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The relationship of the cryoprotectants methanol and dimethyl sulfoxide and hyperosmotic extenders on sperm cryopreservation of two North-American sturgeon species

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Abstract

Successful sperm cryopreservation techniques have been developed for Eurasian sturgeon species; however, there is little information available on these techniques for North-American species. In this study, two sets of sperm cryopreservation experiments were carried out on the endangered shortnose sturgeon (*Acipenser brevirostrum*). In the first set, the cryoprotectants methanol (MeOH) and dimethyl sulfoxide (DMSO) were investigated using three concentrations (5%, 10% and 15%). The highest post-thaw motility was found using 5% DMSO (26±13%) while the use of 5% MeOH resulted in the highest rates for fertilization at the 4-cell stage (40±15%), neurulation (38±13%) and hatching (32±12%). In the second set, the Original Tsvetkova's extender (OT), Modified Tsvetkova's extender (MT) and modified Hanks' balanced salt solution (mHBSS) were investigated in combination with three MeOH concentrations. The highest post-thaw motility (18±10%), fertilization (18±11%) and hatching rates (17±12%) were observed with MT extender used in combination with 5% MeOH. In another set of experiments, the effects of two extenders (MT and mHBSS) and two concentrations of MeOH were investigated for sperm cryopreservation of pallid sturgeon (*Scaphyrinchus albus*). The highest post-thaw motility (70±10%) was observed using MT and 10% MeOH while MT and 5% MeOH yielded the highest rates of fertilization (88±6%) and

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hatching (73±14%). In general we conclude that although hyperosmotic conditions of extenders and cryoprotectants result in higher post-thaw motility, they seem to reduce the fertilizing ability of the sperm.

Keywords: Sturgeon; *Acipenser brevirostrum*; Sperm; Cryopreservation; Extenders; Cryoprotectants; Osmolality

1. Introduction

Sturgeons (Order *Acipenseriformes*) are chondrostean fishes of ancient origin that inhabit only the Northern hemisphere (Birstein and DeSalle, 1998). Several species are restricted to very small populations which in some cases are close to extinction due to exploitation of natural stocks for meat and caviar as well as destruction of habitat (Billard and Lecointre, 2001).

The shortnose sturgeon (*Acipenser brevirostrum*, Lesueur) inhabits the rivers and brackish waters of the North American Atlantic coast from New Brunswick, Canada to northern Florida (Vladykov and Greeley, 1963). Stocks have been declining during the 20th century and it is federally listed as an endangered species since 1967 (U.S. Office of the Federal Register, 1967). Studies on cultivation and stock enhancement have begun in the southern United States and the results are promising (Smith et al., 1995).

The pallid sturgeon (*Scaphyrinchus albus*, Forbes and Richardson) is indigenous to the Mississippi, Missouri and Yellowstone river drainages (Billard and Lecointre, 2001). This fish, first recognized as a separate species from shovelnose sturgeon (*S. platorhynchus*, Rafinesque) only in 1905, has never been abundant and now it is listed as an endangered species in the United States (Kallemeyn, 1983). Recovery plans by the US Fish and Wildlife Service call for the development of sperm banks to aid artificial propagation of this species (DiLauro et al., 2001) with the ultimate goal of supplemental stocking of the species into its natural habitat.

The first experiments on the cryopreservation of sturgeon sperm were carried out in the 1960s by Soviet scientists (Dettlaff et al., 1993). Several protocols have been developed since then (Drokin et al., 1991; Ciereszko et al., 1996) but they all share a common problem: although high post-thaw motility is observed, fertilization rates remain low or non-existent. Methods resulting in satisfactory fertilization and hatching rates have been described for sterlet (*Acipenser ruthenus* L.) and Siberian sturgeon (*A. baeri* Brandt) by several authors (Tsvetkova et al., 1996; Jähnichen et al., 1999; Glogowski et al., 2002). However, little success has been achieved thus far in sperm cryopreservation of North-American sturgeon species.

The objectives of our work were to test the effect of: (1) cryoprotectants in different concentrations on the motility and fertilizing ability of shortnose sturgeon sperm; (2) several extenders in combination with different cryoprotectant concentrations on the motility and fertilizing ability of shortnose sturgeon sperm; (3) several extenders in combination with different cryoprotectant concentrations on the motility and fertilizing ability of pallid sturgeon sperm.

2. Materials and methods

2.1. Shortnose sturgeon

Captive shortnose sturgeon broodstock were maintained at the Bears Bluff National Fish Hatchery (Wadmalaw Island, South Carolina) of the US Fish and Wildlife Service. The fish (age, 13 years) were kept in plastic holding tanks (2864 l or 4820 l) at 16 °C. Spermiation and ovulation were induced using injection of carp pituitary extract (Stoller Fisheries, Spirit Lake, Iowa, USA). The dose of carp pituitary for males was 1 mg per kg of fish that was administered from a stock solution. A stock solution of 68 mg carp pituitary extract per ml of sterile saline solution (0.6% NaCl) was prepared each week. The dose for females was 4 mg of carp pituitary per kg of fish and a stock solution equivalent to 18 mg per ml 0.6% sterile saline was prepared each week. Also, females received two injections. The first was 10% of the total dose, and the second injection delivered 12 h later was the remaining 90%. Males received only one

injection, at the time of the females' first injection. Males were stripped 24 h following injection while females were stripped several times after the injection with the first stripping taking place 12 h following hormonal treatment. The fish were not anaesthetized before the collection of gametes.

Sperm was collected from males by use of a dry syringe with a silicone tube attached to it. The tube was inserted into the urogenital opening of the fishes and gently introduced into the sperm duct. Collected sperm was stored in plastic bags with oxygen (Billard, 1980) at 4 °C until use. Motility (percentage of spermatozoa performing forward movement) was estimated using dark-field microscopy at 200× magnification by mixing 20 μl of hatchery water with 2 μl of sperm on a glass slide. Sperm concentrations were counted in a Makler Counting Chamber (Sefi Medical Instruments, Haifa, Israel).

The belly of females releasing eggs was wiped with a dry cloth to prevent contact of eggs with water. Eggs were collected into dry bowls by gentle pressure on the abdomen.

Two sets of experiments were carried out on shortnose sturgeon sperm. In the first set, sperm from three males (not mixed) were diluted in Modified Tsvetkova's (MT) extender (30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl, pH 8.0, osmolality: 82 mosM/kg) (Glogowski et al., 2002) in 1:1 ratio. Five, ten or fifteen percent (V/V final concentration) methanol (MeOH, 123, 246 or 369 mM) or dimethyl sulfoxide (DMSO, 70, 140 or 210 mM) were used as cryoprotectant. Thus, 1 ml of 5% solution of cryoprotectants contained 450 μl of extender, 50 μl of cryoprotectant and 500 μl of sperm. The sperm samples were loaded into 0.5-ml French straws. These were distributed on a 3-cm styrofoam frame and frozen in the vapor of liquid nitrogen in a styrofoam box. After 3 min, the straws were plunged into liquid nitrogen and stored in plastic goblets attached to canes in shipping dewars for 24–36 h before thawing and use for fertilization. Freezing rates were measured using a thermocouple inserted into a straw filled with extender mixed with either 10% MeOH or 10% DMSO. The thermocouple was connected to a data-logger (OM-550, Omega Engineering, Stamford, Connecticut) that measured the temperature within the straw every 5 s. The straw was placed onto the styrofoam frame and frozen at the same height as the sperm samples (3 cm). The average freezing rate was calculated using the initial and final temperatures and the time elapsed from placing the straws onto the frame until the temperature reached the minimum that can be read by the data-logger (−105 °C).

Two milliliters of eggs (140–310 eggs depending on the size) were distributed into dry plastic bowls. The sperm samples were thawed in a 40 °C water bath for 13 s. Egg batches were fertilized with either a full straw (500 μ*l*) or half straw (250 μl) of thawed sperm. Fresh sperm from the same males were used as a control to monitor egg quality in a volume equal to the volume of frozen sperm prior to dilution (250 μl or 125 μl). Adhesiveness of eggs was eliminated by gently stirring in a suspension of Fuller's earth (Humco, Texarkana, Texas) for 30 min. Eggs were placed into an incubator containing 300-ml hatching jars. Fertilization percentage was recorded at the 4–8 cell stage (5 h post-fertilization at 16 °C), and development was observed at the neurula stage as well as at hatching. The experiment was repeated the following week using the sperm of three different males and eggs from another female.

In the second set, sperm from three males were used separately. The sperm were diluted 1:1 in three different extenders: MT (described above), Original Tsvetkova's (OT) extender (23.4 mM sucrose, 118 mM Tris, pH 8.0, 222 mosM/kg) (Tsvetkova et al., 1996) and modified Hanks' balanced salt solution (mHBSS, H4385, Sigma, St. Louis, Missouri) diluted 24 times from a concentrate with distilled water to an osmolality of 100 mosM/kg. Osmolality of all solutions used in the experiments was measured with a vapor pressure osmometer (Wescor 5500, Logan, Utah). Osmolalities of MT and OT extenders in combination with 5%, 10% and 15% MeOH and DMSO were also measured. Sperm samples were frozen in the presence of 5%, 10% and 15% MeOH as cryoprotectant (V/V final concentration). Conditions for freezing, thawing, motility estimation and fertilization were the same as described above except that eggs were fertilized with the volume of one straw and 250 μl of fresh sperm used as a control, and the percentage neurulation was not recorded. The experiment was repeated the following week using the sperm of three different males and eggs from another female.

2.2. Pallid sturgeon

Pallid surgeon males were collected from the Mississippi River Outflow Channel at the Old River Control Complex, Concordia Parish, Louisiana (31° 4.5′ N, 91° 35.9′ W), and were kept in fibreglass tanks at Natchitoches National Fish Hatchery (Natchitoches, Louisiana) of the US Fish and Wildlife Service. Spermiation was induced at 18 °C by intra-muscular injection of luteinizing hormone releasing hormone (LHRH) analogue (Syndel International, Vancouver, British Columbia, Canada) at 10 μg per kg body weight. Sperm were collected from males by use of a dry syringe with silicone tubing attached to it. The tube was inserted into the urogenital opening of the fishes and gently introduced into the sperm duct. Collected sperm were stored in 15-ml plastic screw-cap tubes at 4 °C until use (less than 30 min after collection). Motility was estimated using light microscopy at 200× magnification by mixing 20 μl of hatchery water with 2 μl of sperm on a glass slide. Sperm concentrations were counted as described above.

Sperm samples from three males were selected for cryopreservation. The samples were diluted 1:1 in two different extenders: MT and mHBSS. Sperm samples were frozen in the presence of 5% and 10% MeOH as cryoprotectant. Conditions for freezing, thawing and motility estimation were the same as described for shortnose sturgeon except that frozen sperm samples were stored in liquid nitrogen for 4 weeks.

Due to the lack of pallid sturgeon females, shovelnose sturgeon eggs were used to test the fertilizing ability of frozen pallid sturgeon sperm. Shovelnose sturgeon females were collected at the same site as pallid sturgeon males and were kept in fibreglass tanks at the Natchitoches National Fish Hatchery. Ovulation was induced at 18 °C with intramuscular injection of 100 μg per kg of body weight of LHRH analogue administered in two doses. Ten percent of the total dose was injected as a priming dose whereas the resolving dose (90%) was injected 12 h later. The belly of females was wiped dry and the eggs were collected into dry bowls by gentle massage of the abdomen. Stripping of eggs was repeated several times at 2-h intervals. Eggs from two females were used in the experiments.

In fertilization experiments, batches of 2 g of eggs (122–187 ova) were used. Each batch was fertilized with one straw of thawed sperm. Gametes were activated by addition of 5 ml of hatchery water. Approximately 2 min following activation, eggs were spread in a monolayer in plastic bowls and were incubated at 18 °C in 1500-l plastic tanks with recirculated water flow. For control fertilization, 250 μl of fresh pallid sturgeon sperm from two males was used. Percent fertilization was counted at the 4-cell stage and later at the heartbeat stage.

2.3. Statistical analysis of data

Percent motility, fertilization and hatching data were subjected to arcsine transformation prior to analyses. Two-way and three-way analysis of variance (ANOVA) (SAS, SAS Institute, Cary, North Carolina) was used on the transformed data as well as osmolality values to investigate the main effects of extenders, cryoprotectants and their concentrations with Duncan's test as post-test at $P>0.05$.

3. Results

3.1. Shortnose sturgeon

Motility of fresh shortnose sturgeon sperm in the first set of experiments was 77±8% (Table 1). The average cooling rate using methanol as cryoprotectant was 70 °C/min while with DMSO as cryoprotectant it was 66 °C/min (Fig. 1). The highest post-thaw motility (26±13%) was observed using 5% DMSO as cryoprotectant (Table 1). Overall there was no significant difference between cryoprotectants yet there was a significant difference ($P=0.0027$) in motility among cryoprotectant concentrations.

The concentration of shortnose sturgeon sperm was $1.3\pm1.1\times10^9$ spermatozoa per ml of sperm. Sperm-to-egg ratio was $1.5\pm1.3\times10^6$ spermatozoa per egg when a half straw was used for fertilization and $3.3\pm2.8\times10^6$ spermatozoa per egg when a full straw was used. Overall the two different sperm to egg ratios did not have a significant effect on the fertilization and hatching rates in this study. Highest fertilization rate at the 4-cell stage (40±15%), highest neurulation percentage (35±17%) as well as highest hatching rate (31±15%) were observed when 5% MeOH was used as the cryoprotectant. Cryoprotectants and their concen-

Table 1
Post-thaw motility, fertilization and hatching rates (mean±S.D.) of cryopreserved and fresh shortnose sturgeon sperm in the first set of experiments (n=6), DMSO: dimethyl sulfoxide; MeOH: methanol; Control: fresh sperm

Cryoprotectant	Cryoprotectant concentration (%)	Sperm volume (μl)	Motility (%)	Fertilization at 4-cell stage (%)	Neurulation rate (%)	Hatching rate (%)
DMSO		125		0 ± 0^{d}	1 ± 1^{d}	1 ± 1^{d}
	5		26 ± 13^{b}			
		250		1 ± 2^{d}	1 ± 1^{d}	1 ± 1^{d}
DMSO		125		2 ± 2^{d}	0 ± 1^{d}	0 ± 0^{d}
	10		17 ± 6^{cb}			
		250		1 ± 1^{d}	1 ± 1^{d}	0 ± 1^{d}
DMSO		125		0 ± 0^{d}	0 ± 0^{d}	0 ± 0^{d}
	15		2 ± 3^{d}			
		250		0 ± 0^{d}	0 ± 0^{d}	0 ± 0^{d}
MeOH		125		40 ± 15^{b}	35 ± 17^{b}	31 ± 15^{b}
	5		16 ± 7^{cb}			
		250		39 ± 11^{b}	38 ± 13^{b}	32 ± 12^{b}
MeOH		125		19 ± 13^{c}	22 ± 17^{c}	18 ± 16^{c}
	10		13 ± 8^{c}			
		250		21 ± 12^{c}	21 ± 11^{c}	16 ± 8^{c}
MeOH		125		3 ± 5^{d}	2 ± 4^{d}	2 ± 2^{d}
	15		10 ± 5^{cd}			
		250		8 ± 9^{d}	7 ± 6^{d}	5 ± 4^{d}
Control		125		78 ± 26^{a}	69 ± 24^{a}	51 ± 22^{a}
	–		77 ± 8^{a}			
		250		79 ± 23^{a}	71 ± 20^{a}	53 ± 21^{a}

Values sharing a superscript letter within a column were not significantly different (P>0.05).

trations had a significant effect on the fertilization and hatching rates (P<0.0001 for 4-cell stage, neurula stage and percent hatching) and there was also a significant interaction between cryoprotectants and cryoprotectant concentrations (P<0.0001 for all three parameters).

In the second set of experiments, the concentration of spermatozoa was $2.5\pm1.4\times10^{9}$ cells per ml of

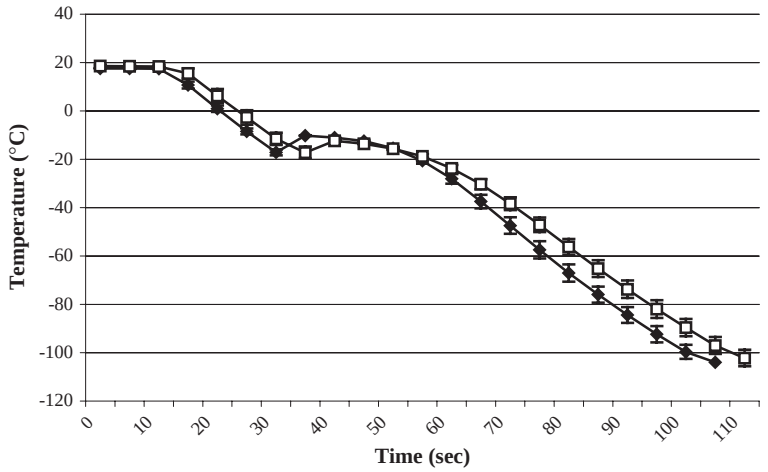

Fig. 1. Cooling curve used in the cryopreservation experiments (n=2). Values are given as mean±S.D. Squares: methanol (MeOH) as cryoprotectant, diamonds: dimethyl sulfoxide (DMSO) as cryoprotectant.

sperm while the motility of fresh shortnose sturgeon sperm was 73±16% (Table 2). The sperm-to-egg ratio was $7.0\pm3.6\times10^6$ spermatozoa per egg in these experiments. Highest post-thaw motility was observed when either MT (18±10%) or OT (12±6%) extenders were used in combination with 5% MeOH. The type of extender did not have a significant effect (P=0.1586) on the post-thaw motility of shortnose sturgeon sperm, however, there were significant differences (P=0.0003) among cryoprotectant concentrations. The highest fertilization and hatching rates were achieved using either MT (18±11% fertilization and 17±12% hatching) or mHBSS (12±10% fertilization and 11±12% hatching) extenders in combination with 5% MeOH. Overall the type of extender had a significant effect on fertilization rate at 4-cell stage (P=0.0197) and on hatching rates (P=0.0500). The concentration of MeOH also had a significant effect on fertilization (P=0.0002) and hatch (P=0.0004).

The effect of osmolality of MT and OT extenders in combination with MeOH and DMSO in different concentrations was investigated (Table 3). Significant differences were found between both extenders (P<0.0001). The use of DMSO significantly increased (P<0.0001) the osmolality of both extenders while all three concentrations of cryoprotectants resulted in significantly different osmolality values (P<0.0001).

Table 3
Osmolalities of Modified Tsvetkova's and Original Tsvetkova's extenders (mean±S.D.) in combination with DMSO or MeOH as cryoprotectants in different concentrations (n=3) for shortnose sturgeon sperm

Extender	Cryoprotectant	Concentration (%)	Osmolality (mosM/kg)
MT	None		73 ± 2^{g}
MT		5	719 ± 9^{e}
MT	DMSO	10	1421 ± 26^{c}
MT		15	1985 ± 12^{a}
MT		5	75 ± 1^{g}
MT	MeOH	10	78 ± 15^{g}
MT		15	84 ± 14^{g}
OT	None		204 ± 8^{f}
OT		5	840 ± 15^{d}
OT	DMSO	10	1481 ± 24^{b}
OT		15	>2000
OT		5	196 ± 3^{f}
OT	MeOH	10	187 ± 3^{f}
OT		15	204 ± 8^{f}

DMSO: dimethyl sulfoxide; MeOH: methanol, OT: Original Tsvetkova's extender; MT: modified Tsvetkova's extender. Values sharing a superscript letter within a column were not significantly different (P>0.05). Osmolality of OT extender in combination with 15% DMSO is not shown as it was higher than the measurable range of the used osmometer.

3.2. Pallid sturgeon

The concentration of pallid sturgeon spermatozoa was $1.3\pm8.9\times10^9$ which resulted in a sperm–egg ratio of $3.9\pm2.2\times10^6$. Motility of fresh pallid sturgeon sperm was 87±6%. There was no significant difference in post-thaw motilities (Table 4) between the two extenders (P=0.2455) or concentrations of cryoprotectants (P=0.5662). While statistical analysis did not show any difference between the extenders at the 4-cell stage (P=0.1366), at the

Table 2
Post-thaw motility, fertilization and hatching rates (mean±S.D.) of cryopreserved and fresh shortnose sturgeon sperm in the second set of experiments (n=6)

Extender	Concentration of MeOH (%)	Motility (%)	Fertilization at 4-cell stage (%)	Hatching rate (%)
OT	5	12 ± 6^{bc}	3 ± 7^{cd}	2 ± 2^{cd}
OT	10	$11\pm2b^{cd}$	2 ± 3^{cd}	3 ± 4^{cd}
OT	15	8 ± 3^{cd}	0 ± 1^{d}	1 ± 1^{d}
MT	5	18 ± 10^{b}	18 ± 11^{b}	17 ± 12^{b}
MT	10	8 ± 5^{cd}	7 ± 11^{cd}	3 ± 4^{cd}
MT	15	6 ± 4^{cd}	2 ± 2^{cd}	1 ± 2^{d}
mHBSS	5	11 ± 7^{bcd}	12 ± 10^{bc}	11 ± 12^{bc}
mHBSS	10	8 ± 7^{cd}	4 ± 5^{cd}	5 ± 6^{cd}
mHBSS	15	3 ± 2^{d}	1 ± 1^{cd}	0 ± 0^{d}
Control	–	73 ± 16^{a}	40 ± 22^{a}	27 ± 13^{a}

OT: Original Tsvetkova's extender; MT: modified Tsvetkova's extender; mHBSS: modified Hanks' balanced salt solution; MeOH: methanol; Control: Fresh sperm. Values sharing a superscript letter within a column were not significantly different (P>0.05).

Table 4
Post-thaw motility, fertilization and hatching rates (mean±S.D.) of cryopreserved and fresh pallid sturgeon sperm in the second set of experiments (n=3)

Extender	Concentration of MeOH (%)	Motility (%)	Fertilization at 4-cell stage (%)	Heartbeat stage embryos (%)
MT	5	60 ± 10^{a}	88 ± 6^{ab}	73 ± 14^{a}
MT	10	70 ± 10^{a}	85 ± 3^{ab}	71 ± 10^{a}
mHBSS	5	60 ± 0^{a}	84 ± 9^{ab}	56 ± 16^{b}
mHBSS	10	57 ± 12^{a}	79 ± 9^{b}	52 ± 10^{b}
Control	–	–	89 ± 6^{a}	80 ± 4^{a}

MT: modified Tsvetkova's extender; mHBSS: modified Hanks' balanced salt solution; MeOH: methanol; Control: fresh sperm. Values sharing a superscript letter within a column were not significantly different (P>0.05).

heartbeat stage the use of MT extender provided significantly higher (P=0.0032) fertilization rates than did mHBSS. Cryoprotectant concentration did not have a significant effect on fertilization rate at 4-cell stage (P=0.2685) or heartbeat stage (P=0.6530).

4. Discussion

The relative ineffectiveness of sperm cryopreservation methods for Eurasian and North-American sturgeon species can in part be attributed to the lack of comparison between different freezing methods. In our experiments we tried to compare the effects of several extenders and cryoprotectants on motility and fertilizing ability of cryopreserved shortnose and pallid sturgeon sperm.

Although the use of 5% DMSO as a cryoprotectant produced the highest post-thaw motility for shortnose sturgeon sperm, the use of MeOH yielded significantly higher fertilization and hatching rates. This corresponds with previous observations on Siberian sturgeon that post-thaw motility does not predict fertilization or hatching success (Glogowski et al., 2002). Dimethyl sulfoxide and methanol were each found to be effective cryoprotectants for sturgeon (Tsvetkova et al., 1996; Glogowski et al., 2002; Urbányi et al., 2003) and paddlefish (*Polyodon spathula*, Mims et al., 2000) sperm, however, the fertilization and hatching success achieved with DMSO as cryoprotectant varied among species. Prior to the present study there has been no direct comparison reported of these two cryoprotectants for sturgeon sperm. Methanol has been used for the cryopreservation of cyprinid species such as zebrafish (*Danio rerio*) (Harvey et al., 1982) and other groups of fishes such as salmonids (Lahnsteiner et al., 1997), catfishes (Steyn, 1993; Steyn and Van Vuren, 1987; Tiersch et al., 1994) and tilapia (Harvey, 1983). Methanol is nontoxic when used as a cryoprotectant and was found to be superior to DMSO or glycerol in a number of cell types such as mammalian tissue-culture cells (Harvey et al., 1982). It is known for its rapid penetration of cells (Ashwood-Smith, 1980), however, the specifics of its action as a cryoprotectant remain unclear. The fact that sperm samples cryopreserved with DMSO had similar or even higher post-thaw motility than those with MeOH indicates that the cells survive the freezing process, and retain the ability to move, but they lose the ability to fertilize eggs.

In the case of DMSO, post-thaw motility depended on the concentration of the cryoprotectant (lower concentrations resulted in higher motility) whereas for MeOH this difference was not observed. Significant differences were found between fertilization and hatching rates in both sets of experiments with varying cryoprotectant concentrations, with 5% methanol yielding the best results in either experiment. Higher doses of methanol yielded lower fertilization and hatching rates which suggests that at higher concentrations it becomes toxic.

In the second set of experiments we found that fertilization success depended on the extender as well as on the concentration of MeOH. While there was no difference between fertilization and hatching rates when MT or mHBSS were used, OT extender yielded significantly lower values than MT. These data and the observations on the osmolality of MT and OT extenders in combination with different concentrations of DMSO and MeOH indicate that osmolality plays a key role in fertilization and hatching success for sturgeon sperm. It is known that in contrast to

teleost sperm, where the osmolality of seminal plasma is around 250–300 mosM/kg (Morisawa et al., 1983), sturgeon sperm has a lower osmolality, in some cases as low as 30 mosM/kg (Gallis et al., 1991).

Our observations show that OT extender (204±8 mosM/kg) had a significantly higher osmolality than did MT extender (73±2 mosM/kg). Addition of DMSO increased the osmolality of both solutions, while methanol did not change it significantly (Table 3). This corresponds with the observations that addition of 5%, 10% or 15% DMSO to Mounib's solution increased its osmolality (originally 289 mosM/kg H_2O) to 1022, 1768 and 2456 mosM/kg H_2O, while addition of MeOH resulted in a slight decrease of osmolality (Tiersch et al., 1994; Ogier de Baulny et al., 1997).

Reduction of osmolality typically results in the activation of spermatozoa of freshwater fishes (Morisawa et al., 1983), however, it is not clear what happens to sperm cells in solutions with higher osmolality values than that of the seminal plasma. Dehydration as a result of increasing extracellular osmotic pressure is an important event in course of freezing of live cells and tissues to ensure cell survival (Denniston et al., 2000). In this study two factors facilitated the dehydration of cells before freezing: dilution in an extender with osmolality higher than that of the seminal plasma (in this study OT extender), and the addition of a cryoprotectant that increases osmolality (DMSO). Thus, higher osmolality values increase the chances of cell survival, which is expressed in higher sperm motility. According to our results, however, sperm samples frozen in either OT extender or in presence of DMSO were not able to fertilize eggs. This problem did not occur when mHBSS was used as an extender with osmolality set to 100 mosM/kg.

Hybridization of endangered species is not desirable and we used the eggs of shovelnose sturgeon to test the fertilizing ability of cryopreserved pallid sturgeon sperm only for experimental purposes. Pallid and shovelnose sturgeon are known to hybridize naturally (Carlson et al., 1985) and the potential of cryopreserved sturgeon sperm for hybridization of different species has been demonstrated previously (Urbányi et al., 2003).

Both MT and mHBSS extenders can successfully be used for the cryopreservation of pallid sturgeon sperm. Although there was no significant difference between the fertilization rates at 4-cell stage, a significantly lower number of embryos survived to heartbeat stage in the batches fertilized with sperm frozen in mHBSS.

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References

Ashwood-Smith, M.J., 1980. Low-temperature preservation of cells, tissues and organs. In: Ashwood-Smith, M.J., Farrant, J. (Eds.), Low-temperature Preservation in Medicine and Biology. Pitman Medical, Turnbridge Wells, pp. 19–45.

Billard, R., 1980. Survie des gamètes de truite arc-en-ciel après dilution dans des solutions salines ou du sucrose. Reprod. Nutr. Dév. 20, 1899–1905.

Billard, R., Lecointre, G., 2001. Biology and conservation of sturgeon and paddlefish. Rev. Fish Biol. Fish. 10, 355–392.

Birstein, B.J., DeSalle, R., 1998. Molecular phylogeny of *Acipenserinae*. Mol. Phylogenet. Evol. 9, 141–155.

Carlson, D.M., Pflieger, W.L., Trial, L., Haverland, P.S., 1985. Distribution, biology and hybridization of *Scaphyrhinchus albus* and *S. platorhynchus* in the Missouri and Mississippi rivers. Environ. Biol. Fishes 14, 51–59.

Ciereszko, A., Toth, G.P., Christ, S.A., Dabrowski, K., 1996. Effect of cryopreservation and theophylline on motility characteristics of lake sturgeon (*Acipenser fulvescens*) spermatozoa. Theriogenology 45, 665–672.

Denniston, R.S., Michelet, S., Godke, R.A., 2000. Principles of cryopreservation. In: Tiersch, T.R., Mazik, P.M. (Eds.), Cryopreservation in Aquatic Species. World Aquaculture Society, Baton Rouge, LA, pp. 59–74.

Dettlaff, T.A., Ginsburg, A.S., Schmalhausen, O.I., 1993. Sturgeon Fishes, Developmental Biology and Aquaculture. Springer-Verlag, Berlin, Heidelberg.

DiLauro, M.N., Walsh, R.A., Peiffer, M., Bennett, R.M., 2001. Sperm-cell ultrastructure of North American sturgeons: IV. The pallid sturgeon (*Scaphyrinchus albus* Forbes and Richardson, 1905). Can. J. Zool. 79, 802–808.

Drokin, S.I., Cherepanov, V.V., Kopeika, E.F., Shilin, N.I., 1991. Sakhalin sturgeon: how to preserve gene fund. Rybnoe Khoziaistvo 7, 38–39 (in Russian).

Gallis, J.L., Fedrigo, E., Jatteau, P., Bonpunt, E., Billard, R., 1991. Siberian sturgeon, *Acipenser baeri*, spermatozoa: effects of dilution, pH, osmotic pressure, sodium and potassium ions on motility. In: Williot, P. (Ed.), Acipenser. Cemagref publ., pp. 143–151.

Glogowski, J., Kolman, R., Szczepkowski, M., Horváth, Á., Urbányi, B., Sieczyñski, P., Rzemieniecki, A., Domagał, J., Demianowicz, W., Kowalski, R., Ciereszko, A., 2002. Fertilization rate of Siberian sturgeon (*Acipenser baeri*, Brandt) milt cryopreserved with methanol. Aquaculture 211, 367–373.

Harvey, B., 1983. Cryopreservation of *Sarotherodon mossambicus* spermatozoa. Aquaculture 32, 313–320.

Harvey, B., Kelley, R.N., Ashwood-Smith, M.J., 1982. Cryopreservation of zebra fish spermatozoa using methanol. Can. J. Zool. 60, 1867–1870.

Jähnichen, H., Warnecke, D., Trölsch, E., Kohlmann, K., Bergler, H., Pluta, H.-J., 1999. Motility and fertilizing capability of cryopreserved *Acipenser ruthenus* L. sperm. J. Appl. Ichthyol. 15, 204–206.

Kallemeyn, L., 1983. Status of the pallid sturgeon. Fisheries (Bethesda) 8, 3–9.

Lahnsteiner, F., Weismann, T., Patzner, R.A., 1997. Methanol as cryoprotectant and the suitability of 1.2 ml and 5 ml straws for cryopreservation of semen from salmonid fishes. Aquac. Res. 28, 471–479.

Mims, S.D., Tsvetkova, L.I., Brown, G.G., Gomelsky, B.I., 2000. Cryopreservation of sperm of sturgeon and paddlefish. In: Tiersch, T.R., Mazik, P.M. (Eds.), Cryopresertvation in Aquatic Species. World Aquaculture Society, Baton Rouge, USA, pp. 123–129.

Morisawa, M., Suzuki, K., Shimizu, H., Morisawa, S., Yasuda, K., 1983. Effects of osmolality and potassium on motility of spermatozoa from freshwater cyprinid fishes. J. Exp. Biol. 107, 95–103.

Ogier de Baulny, B., Le Vern, Y., Kerboeuf, D., Maisse, G., 1997. Flow cytometric evaluation of mitochondrial activity and membrane integrity in fresh and cryopreserved rainbow trout (Onchorynchus mykiss) spermatozoa. Cryobiology 34, 141–149.

Smith, T.I.J., Heyward, L.D., Jenkins, W.E., Collins, M.R., 1995. Culture and stock enhancement of shortnose sturgeon, *Acipenser brevirostrum*, in the Southern United States. Proc. Intern. Sturg. Symp., Moscow, VNIRO, pp. 204–214.

Steyn, G.J., 1993. The effect of freezing rate on the survival of cryopreserved African sharptooth catfish (*Clarias gariepinus*) spermatozoa. Cryobiology 30, 581–590.

Steyn, G.J., Van Vuren, J.H.J., 1987. The fertilizing capacity of cryopreserved sharptooth catfish (*Clarias gariepinus*) sperm. Aquaculture 63, 187–193.

Tiersch, T.R., Goudie, C.A., Carmichael, G.J., 1994. Cryopreservation of channel catfish sperm: storage in cryoprotectants, fertilization trials and growth of channel catfish produced with cryopreserved sperm. Trans. Am. Fish. Soc. 123, 580–586.

Tsvetkova, L.I., Cosson, J., Linhart, O., Billard, R., 1996. Motility and fertilizing capacity of fresh and frozen–thawed spermatozoa in sturgeons *Acipenser baeri* and *A. ruthenus*. J. Appl. Ichthyol. 12, 107–112.

Urbányi, B., Horváth, Á., Kovács, B., 2003. Successful hybridization of *Acipenser* species using cryopreserved sperm. Aquac. Int. 12, 47–56.

U.S. Office of the Federal Register, 1967. Endangered and threatened wildlife and plants; endangered status of the shortnose sturgeon. Fed. Regist. 32, 4001.

Vladykov, V.D., Greeley, J.R., 1963. Order acipenseroidei. Fish of the Western North Atlantic, Sears Foundation Marine Research, vol. 1. Yale University, New Haven, pp. 24–60.