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Cryopreservation of Sperm from the Endangered Colorado Pikeminnow

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Abstract.—We developed methods for the cryopreservation of sperm of the endangered Colorado pikeminnow *Ptychocheilus lucius*. Sperm were collected from a captive broodstock population of Colorado pikeminnow reared and maintained at the Dexter National Fish Hatchery and Technology Center. Our objectives were to (1) evaluate the effects on sperm motility of 24-h storage in Hanks' balanced salt solution (HBSS); (2) characterize sperm motility and duration; (3) examine the relationship between sperm motility and osmotic pressure; (4) examine the effect of four cryoprotectants (dimethyl sulfoxide [DMSO], dimethyl acetamide [DMA], glycerol, and methanol [MeOH] at two concentrations [5% and 10%]) on postthaw motility; and (5) compare the effect of two cooling rates (40°C/min and 4°C/min) on postthaw motility. The sperm samples diluted with HBSS retained higher motility (mean ± SD, 77 ± 22%; $n = 9$) than did undiluted samples (12 ± 30%; $n = 9$) after 24 h of storage. When exposed to HBSS at 274 mosmols/kg or more, few sperm became motile (~1%). Exposure to HBSS at 265 mosmols/kg elicited threshold activation (defined as 10% motility), and maximum motility (>95%) was observed at 93 mosmols/kg. The maximum motility of sperm was observed within 10 s after activation with deionized water, and sperm remained motile for 57 s. The sperm that were cooled at a rate of 40°C/min and cryopreserved with 5% MeOH retained higher postthaw motility (56 ± 13%) than did sperm cryopreserved with DMSO, DMA, or glycerol (at 5% and 10%). When the sperm samples were cooled at a rate of 4°C/min, sperm cryopreserved with MeOH (5% or 10%) or DMSO (5% or 10%) retained the highest postthaw motilities (≤14%). The use of cryopreserved sperm can assist hatchery managers in the production of fish, provide for the long-term conservation of genetic resources, and assist in the recovery of endangered species such as the Colorado pikeminnow.

The endangered Colorado pikeminnow *Ptychocheilus lucius*, formerly known as the Colorado squawfish (Nelson et al. 1998), is a large-river cyprinid that was once abundant throughout the Colorado River and its tributaries in western North America. This species is now restricted to the upper Colorado River basin above Lake Powell (Holden and Stalnaker 1975; Minckley and Deacon 1968), with populations persisting in the Green, White, and Yampa rivers (Holden and Wick 1982; Tiersch et al. 1998). Smaller populations reside in the upper Colorado River, the Gunnison River, and in the San Juan River below the Navajo Dam (Platania et al. 1991). The Colorado pikeminnow was listed as endangered by the U.S. Bureau of Sport Fisheries and Wildlife (now the U.S. Fish and Wildlife Service; USFWS) in 1967 and

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remains protected under the Endangered Species Act of 1973 (U.S. Fish and Wildlife Service 1967). The factors implicated in Colorado pikeminnow population decline are associated with water development and include the interruption of migration routes, the loss of spawning and nursery habitats, suboptimal flow and temperature regimes, and the introduction of nonnative fish species (Holden and Stalnaker 1975; Kaeding and Osmundson 1988; Karp and Tyus 1990; Tyus et al. 1982; Vanicek et al. 1970). Recovery programs for the Colorado pikeminnow (U.S. Fish and Wildlife Service 1987, 1990) include the development and improvement of propagation techniques, and the production of fish in hatcheries for research and reintroduction. The ultimate goal of these recovery efforts is to maintain a diversified gene pool for introduced Colorado pikeminnow populations in the Lower Colorado Basin and other sites within the historical range of the species.

Controlled propagation techniques for hatchery spawning and the production of endangered fishes include the storage and cryopreservation of gametes (Tiersch et al. 1997, 1998). Gamete storage is an effective way of solving hatchery-related problems of noncoincident maturation of broodstock by allowing flexibility in spawning schedules while managing genetic resources. These methods enable the efficient transfer of genes from wild populations to captive populations (Cloud et al. 1990), provide greater control in implementing breeding programs, and permit the storage of sperm for extended periods, which, in turn, create opportunities for recovery and genetic conservation. The Colorado pikeminnow used in this study were from a broodstock population maintained at the Dexter National Fish Hatchery and Technology Center (DNFHTC) in New Mexico. This first-generation broodstock was founded from wild fish captured in 1974 from the Yampa River in northwestern Colorado. As one of only two captive Colorado pikeminnow broodstocks, these fish represent an invaluable genetic resource for recovery efforts.

Our purpose was to develop techniques for the handling and cryopreservation of sperm of Colorado pikeminnow. Our objectives were to (1) evaluate the effects on sperm motility of 24-h storage in Hanks' balanced salt solution (HBSS); (2) characterize sperm motility and duration; (3) examine the relationship between sperm motility and osmotic pressure; (4) examine the effect of four cryoprotectants (dimethyl sulfoxide [DMSO], dimethyl acetamide [DMA], glycerol, and methanol [MeOH] at two concentrations [5% and 10%]) on postthaw motility; and (5) compare the effect of two cooling rates (40°C/min and 4°C/min) on postthaw motility.

Methods

Sperm samples were collected from 10 Colorado pikeminnow in June 1995. The fish were held head-down with the ventral surface exposed and were wiped dry. Semen flow was initiated by rotating the ventral surface of the fish downward and applying gentle pressure behind the pectoral fins. Semen was allowed to flow freely for several seconds before collection in sterile, 50-mL screw-cap tubes (Fisher Scientific, 05-539-8).

Evaluation of sperm dilution.—Sperm samples from each of the 10 males were divided in half: one-half was diluted with an equal volume of HBSS prepared at 300 mosmols/kg (Tiersch et al. 1994, 1998), and the other half was left undiluted. The samples were placed on ice packs and shipped by overnight delivery to the Aquaculture Research Station (ARS) of the Louisiana State University Agricultural Center, where samples were stored at 4°C. Sperm activation was studied (24 h after collection) without use of a coverslip by placing 2 μL of sperm on a microscope slide and diluting it with 20 μL of deionized water. Sperm concentrations were not standardized, but all samples were suitable for motility estimation using these techniques. Fish sperm are typically nonmotile until contact with water, which is associated with activation and vigorous motility (e.g., Bates et al. 1996). The percentage of sperm swimming actively in a forward direction was estimated using darkfield microscopy at 200× magnification. Sperm vibrating in place were not considered to be motile. The motility of the diluted and undiluted samples was compared using a paired-comparison Student's *t*-test (Microsoft Excel 5.0, Microsoft). Because undiluted sperm had low motility, all subsequent studies were performed with sperm samples diluted with HBSS. The osmolality of diluted and undiluted samples was measured with a vapor pressure osmometer (Model 5500, Wescor, Inc., Logan, Utah).

Characterization of sperm motility and duration.—We characterized sperm motility and duration by using sperm samples (>95% motility) from eight fish (replicated twice) and estimated motility at 5-s intervals. The samples were activated and motility estimated as described above. The time to maximum motility (highest percent motility for a sample) and the time to complete

cessation of motility (no motile sperm, some sperm vibrating in place) were determined.

Sperm motility and osmotic pressure.—To investigate the relationship between osmotic pressure and sperm motility in Colorado pikeminnow, sperm samples were exposed to 10 graded dilutions of HBSS ranging in osmotic pressure from 26 to 297 mosmols/kg. Motility was estimated as described above. The osmotic pressure of a 10-μL sample taken from the microscope slide (after motility estimation) was measured with the vapor pressure osmometer. The level of threshold activation was defined as the osmotic pressure that elicited 10% motility. The level of complete activation was defined as the highest osmotic pressure that elicited the maximal motility observed for that sample. Mean values of motility and osmotic pressure were used to identify the levels of threshold and complete activation. Comparison between these levels was tested with a paired-comparison Student's *t*-test.

Sperm cryopreservation.—The effects on postthaw sperm motility were evaluated for 5% and 10% of DMSO, DMA, glycerol, and MeOH. All cryoprotectants were of reagent grade (Sigma Chemical Corporation, St. Louis, Missouri). The cryoprotectants were dissolved in HBSS and added to the samples to yield the appropriate final concentrations. The sperm samples were exposed to the cryoprotectants for 30–32 min at room temperature (23°C) before freezing. Percent motility was obtained for all sperm samples before the addition of cryoprotectants and just prior to the initiation of freezing. At least two sperm samples from each male were cryopreserved using 0.5-mL French straws (IMV International, Minneapolis, Minnesota). The straws were placed into plastic goblets (10 mm diameter, 12 cm long; IMV International), attached to the bottom position on 20-cm aluminum canes (Southland Cryogenics, Inc., Carrollton, Texas), and cooled to −80°C at either of two rates (40°C/min or 4°C/min) using a computer-controlled freezer (Kryo-10, Planer Products Ltd., Middlesex, UK). Cryopreservation was performed on two consecutive days. The fast rate was performed on the first day (mean initial motility = 90%; range = 80–95% for five males), and the slow rate on the second day (mean initial motility = 76%; range = 70–80% for four males). The sperm samples were held in liquid nitrogen (−196°C) for 16 months before thawing in a water bath (40°C for 7 s). Percent motility data were arcsine square root transformed before analyses. One-way analysis of variance (ANOVA; SAS 6.08; SAS Institute, Cary, North Carolina) was used to examine the effects of cryoprotectant exposure on prefreeze sperm motility. The effect of cryoprotectant on postthaw sperm motility was also examined using one-way ANOVA. No statistical comparisons were made between freezing rates because of the potential differences in sperm quality between days.

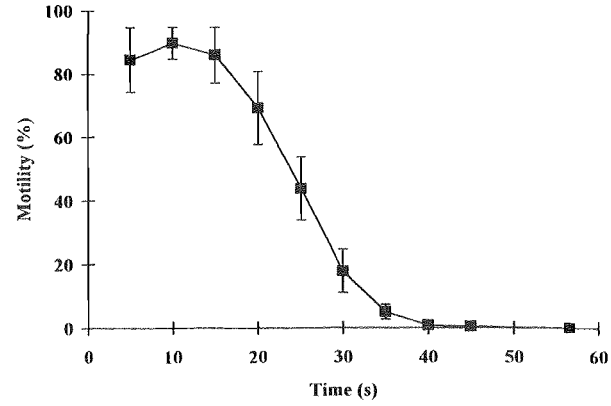

FIGURE 1.—Characterization of sperm motility of the Colorado pikeminnow activated by the addition of 20 μL of water to 2 μL of sperm. Each point represents the mean value (±1 SD) of sperm from eight fish.

Results

Evaluation of Sperm Dilution

The dilution of sperm with 300 mosmols/kg HBSS had a significant effect ($P = 0.0042$) on sperm motility after 24 h of storage. Sperm samples diluted with HBSS retained higher motility (mean ± SD, 77 ± 22%) than did sperm samples left undiluted (12 ± 30%). The osmolality of diluted sperm (260 ± 24 mosmols/kg) was significantly higher ($P = 0.0034$) than that of undiluted sperm (226 ± 37 mosmols/kg).

Characterization of Sperm Motility and Duration

When activated with deionized water, sperm attained maximum motility (95%) within 15 s (Figure 1). Subsequently, the percentage of motile sperm decreased through time until all motility ceased at 57 ± 5 s after activation.

Sperm Motility and Osmotic Pressure

The motility of Colorado pikeminnow sperm increased with decreasing osmolality of the HBSS activating solutions. A few sperm became motile (~1%) at 274 mosmols/kg, threshold activation (10% motility) was observed at 265 mosmols/kg, and maximum motility was attained at 93 mosmols/kg (Figure 2). There was a significant dif-

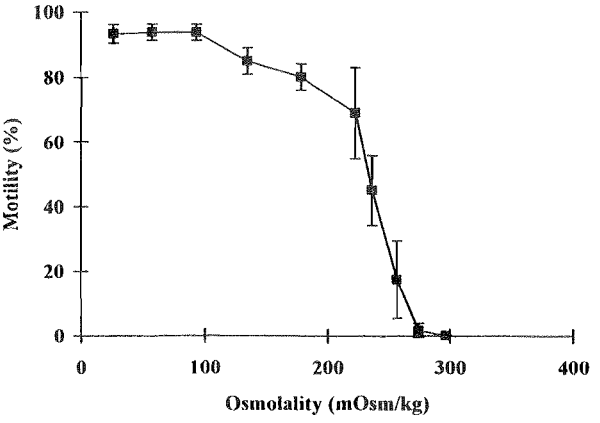

FIGURE 2.—Motility of sperm of the Colorado pikeminnow exposed to Hanks' balanced salt solution prepared at various osmotic pressures. Aliquots of sperm (2 μL) were diluted with 20 μL of test solution, and motility was estimated with darkfield microscopy at 200× magnification. Each point represents the mean value (±1 SD) of sperm from four fish.

ference between the osmotic pressures at threshold and complete activation ($P < 0.0001$).

Sperm Cryopreservation

Exposure to cryoprotectants for 30 min significantly affected sperm motility before freezing. The sperm samples ($n = 10$) used on day 1 containing MeOH (5% or 10%) or DMSO (5%) retained significantly higher prefreeze motility ($P = 0.0001$) than did samples containing either concentration (5% or 10%) of DMA or glycerol, or 10% DMSO (Figure 3). The sperm samples ($n = 8$) studied on day 2 (prefreeze exposure time = 32 min) and containing MeOH (5% or 10%), DMSO (5%), or glycerol (5%) had significantly higher motility ($P = 0.0001$) than did sperm samples containing DMSO (10%), glycerol (10%), or either concentration of DMA (Figure 4).

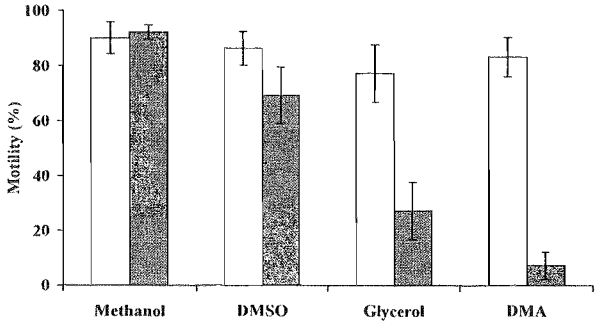

FIGURE 3.—Prefreeze motility of sperm of Colorado pikeminnow after 30-min exposure to concentrations of 5% (white bars) and 10% (hatched bars) of four cryoprotectants (methanol, dimethyl sulfoxide [DMSO], glycerol, or dimethyl acetamide [DMA]). Each bar represents the mean value (±1 SD) of sperm from 10 fish.

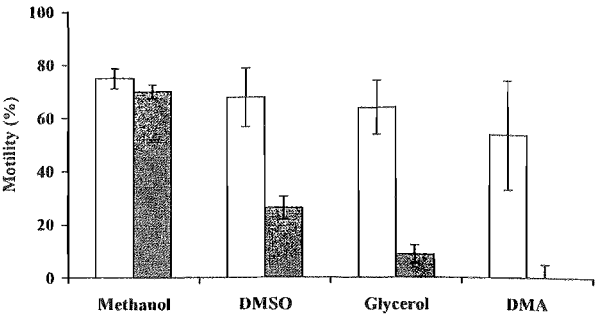

FIGURE 4.—Prefreeze motility of sperm of Colorado pikeminnow after 32-min exposure to concentrations of 5% (white bars) and 10% (hatched bars) of four cryoprotectants (methanol, DMSO, glycerol, or DMA). Each bar represents the mean value (±1 SD) of sperm from eight fish.

Cryoprotectant type and concentration influenced the postthaw motility of sperm frozen at either rate. The sperm samples which contained 5% MeOH and were cooled at 40°C/min had significantly higher ($P = 0.0001$) postthaw motility (56 ± 13%) than did samples cryopreserved with 10% MeOH (41 ± 20%), or either concentration (5% or 10%) of DMSO, DMA, or glycerol (Figure 5). The sperm samples cooled at 4°C/min which contained 5% MeOH (8 ± 3%), 10% MeOH (10 ± 5%), 5% DMSO (8 ± 4%), or 10% DMSO (14 ± 13%) had significantly higher ($P = 0.0001$) postthaw motility than did samples cryopreserved with either concentration (5% or 10%) of DMA or glycerol (Figure 6).

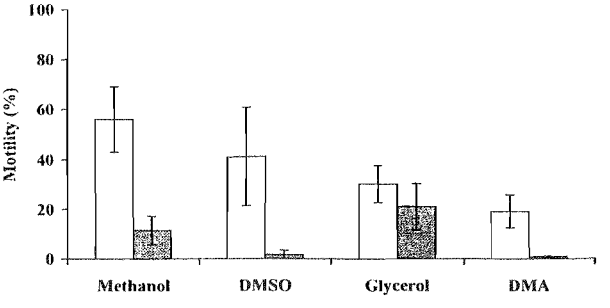

FIGURE 5.—Postthaw motility of sperm from Colorado pikeminnow cooled at 40°C/min with concentrations of 5% (white bars) and 10% (hatched bars) of four cryoprotectants (methanol, DMSO, glycerol, or DMA). Each bar represents the mean value (±1 SD) of sperm from 10 fish. Motility of thawed sperm cryopreserved with 5% methanol was significantly higher ($P = 0.0001$) than that of sperm cryopreserved with other cryoprotectants (at either concentration) or with 10% methanol.

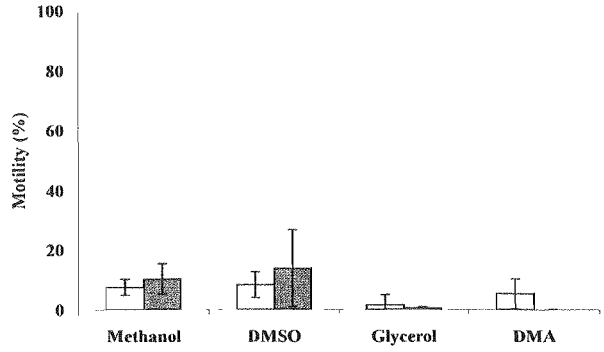

FIGURE 6.—Postthaw motility of sperm from Colorado pikeminnow cooled at 4°C/min with concentrations of 5% (white bars) and 10% (hatched bars) of four cryoprotectants (methanol, DMSO, glycerol, or DMA). Each bar represents the mean value (±1 SD) of sperm from eight fish. Motility of thawed sperm cryopreserved with either concentration of methanol or with DMSO was significantly higher ($P = 0.0001$) than that of samples cryopreserved with either concentration of DMA or glycerol.

Discussion

The activation of sperm of freshwater fishes is typically associated with a decrease in osmotic pressure. The osmolality of the undiluted samples in the present study (226 mosmols/kg) was below the point of threshold activation (265 mosmols/kg), a likely cause of the premature activation of the sperm and reduced storage time. The lowered osmotic pressure of undiluted samples could have been caused by contamination with hypotonic urine during collection. The addition of HBSS raised the osmotic pressure of the diluted samples and appeared to be an effective measure to protect sperm during refrigerated storage. Hanks' balanced salt solution has proven useful in this regard for the sperm of channel catfish *Ictalurus punctatus* (Tiersch et al. 1994), spotted seatrout *Cynoscion nebulosus* (Wayman et al. 1996), and black drum *Pogonias cromis* (Wayman et al. 1997), and when modified by the removal of calcium, for sperm of the endangered razorback sucker *Xyrauchen texanus* (Tiersch et al. 1997, 1998).

The duration of sperm motility in Colorado pikeminnow was brief (57 s) compared with that of other freshwater fishes such as muskellunge *Esox masquinongy* (6–7 min; Lin and Dabrowski 1996) and paddlefish *Polyodon spathula* (4–5 min; Mims 1991) but similar to the duration of sperm motility in walleye *Sander vitreus* (formerly *Stizostedion vitreum*; 51 s; Satterfield and Flickinger 1995) and razorback suckers (70 s; Tiersch et al. 1997). The maximal sperm activity was also brief (10 s) and indicates that care should be taken to ensure the complete, early mixing of gametes during the induced spawning of Colorado pikeminnow. Additionally, the dilution of sperm samples to at least 93 mosmols/kg (complete activation) should be made to obtain the maximal motility of sperm and to ensure sufficient dilution for maximizing fertilization rates (e.g., Bates et al. 1996).

The sperm samples containing 5% methanol and cooled at 40°C/min resulted in the highest postthaw motility. These results are related, in part, to the toxic effects associated with the cryoprotectants (and concentrations) used in our study. The sperm viability of the samples containing methanol was reduced the least before freezing (30-min exposure). Methanol could have provided greater cryoprotection to Colorado pikeminnow sperm than did DMSO, DMA, and glycerol (at the concentrations we tested). Methanol can enter and exit cells rapidly (Ashwood-Smith 1980), and is a sometimes overlooked but useful cryoprotectant for fish sperm (Harvey and Ashwood-Smith 1982; Christensen and Tiersch 1997). Experiments that further address cryoprotectant effects would be useful for the sperm of Colorado pikeminnow.

Colorado pikeminnow sperm cooled at 40°C/min had higher postthaw motility than did sperm cooled at 4°C/min. Cooling rates can be crucial for sperm of fish species, and, in general, higher postthaw motility and fertilization rates are attained using rapid cooling rates compared with slower rates (Kerby 1983; Mounib et al. 1968; Tiersch et al. 1998). High postthaw motility can be directly related to fertilization rates (Harvey et al. 1982; Steyn and Van Vuren 1987), although the relationship between postthaw sperm and fertilization must be examined on a species-by-species basis.

The development of sperm cryopreservation techniques has been reported for less than 1% of all fish species (Billard 1995; Rana 1995), including less than 10 endangered species (Tiersch 2000). It is not unlikely that fish such as the Colorado pikeminnow could cease to be found in the wild within the next 20 years without intervention. Studies that address the specific problems of sperm cryopreservation for threatened and endangered fishes are needed to assist in hatchery production and to provide for the long-term storage of genetic resources (Table 1). Cryopreservation increases control in the induced spawning of fishes, provides access to sperm over long intervals (Ashwood-Smith 1980; Piironen 1993), and protects against the loss of genetic diversity due to changes in aquatic habitats. The use of these techniques will not replace the need for the protection and resto-

TABLE 1.—Problems and possible solutions for the development of sperm cryopreservation programs for endangered species.

Problem	Possible solution
Federal permits are required	Work with agencies that hold or assign permits
Captive broodstock are not always available	Develop procedures for field use (e.g., Wayman et al. 1996, 1997)
Work in field conditions	Refrigerated storage to allow sample shipment to laboratory
Gametes available during limited time period	Work with existing collection programs
Spawning protocols not available for the species	Use methods developed for related or similar species
Lack of cryopreservation expertise	Work with established cryopreservation laboratories

ration of habitats, but offers a powerful tool for the long-term conservation and management of genetic material of endangered species. Future studies will need to address the assessment and management of risks associated with the potential for pathogen transfer across time and distance with cryopreserved samples (Jenkins 2000). This is especially relevant for gamete collections made from wild populations of endangered species.

Acknowledgments

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