

## Cryopreservation of Paddlefish *Polyodon spathula* Milt

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**Abstract.**—A practical procedure for cryopreserving milt of paddlefish *Polyodon spathula* was developed to obtain thawed spermatozoa that would fertilize eggs and permit hatching of normal larvae. Milt was mixed with a cryoprotectant medium containing DMSO (2.4 M) in a ratio of 3:1(milt:medium; final concentration of DMSO 0.6 M), stored in 5.0-mL freezing straws, and frozen in dry ice (15 min) and then in liquid nitrogen. A total of three replicates were made; the milt of a different male was used in each replicate. Motility of the thawed spermatozoa decreased to 50%-25% as compared to 100% motility of the fresh (control) spermatozoa. Hatching of paddlefish ( $16.3 \pm 2.2\%$ ) from eggs fertilized with thawed spermatozoa was significantly lower ( $P \leq 0.01$ ) than the hatch rate ( $90.8 \pm 2.5\%$ ) for the control. It was suggested that an increase in viable motile spermatozoa to egg would result in better fertilization and hatching of paddlefish.

Numerous studies have reported cryopreservation of spermatozoa from many teleostean species, which provided thawed spermatozoa capable of fertilizing eggs and resulting in viable progeny (Stoss 1983; McAndrew et al. 1993). On the contrary, little information is available on the cryopreservation of chondrosteian (i.e., sturgeons and paddlefishes) spermatozoa. Only a few studies on sperm cryopreservation of sturgeons have been performed (Drokin et al. 1991; Tsvetkova et al. 1996; Drokin and Kopeika 1996). No published information exists on the cryopreservation of paddlefish *Polyodon spathula* spermatozoa.

Like sturgeon, the paddlefish is valued for its boneless meat and roe processed into caviar. Most paddlefish that are caught and sold originate from wild populations. In recent years, the paddlefish has been listed as a special concern species, a Category II species under the authority of the Endan-

gered Species Act of 1973 (Allardyce 1992). In response to this special condition, many federal and state agencies are developing mitigation and restoration programs to rebuild the present populations. Further, there is an increasing interest in commercial production of paddlefish as a food fish (Mims 1991; Mims et al. 1997).

Cryopreservation of paddlefish spermatozoa is a critical biotechnology needed for long-term preservation of valuable genetic resources and for extending the use of gametes from broodstock with superior genetic potential (USDA 1988). Recent paddlefish research using ploidy manipulation (Mims et al. 1997) and sex-reversal (Mims et al. 1995) also could greatly benefit the aquaculture industry if commercial application of cryopreservation could be developed. For example, if sex-reversed gynogenetic paddlefish are homogametic, a breeding program should theoretically provide all-female progeny that could be raised for the caviar industry. Cryopreservation of spermatozoa from sex-reversed gynogenetic fish could provide a valuable genetic resource for all-female production.

The objective of this study was to develop a practical cryopreservation technique for paddlefish milt that would provide viable spermatozoa for fertilization of the eggs and hatching of normal larvae. The sturgeon studies mentioned above used either motility of thawed spermatozoa or fertilization capacities (morula stage) but not hatching rate. To make our technique more practical, we chose hatching rate as the main index for evaluation of cryopreservation results. We used dimethylsulfoxide (DMSO) as the cryoprotectant which has been wide-

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ly used for cryopreservation of sperm for both teleostean (Stoss 1983; McAndrew et al. 1993) and sturgeons (Drokin et al. 1991; Tsvetkova et al. 1996; Drokin and Kopeika 1996).

## Materials and Methods

### *Ovulation and Spermiation Induction*

Brood paddlefish (5–14 kg) were captured below McAlpine Dam on the Ohio River, transported to the Aquaculture Research Center of Kentucky State University, Frankfort, Kentucky, USA and held in 2.5-m diameter circular tanks. Each fish was held in a separate tank, which was supplied with dechlorinated water at a flow rate of 12 L/min. Water temperature was maintained at  $18.0 \pm 0.3$  C. Paddlefish females were given a total dose of luteinizing hormone releasing hormone analog (LHRH<sub>a</sub>; des-gly<sup>10</sup>[D-ala<sup>6</sup>]-LHRH ethylamide; Sigma Chemical Co., St. Louis, Missouri, USA) at the rate of 100  $\mu$ g/kg body weight (BW) in two injections (10% and 90% of the total dose) separated by 12 h. Ovulation occurred within 12 to 16 h after the second injection. Males were given an intraperitoneal injection of LHRH<sub>a</sub> at a single dose of 50  $\mu$ g/kg BW. Spermiation occurred within 12 to 18 h after the injection.

### *Sperm Collection and Cryopreservation Technique*

Spermiating paddlefish were blotted dry around the genital opening. Tygon tubing (4 mm inside diameter; 5-cm long) attached to a 10-mL plastic syringe was inserted into the urogenital pore. Using gentle suction of the syringe, milt was collected and immediately placed on the surface of wet ice (surface temperature 0 to 1 C). Spermatozoa from each fish were tested for motility. Sperm only with motility of 100% and forward motion (not vibratory) were used for cryopreservation study. Sperm concentration of each milt sample was measured using a hemacytometer and a corpuscle counting chamber. For each measurement, 25  $\mu$ L of sample was diluted with 25 mL of ex-

tender. Sperm counts were made under a light microscope (100 $\times$ ).

Milt from three males ( $6.63 \pm 0.88$  Kg) was placed into separate 100-mL culture flasks; the volume of milt collected from each male ranged from 20 to 30 mL. Twelve mL of the milt from each male were mixed with 4.0 mL of a cryopreservation medium. The medium was composed of 1.6 mL (1.76 g) of DMSO (99.9 % purity, Sigma Chemical Co., St. Louis, Missouri USA), 4.0 mL of trehalose (100 mg/1 mL extender) and 4.4 mL of extender. The composition of the extender was 0.205 g CaCl<sub>2</sub> H<sub>2</sub>O, 0.440 g MgCl<sub>2</sub> 6H<sub>2</sub>O, 0.470 g NaHCO<sub>3</sub>, 5.115 g KCl, 11.560 g NaCl, 20 g glucose, 0.200 g citric acid, 4.760 g HEPES, 2,000 mL double distilled H<sub>2</sub>O, 20 mL (1.270 g/100 mL) KOH, 20 mL penicillin-streptomycin (5,000 units of penicillin and 5 mg streptomycin per mL of 154 mM saline (NaCl) solution), 300 mOsmol/kg, pH 7.6. The DMSO concentration was 2.4 M in the cryopreservation medium and the final DMSO concentration of the milt/medium mixture was 0.6 M. The mixture was allowed to equilibrate for at least 15 min on ice. The type of extender and the dilution rate of the DMSO were determined in preliminary laboratory experiments. The remaining milt samples were placed on ice and later used as the control to compare with thawed sperm.

Large straws (5.0-mL clear, non-flexible plastic drinking straws) were used for freezing. One end of each straw was heat-sealed using a hemostat (Halstead mosquito forceps) heated on a hotplate. Approximately 3.5 mL of mixture was injected into each freezing straw using a 5.0-mL syringe with 10 cm of polyethylene tube (1.0-mm inside diameter) attached to an 18-gauge needle. An air space was left at each end of the straw to prevent rupture of the straws during the freezing process. The open end was then heat-sealed. One straw per male was placed on ice and later checked for motility after diffusion with medium, but before freezing. The other straws ( $N = 9$ ; three

straws from each male) were covered in crushed dry ice. After at least 15 min, the straws were submerged into liquid nitrogen and stored in a cryobiological storage container. Two straws per male were used for egg insemination experiments, and the other straw per male was used to check motility of thawed sperm.

#### *Sperm Thawing and Examination of Sperm Motility*

To thaw the mixture, the straws were removed from the storage container, immediately immersed into a water bath at 20 C for 15 s. Immediately after thawing, the freezing straws were removed from the water bath, wiped dry, and both ends were cut with scissors. One  $\mu\text{L}$  of mixture was combined with 40  $\mu\text{L}$  of dechlorinated tap water to activate the spermatozoa. Percentage of motile spermatozoa was observed under a light microscope (200 $\times$ ) and was recorded based on a scale of 100–75%, 75–50%, 50–25% and 25–0%.

#### *Eggs Insemination and Incubation*

Eggs were manually stripped (Graham et al. 1986) from one female and collected in a dry 18-cm diameter culture bowl. Approximately 3,500 eggs (64 mL of eggs/replication;  $55 \pm 3$  eggs/mL) were transferred into each of several culture bowls, and the thawed samples of two straws containing milt from the same male (about 5.25 mL of pooled milt and 1.75 cryoprotectant medium) per bowl were mixed with the eggs. The control used 5.25 mL of fresh milt from each male to fertilize a similar number of eggs. For insemination, about 160 mL of dechlorinated tap water were added to the eggs, stirred for 1 min, and a suspended clay (Fuller's earth) solution was poured onto the eggs and stirred for 15 min to prevent adhesiveness. The eggs were incubated in separate 10-L McDonald jars for 6 d at  $18 \pm 0.3$  C. The number of hatched larvae were counted and recorded. Larvae from the thawed treatment and control were held in separate net enclosures for 7 d (until

transition to exogenous feeding) in order to record any differences in deformities or mortalities between the treatment and control. There were three replicates for the thawed sperm treatment and for the corresponding control, in which the milt of different males was evaluated in each replicate.

Hatching percentage was determined from the number of hatched larvae in relation to the total number of eggs. Percentage data were subjected to angular transformation. Data were analyzed by Student *t*-test (Steel and Torrie 1980) at a probability level of  $P < 0.05$  using SAS Version 6.1 software (SAS Institute, Inc. Cary, North Carolina, USA).

#### **Results**

The concentration of spermatozoa from the milt of the three males was  $1.1 \pm 0.4 \times 10^9/\text{mL}$ . During the experiment, the fresh sperm (control) maintained motility of 100%. For the experimental treatment, the sperm that was diluted with cryoprotectant medium before freezing had motility of 100%–75%; thawed samples had motility of 50%–25%.

The yield of hatched larvae after egg insemination with thawed sperm was  $16.3 \pm 2.2\%$  (range 466–648 larvae) and was significantly less ( $P < 0.01$ ) than that of the control of  $90.8 \pm 2.5\%$  (3,080–3,294). No differences in deformities and post-hatch mortalities were observed at transition to exogenous feeding.

#### **Discussion**

The yield of hatched larvae after egg insemination with thawed sperm was about 16% as compared to 91% in the control. This technique appears to be reliable and useful for mass production of paddlefish. It minimized further dilution of the milt by adding a concentrated DMSO (2.4 M) cryoprotectant medium to naturally dilute milt and permitted storage of milt using 5.0-mL straws. Further, the large volume of thawed milt allowed insemination of relatively

large quantities of eggs which could be treated using standard fertilization (coating of adhesive eggs) and incubation (upwelling incubators) techniques to provide proper conditions for hatching paddlefish. This production procedure with large straws (5.0 mL) of thawed milt is useful because males release large volumes of milt and females are highly fecund.

Sturgeon species are known to produce large volumes of milt with low sperm concentrations ( $1-10 \times 10^9$  spermatozoa/mL) as compared to the milt of teleostean fishes that produce smaller volumes of concentrated sperm (Ginzburg 1972). Therefore, the ratio of milt to cryoprotectant (DMSO) medium for sturgeon (Drokin et al. 1991; Drokin and Kopeika 1996; Tsvetkova et al. 1996) is usually 1:1 (sperm:medium) as compared to ratios of 1:3 to 1:9 for teleosts (Stoss 1983; McAndrew et al. 1996). Since the concentration of paddlefish milt was  $1.1 \pm 0.4 \times 10^9$  spermatozoa/mL and was less concentrated than most sturgeon milt, we reduced the dilution of milt to medium to a ratio of 3:1 to provide more concentrated sperm for egg insemination.

Dimethylsulfoxide has most often been used for sperm cryopreservation of several sturgeon species (Drokin et al. 1991; Drokin and Kopeika 1996; Tsvetkova et al. 1996). Dimethylsulfoxide, used as the cryoprotectant for freezing paddlefish sperm in this study, had a final concentration of 0.6 M (about 4%) when mixed with the milt. Thawed paddlefish sperm resulted in a reduction in percentage of motility from 100% (fresh) to <50%–25% which apparently decreased fertilization rate as well as the yield of hatched larvae. Tsvetkova et al. (1996) also reported significant decline in sperm motility after freezing of sperm in medium with a final DMSO concentration of 7.5%. Though sperm motility is often used to initially evaluate the success of thawed sperm from teleosts, it is probably less dependable for evaluation of chondrosteian spermatozoa with acrosomes. In Tsvetkova et al. (1996) and our studies, thawed,

motile spermatozoa were considered viable; however, it is known that motile spermatozoa with damaged acrosomes can render them unable to fertilize eggs (Dan 1956). Fertilization capacity or percentage of hatch should be the main criteria for evaluation of cryopreservation success.

Several studies have indicated the necessity of increasing the spermatozoa to egg ratio when using thawed spermatozoa because morphological, physiological or biochemical alternations resulting from cryopreservation procedures may affect the sperm capability to fertilize eggs (Jamieson 1991; Lahnsteiner et al. 1996). In this study, the ratio of motile, thawed spermatozoa to egg for paddlefish was estimated to be  $6.17 \times 10^5$  motile spermatozoa/egg. For paddlefish the minimum ratio of motile spermatozoa per egg for successful fertilization of eggs is not known. However, it is possible that an increase in number of viable motile spermatozoa per egg would result in better fertilization and hatching of paddlefish. Further research should be continued to improve effectiveness of cryopreservation techniques for large-scale application.

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### Literature Cited

- Allardyce, D. S. 1992. Endangered and threatened wildlife and plants: finding on petition of list the paddlefish. *Federal Register* 57(184):43,676–43,682.
- Dan, J. C. 1956. The acrosome reaction. *International Review of Cytology* 5:365–393.
- Drokin, S. I. and E. F. Kopeika. 1996. Motility of and phospholipid content in cryopreserved spermatozoa of three sturgeon species. *The Sturgeon Quarterly* 4(4):8–10.
- Drokin, S. I., V. V. Cherepanov, E. F. Kopeika, and N. I. Shilin. 1991. Sturgeon Sakhalin: gene pool

- conservation. *Rybnoje Chozjajstvo* 7:38–39 (in Russian).
- Ginzburg, A. S.** 1972. Fertilization in fishes and the problem of polyspermy. Israel Program for Scientific Translations, Jerusalem.
- Graham, L. K., E. J. Hamilton, T. R. Russell, and C. E. Hicks.** 1986. The culture of paddlefish—a review of methods. Pages 78–94 in J. G. Dillard, L. K. Graham, and T. R. Russell, editors. *The paddlefish: status, management, and propagation*. Modern Litho-Print Co., Jefferson City, Missouri, USA.
- Jamieson, B. G. M.** 1991. Fish evolution and systematics: evidence from spermatozoa. Cambridge University Press, Cambridge, United Kingdom.
- Lahnsteiner, F., T. Weismann, and R. Patzner.** 1996. Cryopreservation of semen of grayling (*Thymallus thymallus*) and Danube salmon (*Hucho hucho*). *Aquaculture* 144:265–274.
- McAndrew, B. J., K. J. Rana, and D. J. Penman.** 1993. Conservation and preservation of genetic variation in aquatic organisms. Pages 295–336 in J. F. Muir and R. J. Roberts, editors. *Recent advances in aquaculture IV*. Blackwell Scientific Publications, Oxford, England.
- Mims, S. D.** 1991. Paddlefish: an aquacultural species? *Farm Pond Harvest* 24(2):18–20.
- Mims, S. D., W. L. Shelton, and J. A. Clark.** 1995. Steroid induced sex reversal of paddlefish. Page 129 in F. Goetz and P. Thomas, editors. *Proceedings of the fifth international symposium on reproductive physiology of fish*. University of Texas, Marine Science Institute, Austin, Texas, USA.
- Mims, S. D., W. L. Shelton, O. Linhart, and C. Wang.** 1997. Induced meiotic gynogenesis of paddlefish *Polyodon spathula*. *Journal of the World Aquaculture Society* 28:334–343.
- Steel, R. G. D. and J. H. Torrie.** 1980. Principles and procedures of statistics, 2nd edition. McGraw-Hill, New York, New York, USA.
- Stoss, J.** 1983. Fish gamete preservation and spermatozoa physiology. Pages 305–350 in W. S. Hoar, D. J. Randall, and E. M. Donaldson, editors. *Fish physiology*, volume IX, part B. Academic Press, Inc., New York, USA.
- Tsvetkova, L. I., J. Cosson, O. Linhart, and R. Billard.** 1996. Motility and fertilizing capacity of fresh and frozen-thawed spermatozoa in sturgeons *Acipenser baeri* and *A. ruthenus*. *Journal of Applied Ichthyology* 12:107–112.
- USDA.** 1988. Aquacultural genetics and breeding: national research priorities, volume 1. USDA Office of Aquaculture.