Journal of Applied Ichthyology

J. Appl. Ichthyol. 27 (2011), 673–677

ISSN 0175–8659

Received: June 10, 2010
Accepted: November 25, 2010
doi: 10.1111/j.1439-0426.2010.01634.x

Physico-chemical properties and protein profiles of sperm from three freshwater chondrostean species: a comparative study among Siberian sturgeon (*Acipenser baerii*), sterlet (*Acipenser ruthenus*) and paddlefish (*Polyodon spathula*)

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Summary

Physico-chemical properties (spermatozoa motility, spermatozoa concentration, seminal plasma protein concentration, osmolality, pH, ions) and sperm protein profiles were evaluated in three Acipenseriforme species [Siberian sturgeon (*Acipenser baerii*), sterlet (*Acipenser ruthenus*) and paddlefish (*Polyodon spathula*)]. The spermatozoa velocity was observed significantly highest in sterlet and lowest in Siberian sturgeon ($P < 0.05$). Spermatozoa velocity was positively correlated to pH and the concentration of Na^+ in seminal plasma in all species examined. Other tested parameters, in particular the concentration of K^+, expressed species specific patterns, which could be linked to taxonomical status of species. The protein profiles of seminal plasma indicated a protein band with a molecular weight of 32.6 kDa exclusively detected in paddlefish. In spermatozoa, 95 protein spots were detected in Siberian sturgeon and sterlet, respectively, whereas only 70 spots were observed in paddlefish. Each spermatozoa protein map expressed a strong species specific pattern, which offers a basic benchmark to trace molecular differences among Acipenseriformes. It can be concluded that results presented in this study could be applied for successful reproduction management and cryopreservation protocols of these endangered species.

Introduction

The order Acipenseriformes belongs to the class Actinopterygii (ray-finned fishes), and includes two families: Acipenseridae (sturgeons) and Polyodontidae (paddlefishes). Fisheries of almost all Acipenseriformes have collapsed due to overexploitation, either independently or in combination with factors such as habitat loss/fragmentation. Increasing harvest restrictions, and the rapid decline of wild populations, undoubtedly have created a demand for artificial reproduction and stocking of early life stages into river systems (Wei et al., 1997; Pikitch et al., 2005). Therefore, better understanding of gamete biology of Acipenseriformes could provide alternatives to managers for improving reproduction strategies of these endangered species.

Until now, most studies on sperm biology of Acipenseriformes have concentrated on ultrastructure and morphological functions (e.g. Psenicka et al., 2007; Wei et al., 2007), characterization of motility and fertilization ability (Cosson and Linhart, 1996; Billard et al., 1999; Cosson et al., 2000), and cryopreservation (e.g. Billard et al., 2004; Horvath et al., 2005; Linhart et al., 2006; Li et al., 2008). Limited information is available on biophysical and biochemical characteristics (e.g. Piros et al., 2002), sperm protein composition and protein functions (Fabrik et al., 2008; Sarosiek et al., 2008).

In the present study we determined the physico-chemical properties and protein profiles of sperm in Siberian sturgeon (*Acipenser baerii*), sterlet (*Acipenser ruthenus*), and paddlefish (*Polyodon spathula*), with the aim to explore inter-species specific characteristics of Acipenseriforme sperm. This information could aid to improve reproduction and cryopreservation protocols of these endangered species.

Materials and methods

Induction of spermiation and sperm collection

Ten Siberian, 10 sterlet sturgeons and three paddlefish males were used for the experiment. The spermiation was stimulated by intra-peritoneal injection of carp pituitary powder dissolved in 0.9% NaCl solution at doses of 4–5 mg kg^{-1} of body weight, 48 h before sperm collection. Sperm was collected in 250 ml cell culture containers and kept on ice (0–4°C) until processing.

Ionic contents, osmolality and pH of the seminal plasma

Sperm samples were initially centrifuged at 300 ***g*** for 30 min, followed by 10 min at 10 000 ***g*** and 4°C. The supernatant of the seminal plasma was collected and stored at −80°C until analysed. Ionic contents (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-) were determined by flame photometry (ISE; Bayer HealthCare, NY, USA). A Vapour Pressure Osmometer (Wescor, Logan, UT, USA) was used to measure osmolality, whereas pH determinations were done with a laboratory pH meter (WTW, pH 320, Weilheim, Germany).

Evaluation of sperm concentration

Prior analysis, sperm was additionally diluted by an immobilizing solution (20 mM Tris, 10 mM KCl, pH 8), and concentration was measured according to methods described by Caille et al. (2006).

Observation and evaluation of spermatozoa velocity

Spermatozoa velocity ($\mu m\ s^{-1}$) was determined after triggering sperm motility under dark-field microscopy (Olympus BX 50;

Tokyo, Japan) (20× objective magnification). For triggering, sperm was diluted in Tris–HCl 30 mM, 5 mM $CaCl_2$, pH 8.0 at a dilution ratio of 1 : 50. To avoid sperm sticking to the slide, 0.1% BSA was added. Spermatozoa motility was observed under dark-field microscopy (Olympus BX 50, objective magnification 20×) with mounted CCD video camera (SONY SSCDC50AP; Tokyo, Japan) and recorded using a video-recorder (SONY SVHS, SVO-9500 MDP). The successive positions of the heads of spermatozoa were measured from five frames using a video-recorder (SONY SVHS, SVO-9500 MDP). The positions of sperm heads were captured from videosequence in five successive frames, and analyzed with a micro image analyzer (Olympus Micro Image 4.0.1. for Windows, Japan). Analysis of sperm motility was done in triplicate for each sample.

Preparation of protein samples

Seminal plasma proteins were separated by centrifugation at 300 ***g*** and 4°C for 30 min, followed by 10 min at 16 000 ***g***. Supernatants were stored at −80°C. The resulted pellets of spermatozoa were washed twice in the sperm immobilizing solution for each species at 300 g and 4°C for 30 min. Pellets were re-suspended in the protein extraction buffer (immobilizing solution with 1% Triton) and incubated for 1 h on ice, whereafter centrifugated for 10 min at 16 000 ***g*** and 4°C. Collected supernatants were stored at −80°C. Finally, the bicinchoninic acid assay (BCA), using an Infinite M200 (Tecan, Switzerland), was applied to determine protein concentration.

SDS-gel electrophoresis (SDS-PAGE)

Seminal plasma proteins were separated by 12% polyacrylamide-bisacrylamide gel electrophoresis. Samples were re-suspended in buffer containing 65 mM Tris, 10% glycerol, and 2% sodium dodecyl sulphate (SDS), 5% beta-mercaptoethanol and denaturated for 3 min at 95°C prior to loading to gels. The gels were visualised by silver staining.

Two-dimensional gel electrophoresis (2DE)

Spermatozoa proteins were analysed by 2DE. Isoelectric focusing (IEF) was performed on ReadyStrip IPG strips (pH 3–10, 7 cm) with PROTEAN® IEF (Bio-Rad, CA, USA). A total of 50 μg of protein was used for preliminary runs to a total volume of 125 μl of rehydration buffer (8 M urea, 2 M thiourea, 4% Chaps, 50 mM dithiothreitol, 0.4% IPG buffer). The IPG strips were rehydrated at 50 V for 14 h and focused for 10 000 volt-hour (Vh). After IEF, the IPG strips were equilibrated as follow: in the first step with a solution containing 6 M urea, 29.3% glycerol, 2% SDS, 75 mM Tris-HCl pH 8.8 and 2% dithiothreitol for 15 min, and in the second step with a solution containing 2.5% iodacetamide instead of dithiothreitol for another 15 min. Each IPG strip was thereafter laid onto a 12% SDS-PAGE gel for second dimension electrophoresis. Protein spots were visualized by Coomassie Blue R-250. The stained two-dimensional gels were scanned and analyzed using Nonlinear's 2D software (Nonlinear Dynamics Ltd, NC, USA). Each specimen was analyzed in duplicate. The representative map was picked up from each species.

Data analysis

Differences in physico-chemical properties among species were analysed by one-way analysis of variance (ANOVA). Relationships among physico-chemical parameters within species were examined by a partial principal component analysis (PCA). Stepwise multiple regression analysis with forward selection was used to quantify relationships between spermatozoa velocity and other parameters. The influence of parameters was evaluated by means of standardized partial regression coefficient (beta). All statistical analyses were conducted with Statistica for Windows, Version 7.1 (StatSoft, Tulsa, OK, USA). Statistical significance was declared at $P < 0.05$. Results are presented as means ± standard deviation (SD).

Results

Physico-chemical properties

Spermatozoa velocity, seminal plasma protein concentration, spermatozoa concentration, pH, and Na^+, K^+ and Cl^- concentrations of seminal plasma differed ($P < 0.05$) among species (Table 1). Seminal plasma protein concentration of Siberian sturgeon and sterlet were significantly higher than those of paddlefish, while Na^+ and Cl^- concentrations in seminal plasma of Siberian sturgeon and sterlet were lower ($P < 0.05$) than in paddlefish. The pH of seminal plasma of Siberian sturgeon was significantly lower than that of sterlet and paddlefish. Moreover, the spermatozoa velocity and K^+ concentration of seminal plasma were shown variances among three species ($P < 0.05$). However, no species effect ($P > 0.05$) was observed for osmolality, Ca^{2+} and Mg^{2+} concentrations.

Based on physico-chemical properties, three species with 68.47% of total accumulated variance were distinguished by principal components analysis (PCA) (Fig. 1). Two groups of inter-correlated properties were revealed by a PCA plot (Fig. 2). One group comprised spermatozoa velocity and concentration, seminal plasma osmolality, pH, Na^+ and Cl^- concentrations, with seminal plasma protein concentration, and K^+, Ca^{2+}, Mg^{2+} concentrations forming the second group. Only pH and Na^+ concentration were selected by stepwise multiple regression analysis as been positively correlated with spermatozoa velocity ($\beta = 0.68$ and 0.25, respectively; $R^2 = 0.93$, $P < 0.01$).

Protein profiles

Using SDS-PAGE, five mutual protein bands, with molecular weight (MW) ranging from 29 to 71.3 kDa, were detected in the seminal plasma of all three specimens examined. A specific band with a MW of 32.6 kDa was found exclusively in paddlefish (Fig. 3).

Two dimensional plots from gel electrophoresis presented 95 protein spots in samples from Siberian sturgeon and sterlet, respectively, whereas only 70 protein spots were detected in paddlefish. For comparison of differences in each plot, 2DE images were divided in five parts (Fig. 4). Protein spots detected in parts A and F were all comparable, indicating highly conserved proteins in all three specimens examined. The protein spots depicted in part B, C and E were exclusively comparable between Siberian sturgeon and sterlet, but not paddlefish. Spots with high expression in all samples (marked by arrows in part D) did not agree among the three different gels.

Discussion

The present study presented evidence of strong interspecies variation in physico-chemical properties of sperm among

Table 1
Comparisons of sperm physico-chemical properties among *Acipenser baerii*, *A. ruthenus* and *Polyodon spathula*

Species	Velocity ($\mu m\ s^{-1}$)	PC ($mg\ ml^{-1}$)	SC ($\times 10^9$ spz ml^{-1})	Osmo ($mOsmol\ kg^{-1}$)	pH	Na^+ (mM)	K^+ (mM)	Ca^{2+} (mM)	Mg^{2+} (mM)	Cl^- (mM)
A. baerii	123.76 ± 42.59^c	0.50 ± 0.11^a	0.20 ± 0.17^b	46.25 ± 11.60	7.97 ± 0.43^b	14.59 ± 6.11^b	4.55 ± 1.12^a	0.27 ± 0.09	0.48 ± 0.22	6.20 ± 1.23^b
A. ruthenus	171.85 ± 8.56^a	0.41 ± 0.05^a	0.72 ± 0.32^a	50.83 ± 10.07	8.32 ± 0.10^a	17.71 ± 3.49^b	2.76 ± 0.99^b	0.15 ± 0.09	0.21 ± 0.15	7.60 ± 1.50^b
P. spathula	144.67 ± 7.35^b	0.29 ± 0.11^b	0.26 ± 0.37^b	52.11 ± 9.57	8.23 ± 0.07^a	23.60 ± 5.49^a	0.78 ± 0.56^c	0.14 ± 0.04	0.43 ± 0.04	10.67 ± 4.62^a

PC, seminal plasma protein concentration; SC, spermatozoa concentration; Osmo, osmolality.
Values with the same superscript (a, b, c) within columns are not significantly different (ANOVA, $P > 0.05$).

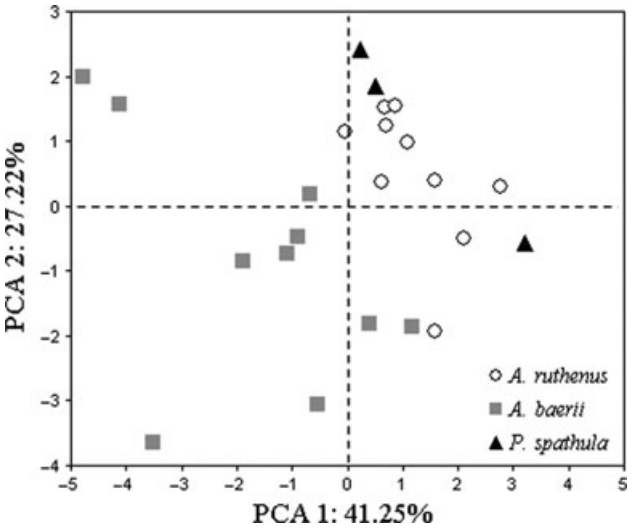

Fig. 1. Partial PCA plot depicting variability of sperm physico-chemical properties among *A. baerii*, *A. ruthenus* and *P. spathula*. The first and second axes displayed accounted for 41.25 and 27.22% of total variation in data, respectively

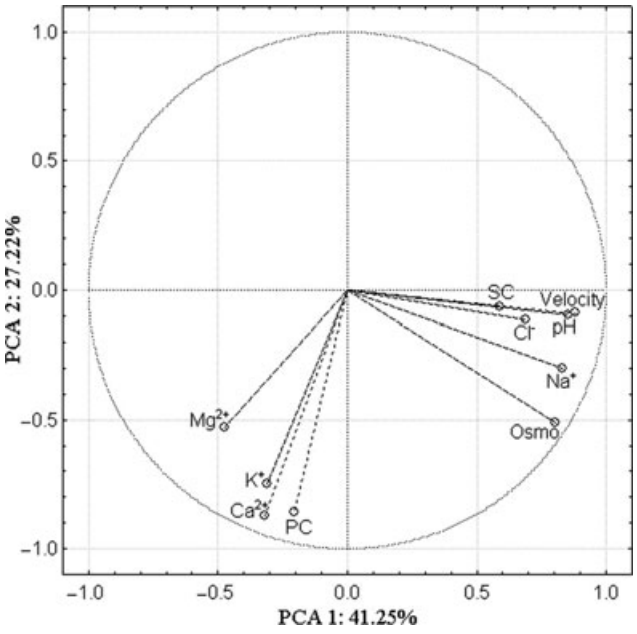

Fig. 2. Partial PCA plot depicting correlations of physico-chemical properties among *Acipenser baerii*, *A. ruthenus* and *Polyodon spathula*. The first and second axes displayed accounted for 41.25 and 27.22% of total variation in data, respectively. PC, seminal plasma protein concentration; SC, spermatozoa concentration; Osmo, Osmolality

species of the order Acipenseriforme. Some properties (e.g. K^+ concentration in seminal plasma) expressed species specific patterns, which could be linked to the taxonomical status of species examined. This is agreement with a recent review by Alavi and Cosson (2006), which clarified that sperm biosensitivity to ions such as K^+ is a species-specific characteristic. However, other properties, such as osmolality, and concentrations of Ca^{2+} and Mg^{2+}, demonstrated relatively constant values among species. Differences to published (Gallis et al., 1991; Piros et al., 2002; Psenicka et al., 2008) values could probably be related to secretory activity of the sperm duct, which could depend on the origin of broodfish, feeding regime,

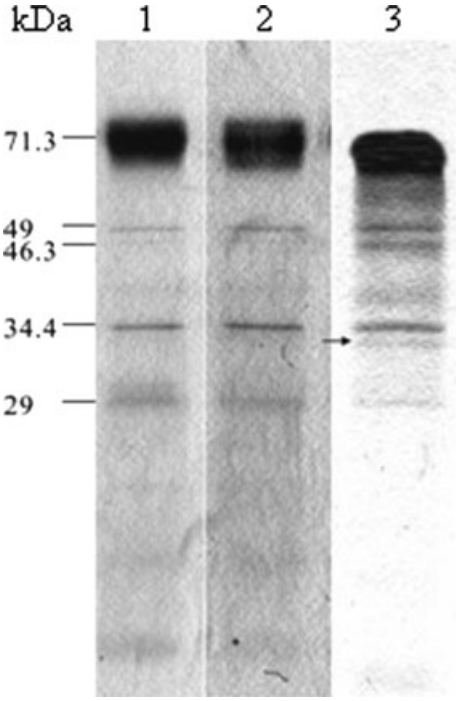

Fig. 3. The SDS-PAGE analysis of seminal plasma proteins in *Acipenser baerii* (lane 1), *A. ruthenus* (lane 2) and *Polyodon spathula* (lane 3). Arrow indicates the specific protein band (32.6 kDa) detected exclusively in paddlefish

and other factors (Alavi et al., 2008). A significant lowest spermatozoa velocity and pH in Siberian sturgeon observed in present study might have resulted from contamination with urine, as found with paddlefish when the pH of fresh sperm was lower than 7 (Cosson and Linhart, 1996).

Positive correlations among spermatozoa velocity and concentration, seminal plasma osmolality, pH, Na^+ and Cl^- concentrations within the three species evaluated are in agreement to results obtained (Lahnsteiner et al., 1996) with Common Bleak (*Alburnus alburnus*). Correlations could be applied to elucidate the effects of several components of seminal plasma that has an influence on spermatozoa motility, and would provide further knowledge on the metabolic pathways important for fertilization capacity of spermatozoa (Lahnsteiner et al., 1996).

Information on the protein composition of Acipenseriformes sperm is lacking (reviewed by Li et al., 2009), mostly due to a lack of data on cDNA and gene sequences. The present study was designed aiming to identify the protein composition of Acipenseriformes species where species specific differences between protein maps could be expected, at least from an evolutionary point of view and taxonomical status of specimens. From present results it can be hypothetised that the detected protein band with a molecular weight of 32.6 kDa might be a specific protein for seminal plasma of paddlefish. To confirm this it will be necessary to identify the amino acid sequence of this protein. The protein band of 71.3 kDa identified in seminal plasma of all three species can be classified, based on MW as a β-Nacetylglucosaminidase, the protein which was isolated and characterized (Sarosiek et al., 2008) in seminal plasma of Siberian sturgeon (*Acipenser baerii*). Protein maps of spermatozoa were constructed by 2DE analysis, presenting background information to locate molecular differences for species specific, and could be applied

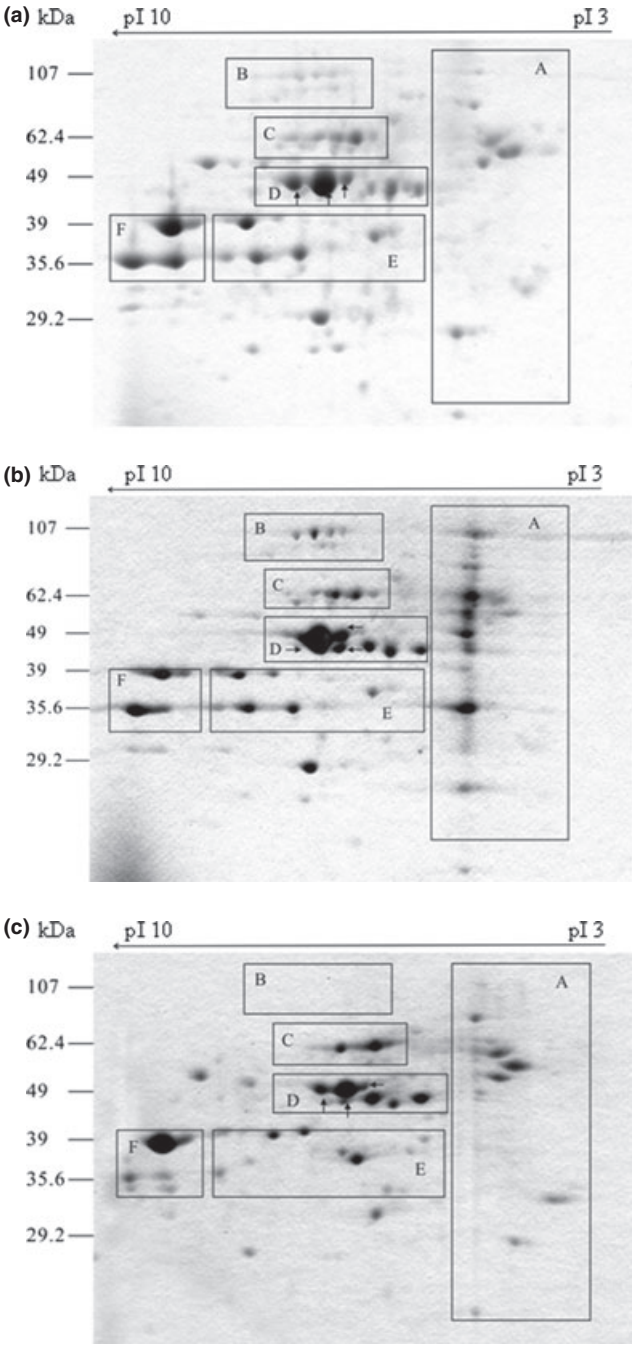

Fig. 4. The 2DE protein maps of spermatozoa in *Acipenser baerii* (a), *A. ruthenus* (b) and *Polyodon spathula* (c). In order to compare gels, each gel was divided into six parts (A–F). Arrows present in part D represent unmatched spots among species according to molecular weigh and isoelectric point

to develop reproduction procedures and sperm cryopreservation protocols. Protein maps expressed strong species specific patterns, with some agreement between Siberian sturgeon and sterlet. However, some acidic proteins in part A were not detected due to vertical streak which may be because those proteins were not easily solved in sample buffer during IEF. The narrow pH range (e.g. pH 4–7) IPG strip should be used to consider those acidic proteins in the future.

It can be concluded that the physico-chemical properties, together with comparative proteomics, could be applied as a powerful analytical tool for better understanding of

species-specific characters among Acipenseriformes. In addition, observed physico-chemical differences should be considered in development of controlled reproduction procedures and sperm cryopreservation protocols.

Acknowledgements

This work was supported by Czech Republic grants CZ.1.05/2.1.00/01.0024, MSM 6007665809, LC06073, IAA608030801, 523/08/0824, ME10015, QH92308, QH82119, GAJU 003/2010/Z, 046/2010/Z and 047/2010/Z, and China grant 200701029.

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