season in paddlefish (*Polyodon spathula*) and in white sturgeon (*Acipenser transmontanus*), therefore to artificial propagate, spermiation must be stimulated. The LHRH analogue (LHRHa) and carp pituitary extract (CPE) have been effective to enhance spermiation (Mims 1991; Van Eenennaam et al. 1996). Injection of single dose of LHRHa at 200 $\mu g.kg^{-1}$ of body weight (b.w.) increased the number of spermatozoa per kilogram of b.w. and prolonged spermiation up to 96 hours after application of treatment (Linhart et al. 2000).

In the present study, differences in ionic composition as Na^+, K^+, Ca^{2+} and Mg^{2+}, osmolality in seminal fluid, percentage of motile spermatozoa and velocity were investigated in response to a single dose of carp pituitary powder (CPP) and different dosages of LHRHa. A preliminary study on the potency of LHRHa at three different doses and CPP in male paddlefish to stimulate spermiation was published by Linhart et al. (2000).

Materials and methods

The experiments were conducted at the Aquaculture Research Center, Kentucky State University (KSU), Frankfort, Kentucky, USA. Paddlefish males from 4.0 to 8.0 kg were captured in Ohio River, Kentucky. Broodfish

were transported to the ponds of the Aquaculture Research Center at KSU and used within four weeks for experiments. Males were selected for good condition and held randomly in five groups in circular metal tanks (6 000 l) with a water flow rate of 12 l.min^{-1}, dissolved oxygen of 9.0 mg 0_2.l^{-1} and at water temperature between 15 and 19 °C. Maturity of males was judged presence of milt after abdominal compression. Males of paddlefish were treated as follows:

Group 1: LHRH analogue, des-Gly^{10} (D-Ala^6) LHRH ethylamide (Sigma Chemical Company) injected intramuscularly at the dose of 50 μg.kg^{-1} b.w. The fish weights were 4.1, 5.7 and 5.5 kg with an average of 5.1 $\pm$ 0.9 kg.

Group 2: LHRH analogue as in group 1, but at 100 μg.kg^{-1} b.w. The weights were 6.4, 7.5 and 6.4 kg with an average of 6.8 $\pm$ 0.6 kg.

Group 3: LHRH analogue as in group 1, but at 200 μg.kg^{-1} b.w. The weights were 5.4, 7.1 and 7.46 kg with an average of 6.6 $\pm$ 1.1 kg.

Group 4: Carp pituitary acetone powder (CPP; Sigma, no. P3034) dissolved in 102 mmol.l^{-1} NaCl solution and injected intramuscularly at a dose of 4 mg.kg^{-1} b.w. The weights were 4.7, 5.7 and 6.0 kg with an average of 5.5 $\pm$ 0.7 kg.

Group 5 (control): Solution of 102 mmol.l^{-1} NaCl injected intramuscularly with no LHRHa or CPP. The weights were 7.7, 6.0 and 5.0 kg with an average of 6.23 $\pm$ 1.11 kg.

For stripping, males were fished out from the tank, fixed in dorsal position and urogenital pore was wiped. A 10-mL plastic syringe with 5 cm of Tygon tubing was used to collect sperm. The tube was inserted into the urogenital pore to fill with milt, then transferred to 100 ml containers which was stored on ice until examination. The sperm was collected 24 hours after injection, then every 12 hours over the next 2 days (Linhart et al. 2000).

Observation of sperm motility and velocity

Spermatozoa were evaluated for the percentage of motility and velocity. Measurements used dark field microscopy and a Nikon camera setup as described by Billard et al. (2000), Cosson et al. (2000) and Linhart et al. (2000). Percentage of motility and velocity was examined under 200x magnification immediately after mixing 0.5 μl of sperm with 49.5 μl of swimming medium (SM = 20 mmol.l^{-1} NaCl + 20 mmol.l^{-1} TRIS-HCl, pH 8.2), on a glass slide previously prepositioned on the microscope stage. The final dilution was 1:100. Within 10 s after mixing, a video recording was made for 5 mins to be used in the evaluation of spermatozoan swimming activity.

The focal plane was always positioned near the glass slide surface. The movements of spermatozoa were recorded at 60 frames.s^{-1} using a 3CCD video camera (SONY DXC-970MD) mounted on a dark-field microscope (NIKON Optiphot 2, Japan) and visualized on a video monitor illuminated with stroboscopic lamp of Strobex (Chadvick-Helmut, 9630, USA). The adjustable frequency stroboscopic flash illumination was set in automatic register with video frames (60 Hz) for sperm velocity measurement.

Evaluation of the velocity and percentage of motility

The successive positions of the recorded sperm heads were measured from video frames using a video-recorder (SONY SVHS, SVO-9500 MDP) at 30 s after activation from three frames (25 frames/second were recorded) analyzed by micro-image analysis (Version 4.0.1. for Windows with special macro from the Czech Republic Olympus). The velocity and percentage of motility were measured by evaluating spermatozoa head positions on three successive frames with three different colors (blue, green and red). The analyses were repeated 3 times at 30-second intervals, i.e. frames 1–3, then frames 11–13, and finally from frames 21–23. Thirty to forty spermatozoa were evaluated from each frame. Spermatozoon that moved were visible in three colors, while non-moving spermatozoa were white. Percentage of motile spermatozoa was easily calculated from white versus red cells. Velocity of spermatozoa was calculated as μm.s^{-1} based on length traces of spermatozoa from blue to green and red heads, calibrated for magnification. Excel 97 automatically calculated both values.

Osmolality and ionic composition

Seminal fluid was centrifuged at 3000 RPM for 5 min at room temperature. The osmolality of seminal fluid was measured using a Vapour Pressure Osmometer (Wescor, USA) and expressed as mOsmol.kg^{-1}. Ionic composition (Na^+, K^+, Ca^{2+} and Mg^{2+}) of seminal fluid were measured in flame spectrometer SpectrAA 640 (Varian Techtron, Australia) and expressed as mmol.l^{-1}.

Data analysis

The data were acquired from the following replications: percentage of spermatozoa motility and velocity 9 times per group and time, ionic composition and osmolality 3 times per group. Data were evaluated and statistical significance was assessed using multiple analysis of variance (ANOVA, Statgraphics version 5), followed by multiple comparison Tukey HSD range tests (Tables 1–2). Linear or logarithmic regression analysis and correlation were obtained using Microsoft Excel 97 for establishment of the relationship in Table 3 and Figure 1. Probability values < 0.05 were considered significant.

Results

Spermatozoa motility was observed over a 3-day period for each experimental group. The percent motility did not differ significantly between groups nor over the 2-day time period of sperm collection. The percentage sperm motility at 30 s after activation was similar in all groups over the duration of the experiment with a slight decline on day 3 (Table 1). The velocity of spermatozoa was highest on the first day of sperm collection in the groups treated with LHRHa at 50 μg.kg^{-1} b.w. and for the 50 and 100 μg.kg^{-1} b.w. LHRHa treatments at 1.5 day of sperm collection. The velocity of sperm at 30 s after activation declined on day 3. The lowest velocity was observed in the CPP treatment on day 2 of sperm collection (Table 1). The main treatment effects were the velocity of spermatozoa ($P < 0.0001$) and the time of sperm collection ($P < 0.0104$).

Osmolality of seminal fluid was significantly different between experimental groups of LHRHa (48.0–62.7 mOsmol.kg^{-1}) and CPP (33.0–46.3 mOsmol.kg^{-1}) and over time (1st and 3rd day of sperm collection). Osmolality was highest on the first day of sperm collection in the LHRHa treatment at dose of 100 μg.kg^{-1} b.w., declining by day 3 ranging from 33.0 to 62.7 mOsmol.kg^{-1} (Table 1). Analysis of variance showed significant main treatment effects ($P < 0.0001$) were the time of sperm collection ($P < 0.0002$) on osmolality of seminal fluid.

Ionic composition (Na^+, K^+, Ca^{2+} and Mg^{2+}) from seminal fluid was analyzed in each experimental group every 12 hours during a 3-day period (Table 2). The levels of Na^+ with K^+ and Ca^{2+} with Mg^{2+} did not differ significantly between the times of sperm collection in the experimental groups. Analysis of variance showed significant main effect of the treatment ($P < 0.0001$) for the level of Na^+ and K^+ ions and also significant main effect of the time of sperm collection for the level of the K^+, Mg^{2+} and Ca^{2+} ions, respectively ($P < 0.02$, $P < 0.0001$ and $P < 0.0004$). The ionic

Table 1. Evolution of the velocity (μm.s^{-1}) of spermatozoa, percentage of motile spermatozoa (%) and osmolality (mOsmol.kg^{-1}) from sperm collected after paddlefish hormonal stimulation with LHRHa and CPP. Groups with a common superscript in each column and in each line do not differ significantly ($P < 0.05$).

	Treatment	Dosage	Collection of sperm (days, means ± SD)				
			1	1.5	2	2.5	3
Velocity	LH-RHa	50 μg.kg^{-1}	144.8 ± 14.5^{b}	150.5 ± 6.7^{b}	119.8 ± 6.6^{ab}	120.7 ± 25.2^{ab}	123.8 ± 3.9^{ab}
		100 μg.kg^{-1}	138.1 ± 20.9^{ab}	148.3 ± 3.8^{b}	121.0 ± 4.7^{ab}	139.7 ± 16.7^{ab}	136.2 ± 4.2^{ab}
		200 μg.kg^{-1}	117.8 ± 32.7^{ab}	134.7 ± 13.2^{ab}	109.8 ± 15.9^{ab}	124.6 ± 35.5^{ab}	110.0 ± 19.6^{ab}
	CPP	4 mg.kg^{-1}	106.8 ± 5.8^{ab}	104.0 ± 27.3^{ab}	91.9 ± 8.1^{a}	108.0 ± 0.5^{ab}	106.4 ± 4.0^{ab}
Motility	LH-RHa	50 μg.kg^{-1}	99.1 ± 1.6^{a}	98.4 ± 2.8^{a}	97.2 ± 2.4^{a}	78.8 ± 27.6^{a}	90.9 ± 15.7^{a}
		100 μg.kg^{-1}	95.9 ± 5.0^{a}	98.7 ± 2.2^{a}	98.8 ± 1.2^{a}	94.1 ± 2.0^{a}	93.2 ± 6.0^{a}
		200 μg.kg^{-1}	83.4 ± 20.9^{a}	88.7 ± 13.9^{a}	73.7 ± 24.6^{a}	95.8 ± 3.9^{a}	95.3 ± 4.1^{a}
	CPP	4 mg.kg^{-1}	85.8 ± 1.2^{a}	93.2 ± 4.2^{a}	94.9 ± 8.9^{a}	79.8 ± 8.4^{a}	90.3 ± 9.7^{a}
Osmolality	LH-RHa	50 μg.kg^{-1}	56.0 ± 4.6^{cde}	59.0 ± 6.3^{cde}	57.3 ± 1.5^{cde}	56.3 ± 4.9^{cde}	48.0 ± 2.7^{bcd}
		100 μg.kg^{-1}	62.7 ± 3.5^{e}	57.0 ± 4.6^{cde}	56.7 ± 1.5^{cde}	55.3 ± 1.2^{cde}	52.3 ± 3.1^{cde}
		200 μg.kg^{-1}	60.3 ± 2.1^{de}	58.7 ± 5.7^{cde}	55.0 ± 2.0^{cde}	55.7 ± 5.5^{cde}	53.7 ± 1.5^{cde}
	CPP	4 mg.kg^{-1}	38.7 ± 7.2^{ab}	46.3 ± 7.5^{abc}	33.3 ± 4.2^{a}	35.7 ± 5.8^{ab}	33.0 ± 4.0^{a}

concentration differed; Na^+ was highest at 11.11 mmol.l^{-1}, while K^+, Ca^{2+} and Mg^{2+} concentrations were 1.56, 0.52 and 0.36 mmol.l^{-1}, respectively. Ratio between Na^+:K^+:Ca^{2+}:Mg^{2+} was 30.9:5:1.7:1.

In paddlefish, there were highly positive logarithmic or linear correlations between osmolality of seminal fluid and dosage of LHRHa (r = 0.84, Figure 1A), velocity of spermatozoa and osmolality of seminal fluid (r = 0.57, Figure 1B), osmolality of seminal fluid and Na^+ concentration in seminal fluid (r = 0.70, Figure 1C), Na^+ and K^+ concentrations in seminal fluid (r = 0.65, Figure 1D), and Ca^{2+} and Mg^{2+} concentrations in seminal fluid (r = 0.53, Figure 1E). Correlations were low between percentage of sperm motility, osmolality of seminal fluid, day of sperm collection, dosage level of the treatment and ionic concentrations (Table 3).

Discussion

Ionic concentration in seminal fluid of paddlefish was lower than for teleosts (Linhart et al. 1991). In paddlefish the ionic concentration and osmolality in seminal fluid declined during time of sperm collection. Munkittrick and Moccia (1987) also reported on decline of osmolality in seminal fluid of rainbow trout. In the present study, low osmolality of seminal fluid decreased velocity of paddlefish sperm but there was no relation between percentage of sperm motility and osmolality. No relation between percentage of sperm motility and osmolality of seminal fluid was observed in Siberian sturgeon (Williot et al. 2000). The concentrations of Na^+ and K^+ in the seminal fluid was two times lower than that reported by Mims (1991), and of Ca^{2+} and Mg^{2+} ions were three times higher. The ratio between Na^+:K^+:Ca^{2+}:Mg^{2+} was also different from the levels reported in the previous study; especially between Na^+ and K^+ which was 6.18 and 8.8. Differences between ionic levels from present study and Mims (1991) may be related to contamination of samples by urine (Linhart et al. 1995). The level and ratio of ions in seminal fluid is very important to the maintenance of energetic content. When the quantity or ratio between Na^+ and K^+ change, the sperm motility can be initiated (Cosson et al. 1999; Linhart et al. 2002). The concentration of K^+ that triggers motility of sturgeon and paddlefish sperm is 0.5 mmol.l^{-1} (Toth et al. 1994) and 0.125–1 mmol.l^{-1} (Cosson and Linhart 1996; Linhart et al. 2002), respectively. Consequently, paddlefish sperm motility can be initiated by a dilution of about four times that which was tested in the present experiment, or eight times that reported by Mims (1991). The potential for sperm storage at 4 °C can be longer with a high concentration of K^+.

Osmolality of seminal fluid in the present study (33.0–62.7 mOsmol.kg^{-1}) was higher than that for Siberian sturgeon (Gallis et al. 1991); however,

Table 2. Evolution of the ions concentration (Na^+, K^+, Ca^{2+} and Mg^{2+}) at $mmol.l^{-1}$ of seminal fluid from sperm collected after paddlefish hormonal stimulation with LHRHa and CPP. Groups with a common superscript in each column and in each line do not differ significantly ($P < 0.05$).

	Treatment	Dosage	Collection of sperm (days, means ± SD)				
			1	1.5	2	2.5	3
Na^+	LH-RHa	50 $\mu g.kg^{-1}$	13.1 ± 2.4^{abc}	13.6 ± 2.3^{abc}	13.2 ± 2.0^{abc}	14.5 ± 1.0^{abc}	11.5 ± 0.6^{abc}
		100 $\mu g.kg^{-1}$	15.3 ± 0.9^{bc}	12.1 ± 1.0^{abc}	16.9 ± 4.0^{c}	13.9 ± 0.5^{abc}	14.8 ± 2.7^{abc}
		200 $\mu g.kg^{-1}$	9.3 ± 7.6^{abc}	14.3 ± 1.8^{abc}	14.2 ± 0.6^{abc}	10.4 ± 7.9^{abc}	8.1 ± 7.2^{abc}
	CPP	4 $mg.kg^{-1}$	4.0 ± 0.4^{a}	6.2 ± 4.5^{abc}	4.7 ± 2.1^{ab}	6.2 ± 2.1^{abc}	6.0 ± 1.8^{abc}
K^+	LH-RHa	50 $\mu g.kg^{-1}$	1.9 ± 1.1^{a}	1.5 ± 0.2^{a}	1.8 ± 0.7^{a}	1.9 ± 0.6^{a}	2.2 ± 0.3^{a}
		100 $\mu g.kg^{-1}$	1.3 ± 0.1^{a}	1.6 ± 0.4^{a}	2.2 ± 0.4^{a}	1.8 ± 0.5^{a}	2.4 ± 0.3^{a}
		200 $\mu g.kg^{-1}$	1.2 ± 0.6^{a}	1.6 ± 0.1^{a}	2.0 ± 0.6^{a}	1.7 ± 1.3^{a}	1.9 ± 1.2^{a}
	CPP	4 $mg.kg^{-1}$	0.4 ± 0.2^{a}	0.6 ± 0.2^{a}	1.1 ± 0.8^{a}	0.9 ± 0.5^{a}	1.3 ± 1.3^{a}
Ca^{2+}	LH-RHa	50 $\mu g.kg^{-1}$	0.7 ± 0.4^{ab}	0.3 ± 0.1^{a}	0.8 ± 0.1^{ab}	0.5 ± 0.3^{ab}	0.5 ± 0.2^{ab}
		100 $\mu g.kg^{-1}$	0.7 ± 0.1^{ab}	0.5 ± 0.1^{ab}	0.5 ± 0.0^{ab}	0.3 ± 0.1^{ab}	0.8 ± 0.4^{ab}
		200 $\mu g.kg^{-1}$	0.4 ± 0.3^{ab}	0.3 ± 0.2^{ab}	0.6 ± 0.1^{ab}	0.4 ± 0.2^{ab}	0.4 ± 0.2^{ab}
	CPP	4 $mg.kg^{-1}$	0.9 ± 0.3^{b}	0.3 ± 0.0^{a}	0.7 ± 0.2^{ab}	0.4 ± 0.1^{ab}	0.5 ± 0.3^{ab}
Mg^{2+}	LH-RHa	50 $\mu g.kg^{-1}$	0.3 ± 0.2^{ab}	0.3 ± 0.1^{ab}	0.5 ± 0.1^{ab}	0.4 ± 0.3^{ab}	0.3 ± 0.1^{ab}
		100 $\mu g.kg^{-1}$	0.3 ± 0.0^{ab}	0.3 ± 0.1^{ab}	0.7 ± 0.2^{b}	0.3 ± 0.0^{ab}	0.3 ± 0.2^{ab}
		200 $\mu g.kg^{-1}$	0.3 ± 0.2^{ab}	0.4 ± 0.1^{ab}	0.6 ± 0.1^{ab}	0.2 ± 0.2^{a}	0.3 ± 0.2^{ab}
	CPP	4 $mg.kg^{-1}$	0.4 ± 0.2^{ab}	0.3 ± 0.1^{ab}	0.4 ± 0.1^{ab}	0.3 ± 0.1^{ab}	0.2 ± 0.1^{a}

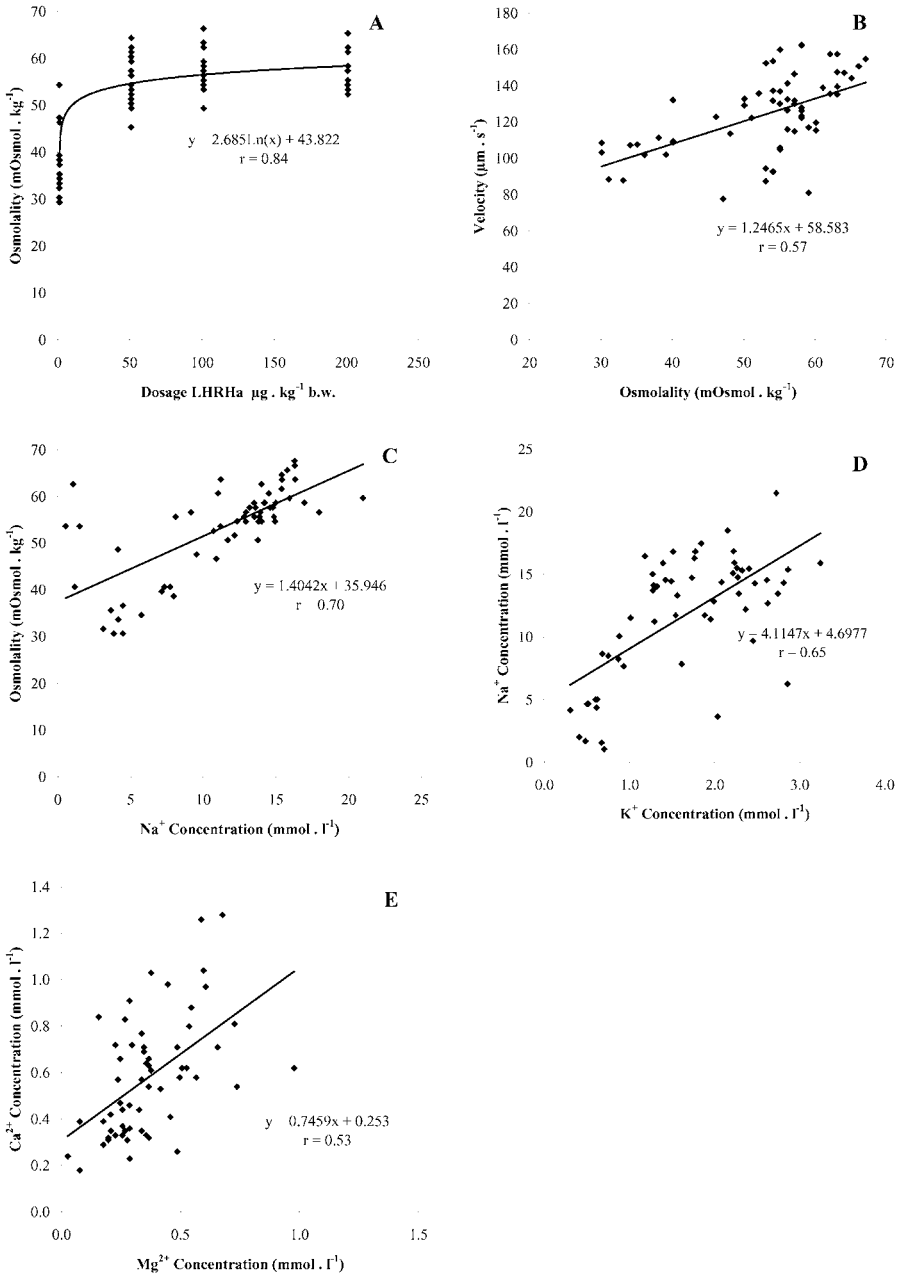

Figure 1. Relationship between osmolality of seminal fluid and dosage of LHRHa (A), velocity and osmolality (B), osmolality and Na^+ concentration (C), Na^+ concentration and K^+ concentration (D), Ca^{2+} concentration and Mg^{2+} concentration (E).

Table 3. Logarithmic or linear correlations (r) between velocity of spermatozoa, percentage of sperm movement, osmolality, day of sperm collection, dosage level of the LHRHa and ionic concentrations (r with very gray panel – very high or high correlation, r with white panel – low or very low correlation).

	Velocity of spermatozoa	Percentage of sperm motility	Osmolality of seminal fluid	Day of sperm collection	Dosage of LHRHa	Concentration			
						Na^+	K^+	Ca^{2+}	Mg^{2+}
Velocity of spermatozoa	1.00	0.46	0.57	–0.18	0.17	0.46	0.29	–0.10	–0.10
Percentage of sperm motility	0.46	1.00	0.19	–0.06	–0.07	–0.01	0.02	0.03	–0.12
Osmolality of seminal fluid	0.57	0.19	1.00	–0.29	0.84	0.70	0.37	–0.17	0.12
Day of sperm collection	–0.18	–0.06	–0.29	1.00	0.00	–0.03	0.34	–0.07	–0.13
Dosage of LHRHa	0.17	–0.07	0.84	0.00	1.00	0.33	0.30	–0.21	0.06
Concentration Na^+	0.46	–0.01	0.70	–0.03	0.33	1.00	0.65	0.05	0.44
Concentration K^+	0.29	0.02	0.37	0.34	0.30	0.65	1.00	0.22	0.33
Concentration Ca^{2+}	–0.10	0.03	–0.17	–0.07	–0.21	0.05	0.22	1.00	0.53
Concentration Mg^{2+}	–0.10	–0.12	0.12	–0.13	0.06	0.44	0.33	0.53	1.00

when CPP was used osmolality values were similar between the present study and that in the Siberian sturgeon at 33.0–46.3 mOsmol.kg^{-1} and 38.0 mOsmol.kg^{-1}, respectively. Sperm in seminal fluid with higher osmolality can also have longer storage, similar to the effect with K^+.

Conclusions

Linhart et al. (2000) found that injection of LHRHa compared to CPP, increased the quantity of sperm. The present study demonstrated that the quality was also better in LHRHa injected fish compared to treatment with CPP. Velocities of sperm were increased as was the levels of Na^+, K^+, and osmolality at seminal fluid.

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