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[Management Brief]

Cryopreservation of Sperm from Endangered Pallid Sturgeon

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Abstract.—We sought to develop sperm cryopreservation methods for the pallid sturgeon *Scaphirhynchus albus*, a federally listed endangered species. Males were injected with synthetic luteinizing hormone releasing hormone at 50 μg/kg of body weight. After 24 h, sperm were collected, diluted at a ratio of 1:4 (sperm : extender) with Hanks' balanced salt solution (HBSS; diluted to 100 milliosmoles/kg), and kept refrigerated until use. Methanol was used as a cryoprotectant at concentrations of 5, 10, and 15% (volume per volume) and was mixed 1:1 with HBSS before the experiment to reduce effects of initial mixing. Sperm were mixed with the cryoprotectant, loaded into 0.5-mL straws, packed into goblets (5 straws/goblet), and placed in the lower position on aluminum canes. Motility was estimated before freezing to determine the effects of cryoprotectant toxicity; there was no significant difference in motility at the concentrations tested ($P = 0.4828$). After a 2-min equilibration period, the canes were lowered into a nitrogen vapor shipping dewar. The cooling rate of −22°C/min was recorded by thermocouples inserted into 0.5-mL straws filled with extender and cryoprotectant. After 1 year of storage in liquid nitrogen, straws were thawed in a 40°C water bath for 9 s and motility was estimated. Postthaw motility did not differ among the cryoprotectants tested ($P = 0.4880$). Each sample was used to fertilize approximately 150 eggs, which were incubated at 21°C. Sperm that was cryopreserved with 5% or 10% methanol produced eggs with significantly higher hatch rates did sperm cryopreserved with 15% methanol ($P < 0.0001$). The development of techniques for cryopreserved sperm of pallid sturgeon allows for the creation of germplasm repositories that will aid in the recovery of this endangered species.

Three riverine sturgeons—the pallid sturgeon *Scaphirhynchus albus*, shovelnose sturgeon *S. platorynchus*, and Alabama sturgeon *S. suttkusi*—constitute one-third of the North American acipenserid species. The pallid sturgeon is one of four sturgeon species listed as endangered in the United States (USFWS 1993). This species was originally described by Forbes and Richardson (1905) as being distributed throughout the Missouri and Mississippi River systems. At the time of the species' original description, pallid sturgeon were relatively rare and accounted for 1 in 500 of the sturgeon captured in the Mississippi River at Grafton, Illinois (Forbes and Richardson 1905). More recently, only 11 pallid sturgeon were reported among the 4,355 sturgeon (i.e., ratio of 1:400) captured in the Missouri and Mississippi rivers (Carlson et al. 1985). Moreover, no pallid sturgeon was captured in the Mississippi River upstream from the Missouri River confluence, suggesting significant declines in numbers and distribution of this species.

Destruction and alteration of habitat, such as dam construction and channelization, are believed to be the primary causes of pallid sturgeon population decline (USFWS 1993). On the Missouri River, six earthen dams have eliminated 36% of the riverine habitat within the range of the pallid sturgeon. Another 40% of the river has been channelized, and the remaining stretches of river possess altered flow regimes. Pallid sturgeon prefer living over sand bottoms in highly turbid waters with strong current (Bailey and Cross 1954), and the reduced flow and turbidity may be causing reduced survival and reproduction.

Another potential threat to the survival of pallid sturgeon is hybridization with shovelnose sturgeon. Putative pallid sturgeon × shovelnose sturgeon hybrids (as determined based on morphometric assessment) have been collected throughout the Mississippi and Missouri River drainages. Putative hybrids had not been reported before 1985 (Carlson et al. 1985; Keenlyne et al. 1994). The morphological measurements used to distinguish hybrids in these reports have been disputed based on studies with larger sample sizes; these larger studies indicate that measurements for the parent species are not distinct but actually overlap (Keenlyne et al. 1994). However, recent

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analysis of mitochondrial DNA (Simons et al. 2001) and microsatellite data (Tranah et al. 2001) suggests that hybridization is occurring and that continued hybridization will result in the loss of genetic integrity (Simons et al. 2001). Furthermore, pallid sturgeon occupying the northern and southern parts of the species' range are genetically distinct and thus should be managed as separate populations (Tranah et al. 2001). Cryopreservation of sperm could be used to preserve the remaining diversity and aid in the recovery of this endangered species (USFWS 1993).

Sperm cryopreservation also offers advantages for artificial spawning and recovery of endangered species (Tiersch et al. 1997, 1998). The use of cryopreserved sperm reduces the need for collection and maintenance of male broodstock, thus decreasing the amount of stress applied to wild fish captured for annual captive spawning efforts. In the case of pallid sturgeon, which can weigh as much as 30 kg each (Pflieger 1975), use of cryopreserved sperm reduces the demand for male broodstock holding facilities. Another major obstacle encountered during induced spawning of sturgeon is noncoincident maturation (DiLauro et al. 1994). Often, males and females do not produce gametes at the same time of the year or only one sex is collected at a given time. Cryopreservation of sperm allows for spawning whenever ripe females are ovulating. A third advantage of cryopreserved sperm is its ability to increase efficiency and control of genetic management (e.g., Cloud et al. 1990; Van der Walt et al. 1993; Tiersch et al. 1998).

Accordingly, the goal of this study was to improve methods for cryopreservation of pallid sturgeon sperm. The most common cryoprotectants used in cryopreservation of sturgeon sperm have been glycerol, dimethyl sulfoxide, and methanol (Mims et al. 2000; Billard et al. 2004). Previous research on pallid sturgeon has only investigated the use of methanol as a cryoprotectant at concentrations of 5% and 10% (Horvath et al. 2005). Our objectives were to evaluate the effects of additional cryoprotectant concentrations on (1) motility of sperm exposed to cryoprotectants before freezing, (2) postthaw motility, (3) fertilization rate, and (4) hatch rate. Development of cryopreservation procedures for pallid sturgeon sperm could aid in the creation of germplasm repositories for conservation of genetic resources and recovery of this endangered species.

Methods

Collection and identification of pallid sturgeon.—Pallid sturgeon were collected by members of the Upper Basin Pallid Sturgeon Recovery Team from the upper Missouri River near the confluence with the Yellowstone River in Montana. Pallid sturgeon from the upper basin can be easily distinguished morphologically from shovelnose sturgeon. Also, 15 microsatellite loci (based on Tranah et al. 2004) were used to identify the males used in this study as pallid sturgeon (Patrick DeHaan, U.S. Fish and Wildlife Service, personal communication).

Collection of sperm.—Four male pallid sturgeon held at the Garrison Dam National Fish Hatchery (NFH), North Dakota, were used in the study. Each male was injected with luteinizing hormone releasing hormone analog (LHRHa; Syndell Laboratories, Vancouver, British Columbia) at a concentration of 50 μg/kg of body weight. After 24 h, a 60-mL sample of sperm was collected by inserting a small-diameter tube (outer diameter = 6 mm; internal diameter = 4 mm) connected to a 60-mL syringe into the urogenital opening and drawing on the plunger. Each sample was maintained separately throughout the study. Mean (±SD) seminal plasma osmolality was 65 ± 10 milliosmoles (mOsm) per kilogram. The sperm were extended at a ratio of 1:4 (sperm : extender) with modified Hanks' balanced salt solution (HBSS; Sigma Chemical Corp., St. Louis, Missouri; H4385) that was diluted to 100 mOsm/kg and verified by use of a vapor pressure osmometer (Wescor, Logan, Utah; Model 5500). Samples were stored in 50-mL centrifuge tubes supplemented with oxygen and were refrigerated until use (~12 h).

Motility estimation.—Sperm motility was estimated at four times during the study. Motility was estimated when samples were extended, and all samples were found to be nonmotile in the extender. Initial motility was estimated just before initiation of the freezing protocol. Equilibration motility was estimated 2 min after the addition of cryoprotectants. Postthaw motility was estimated immediately after sperm was thawed. Motility was estimated by activating 2 μL of sperm with 20 μL of deionized water. Sperm were observed at 100× magnification under dark-field illumination. Only sperm that were actively swimming with a progressive motion were classified as motile (sperm that vibrated in place were considered nonmotile).

Freezing.—Reagent-grade methanol (Sigma) was evaluated as cryoprotectant at concentrations of 5, 10, and 15% (volume per volume [v/v]). Cryoprotectants were first mixed 1:1 with HBSS to minimize effects on sperm from direct exposure to the cryoprotectants. The cryoprotectants were added to the sperm and allowed to equilibrate for 2 min. The mixtures were loaded into 0.5-mL French straws (IMV International, Minneapolis, Minnesota), and the straws were sealed with polyvinyl chloride (PVC) powder. The straws were placed into goblets at the bottom position on aluminum canes and placed into a shipping dewar (Taylor-

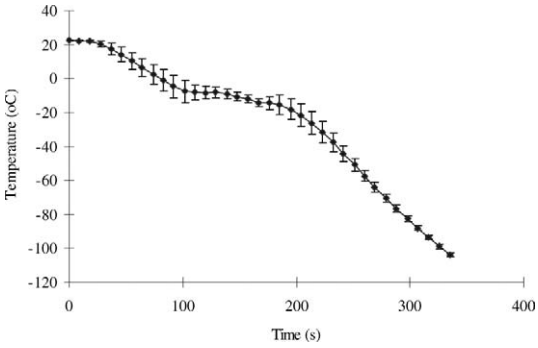

FIGURE 1.—Mean (±SD) cooling rate of 0.5-mL straws ($n = 3$) frozen at the lower position in a nitrogen vapor shipping dewar; similar straws were used in the cryopreservation of pallid sturgeon sperm. Straws were loaded into plastic goblets (5 straws/goblet) and placed on aluminum canes. Type-T thermocouples were inserted into the straws, and the cooling rate was recorded every 9 s.

Wharton, Theodore, Alabama; CP-100). Equilibration motility was estimated immediately after the canes were lowered into the dewar. The cooling rate was measured using a data logger (Omega Engineering, Inc., Stamford, Connecticut; OM-550) and type-T thermocouples inserted into 0.5-mL straws that were filled with extender and cryoprotectant. The average cooling rate was −22°C/min (Figure 1). After freezing, the sperm were transported in shipping dewars back to the Warm Springs Fish Technology Center, Warm Springs, Georgia, and placed in a liquid nitrogen storage dewar.

Fertilization and hatching trials.—Twelve months later, the sperm were transported back to Garrison Dam NFH in shipping dewars for fertilization trials. A female pallid sturgeon was injected with LHRHa at 100 μg/kg and domperidone (a dopamine antagonist; Sigma D-122) at 3 mg/kg. The hormones were administered in two injections. The first injection was 10% of the total dosage; the second injection, given 12 h later, was the remaining 90%. Eggs were collected approximately 24 h after the second injection. Sperm were thawed in a water bath for 9 s at 40°C. Postthaw motility was estimated immediately after the straws were thawed. Each straw was used to fertilize approximately 150 eggs. Four replicates were used per treatment. Fresh sperm from a different male were used as a control of egg quality. After 5 min, the eggs were mixed with Fuller's earth (Humco, Texarkana, Texas) for 15 min to prevent clumping. The residual Fuller's earth was decanted, and the eggs were transferred to an incubation system and held at 21°C. After 4 h, approximately 60 eggs were removed and preserved in 10% buffered formalin for determination of fertilization percentages. Eggs were at the four- to eight-cell stage. After 5 d of incubation, hatching was completed and percent hatch was determined by counting the number of fry and dividing this count by the total number of eggs.

Statistical analysis.—All percentage data were arcsine–square-root transformed before analysis. Equilibration motility data were analyzed by a Kruskal–Wallis analysis of variance (ANOVA; Number Cruncher Statistical Software, Kaysville, Utah), which was corrected for equal values. Data on postthaw motility, fertilization percentage, and hatch percentage were analyzed by one-way ANOVA. Differences were considered to be significant at P-values less than 0.05. The Tukey–Kramer multiple comparison test was used to separate the means.

Results

Motility

The initial motility (mean ± SD) of sperm was 95 ± 0% for all males. There were no differences in equilibration motility ($P = 0.4828$) or postthaw motility

TABLE 1.—Motility characteristics of pallid sturgeon sperm cryopreserved in methanol (MeOH) at three concentrations, and egg fertilization and hatch rates produced by sperm from each treatment. Equilibration of sperm in cryoprotectant occurred for 2 min prior to freezing in a shipping dewar (cooling rate = −22°C/min). After cryopreservation for 12 months, straws were thawed in a water bath for 9 s at 40°C. Postthaw motility was estimated immediately after thawing. Four replicate straws per treatment were used to fertilize 150 eggs each. Eggs were incubated at 21°C, and fertilization rates were estimated at 4 h after fertilization (four- to eight-cell stage). At 5 d (when all eggs had hatched), hatch rates were determined ([number of fry/total number of eggs] ×100). Within a column, values with the same letter are not significantly different ($P > 0.05$).

Cryoprotectant	Equilibration motility (%)	Postthaw motility (%)	Fertilization (%)	Hatch (%)
Control	95 ± 0 z	95 ± 0[a]	97 ± 2 z	69 ± 5 z
5% MeOH	94 ± 2 z	28 ± 6 z	91 ± 5 z	77 ± 6 z
10% MeOH	91 ± 2 z	25 ± 12 z	92 ± 5 z	77 ± 8 z
15% MeOH	90 ± 0 z	20 ± 6 z	12 ± 12 y	10 ± 12 y

[a] Fresh sperm was used as a control of egg quality.

($P = 0.4880$) among the cryoprotectant concentrations (Table 1).

Fertilization Trials

Fertilization rates for sperm cryopreserved with methanol at 5% or 10% were significantly higher ($P < 0.0001$) than for sperm cryopreserved with 15% methanol (Table 1). Fertilization rates did not differ between sperm cryopreserved with methanol at 5% or 10% and fresh sperm. Hatching rates were significantly higher ($P < 0.0001$) for sperm cryopreserved with 5% or 10% methanol than for 15% methanol (Table 1). Hatch rates of eggs fertilized with 5% or 10% methanol cryopreserved sperm were not different from hatch rates of eggs fertilized with fresh sperm ($P > 0.05$).

Discussion

This study reports motility, fertilization, and hatch data for pallid sturgeon sperm cryopreserved with three concentrations of methanol. Cryopreservation of sturgeon sperm has received less attention than sperm cryopreservation for other species (see reviews by Scott and Baynes 1980; Stoss 1983; Rana 1995). Of the few papers that have been published, the majority of sturgeon work was done in the former Soviet Union and is published in Russian (Billard et al. 2004), making access to this information difficult for English-speaking researchers. North American sturgeon species have received minimal attention (i.e., only two peer-reviewed publications). Postthaw sperm motility characteristics in lake sturgeon *Acipenser fulvescens* have been reported, but fertilization trials have not been conducted (Ciereszko et al. 1996). Postthaw motility and fertilization results for shortnose sturgeon *A. brevirostrum* and pallid sturgeon have been investigated more recently (Horvath et al. 2005).

The study by Horvath et al. (2005) investigated the effects of different extenders and concentrations of methanol on postthaw motility and fertilization rates at the four-cell and heartbeat stages. Their study and ours investigated the effects of methanol at 5% and 10% as a cryoprotectant and used modified HBSS as an extender. Egg volumes (150 eggs/treatment; 1 straw/treatment; Horvath et al. 2005) were similar to those used here. Fertilization rates at the four-cell stage were also similar in the two studies. However, the studies differed in some aspects. The sperm : egg ratio reported for pallid sturgeon (Horvath et al. 2005) was $3.9 \pm 2.2 \times 10^6$. Although sperm concentrations were not determined here, the mean sperm concentration for pallid sturgeon ($n = 23$), including the same males collected 5 d later and in future years, was $8.0 \pm 3.1 \times 10^7$. The dilution rate was 1:4 (sperm : extender; v/v), or four times greater than the 1:1 dilution used by Horvath et al. (2005), and would produce a sperm : egg ratio of $5.5 \pm 2.2 \times 10^4$. This suggests that sperm : egg ratios lower than those reported by Horvath et al. (2005) can be used for pallid sturgeon sperm cryopreservation. Also, in the present study, sperm were cooled at a rate of 22°C/min using a nitrogen vapor shipping dewar. Sperm were cooled at a rate of 70°C/min by Horvath et al. (2005) using a Styrofoam platform and liquid nitrogen. Postthaw motility was higher in their study (60–70%) than in ours (20–28%), which suggests that faster freezing is more suitable. However, as noted earlier, there was no difference in fertilization ability between studies, despite the higher sperm dilution rate used here.

Methanol concentrations used here did not significantly reduce sperm motility within 2 min. Methanol has been used for sperm cryopreservation in Siberian sturgeon *A. baeri* (Glowgowski et al. 2002; Urbanyi et al. 2004), sterlet *A. ruthenus* (Urbanyi et al. 2004), Russian sturgeon *A. gueldenstaedti* (Urbanyi et al. 2004), European sturgeon *A. sturio* (Urbanyi et al. 2004), shortnose sturgeon (Horvath et al. 2005), and pallid sturgeon (Horvath et al. 2005). However, the effects of toxicity before freezing were not reported in those publications. Methanol appears to have varied toxicity to fish sperm cells, and in some cases it can even enhance motility during refrigerated storage. Sperm motility of gilthead seabream *Sparus aurata* was reduced to below 50% by exposure to 5% methanol for 10 min, and 10% methanol reduced motility to about 10% within 2 min (Fabbrocini et al. 2000). However, sperm of channel catfish *Ictalurus punctatus* stored in HBSS with 5% methanol retained motility significantly longer (17 d) than did sperm stored in HBSS without methanol (11 d; Christensen and Tiersch 1996).

In this study, fertilization and hatch rates for sperm cryopreserved with 5% and 10% methanol were equal to those with fresh sperm. It should be noted that no attempt was made to standardize the sperm : egg ratio during this study and that the number of eggs used per treatment was low (~150). To apply this method at a production scale, research into optimal ratios of cryopreserved sperm and eggs should be undertaken to increase efficiency, and techniques should be developed for handling of larger volumes of sperm. However, the development of cryopreservation procedures for pallid sturgeon sperm has allowed the collection, long-term storage, and possible shipping (e.g., Tiersch et al. 2004) of genetic resources from this endangered species, and the development of a germplasm repository for pallid sturgeon has been integrated into existing recovery efforts for this species.

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