Cryopreservation of Sperm of the Endangered Razorback Sucker

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Introduction

The native fishes of the Colorado River system of the southwestern United States have declined substantially during the past century and their prospects for survival are not good. Numerous factors including introduction of exotic species and construction of dams have extensively disrupted the natural habitat of these fishes and has resulted in major changes in the aquatic fauna. The razorback sucker *Xyrauchen texanus* was once abundant and widely distributed throughout the Colorado River and its tributaries, but is now found commonly only in the middle Green River and Lake Mohave, below Hoover Dam (Minckley et al. 1991, Modde et al. 1996). Razorback suckers are threatened with extinction because of limited recruitment into the adult size classes, and in 1991, received a formal listing under the Endangered Species Act (U.S. Fish and Wildlife Service 1991). Because of the lack of natural recruitment, artificial propagation and reintroduction are emphasized for the conservation, management and recovery of this species. Methods that enable the transfer of gametes from wild populations to hatchery broodstock (e.g. Cloud et al. 1990) are needed to aid in this recovery program.

Recommendations identified by a multi-agency management team include the stocking of Lake Mohave with razorback suckers to replace the aging population (Figure 1). Razorback suckers are currently maintained for research and stocking at the U.S. Fish and Wildlife Service (USFWS) Dexter National Fish Hatchery and Technology Center (Johnson and Jensen 1991) and at the Willow Beach National Fish Hatchery (WBNFH). Management of razorback suckers has included production and distribution of fertilized eggs and larval fish in protected backwaters adjacent to Lake Mohave (Mueller 1995).

Photograph by T. Tiersch

Figure 1. Male razorback sucker collected from Lake Mohave in 1995. Note the distinctive dorsal keel. Many razorback suckers in this population carry lesions or growths (such as on the tail of this fish) that may be related to advanced age.

Storage and cryopreservation of sperm is an effective management tool for conserving genetic resources of threatened and endangered populations. This technology

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offers advantages by providing genes from wild populations for hatchery broodstock, greater control in breeding programs, and the ability to store large amounts of valuable germplasm for long periods. Gamete storage is an effective way of solving hatchery-related problems of differential maturation of broodstock by allowing flexibility in spawning time. Our purpose in this work was to improve and integrate gamete collection, storage and cryopreservation for enhancement of recovery efforts for razorback suckers (Carmichael et al. 1996, Figiel et al. 1996, Tiersch et al. 1997, 1998). The objectives were to: 1) develop methods for collection of sperm allowing integration with established sampling programs; 2) characterize sperm motility and duration; 3) develop methods for refrigerated storage of sperm; 4) develop methods for the cryopreservation of sperm; 5) fertilize eggs with cryopreserved sperm, and 6) investigate methods for incubation of eggs.

Collection of Gametes

We collected fish from a 17-km section of the Colorado River between WBNFH and Hoover Dam on upper Lake Mohave during the 1994 to 1996 spawning seasons. We integrated our procedures with sampling protocols established by the USFWS: fish were weighed, measured, tagged with passive integrated transponders and general condition was recorded. Sperm were collected immediately after USFWS protocols were completed or fish were kept in live-wells and hatchery tanks. To collect sperm, males were held head down with ventral surface up, and were wiped to remove excess water and debris. The head was raised, and the anal fin was held against the caudal peduncle, exposing the vent. To initiate semen flow, we rotated the ventral surface of the fish downward, and applied gentle pressure behind the pectoral fins, which minimized handling of fish and the contamination of semen with feces and urine often associated with application of pressure along the belly toward the vent. Unless kept undiluted for use in other studies, sperm were diluted with calcium-free Hanks' balanced salt solution (C-F HBSS) and stored on ice for transport to WBNFH. In the laboratory, we stored refrigerated (4 °C) sperm samples in loosely capped tubes. Sperm collection was completed within 1 min and did not interrupt the established sampling protocols.

Characterization of Sperm Motility and Duration

For estimation of motility, 2 µL of sperm were placed on a microscope slide and diluted with 20 µL of water collected from Lake Mohave (21 mOsmol/Kg). Sperm became motile and initiated rapid swimming when diluted. The percentage of sperm swimming actively in a forward direction was estimated using dark-field microscopy at 200-X magnification. The duration of motility was divided into three periods: 1) time required to reach maximum motility after addition of water; 2) duration of maximum motility, and 3) time until complete cessation of motility.

The time required to reach maximum motility was 3 sec after the addition of water (the minimum time at which accurate estimates of motility could be made was 2 sec). Maximum motility was maintained for 16 ± 8 sec (mean ± SD), and the time until complete cessation of motility was 70 ± 32 sec after the addition of water.

Refrigerated Storage of Sperm

We performed two experiments on the motility retention of razorback sucker sperm during storage at 4 °C. In the first, we compared motility of undiluted sperm and sperm diluted with an equal volume of C-F HBSS. In the second, we compared motility of sperm stored in three dilutions: 1 part semen to 1, 3, or 7 parts C-F HBSS. Sperm were collected as described above, diluted 1:1 in the field, delivered within 1.5 hr, and aliquots were diluted beyond 1:1 at WBNFH. We chose six high quality samples (>95% motility) and placed these in loosely capped 15-mL tubes and stored them upright at 4 °C. We estimated motility immediately after final dilution, and daily for 3 d until samples were shipped by commercial airline to Louisiana State University, where daily estimates were continued until all samples became non-motile.

In the first experiment, there was a significant difference in sperm motility within 24 hr between sperm diluted (1:1) with C-F HBSS and undiluted sperm (t-test; $t = -6.45$, $P < 0.0001$). All undiluted sperm samples became non-motile within 72 hr. About half (45%) of the diluted sperm samples retained at least 60% motility for 5 d. In the second experiment, dilution of sperm with different proportions of C-F HBSS did not affect sperm motility on day 3 (ANOVA, $P = 0.1815$), or day 8 of the experiment (ANOVA, $P = 0.5958$). Overall, sperm motility was highest on d 1 (73 ± 10%) and decreased through d 8 (2 ± 6%). Sperm samples retained an average motility of greater than 15% motility for 6 d, although nine of the fifteen samples appeared degraded and were non-motile after 3 d.

Cryopreservation of Sperm

We examined the effects of six cryoprotectants on sperm motility: dimethyl sulfoxide (DMSO), dimethyl acetamide (DMA), glycerol, methanol (MeOH), propylene glycol and ethylene glycol. Cryoprotectants minimize damage to sperm cells during the freezing and thawing processes (Jamieson 1991), but cryoprotectants can have toxic effects on sperm as well. In the first experiment, we examined the effects of 5 and 10% of DMSO, MeOH, DMA and glycerol on post-thaw sperm motility of four males. Cryoprotectants were dissolved to the appropriate concentration and added to sperm samples. Samples were allowed to equilibrate for 90 min at room temperature (~25 °C) before the straws (0.5-mL) were placed into goblets and into shipping dewars (Taylor-Wharton models CP-35 and CP-65) for freezing. In the second experiment, we examined the effects of 5 and 10% of DMSO, ethylene glycol, and propylene glycol, and 10 and 20% of MeOH on post-thaw sperm motility of five males. We followed the same procedures as above except that sperm samples were allowed to equilibrate for 8 min before the straws (0.5-mL) were placed into the dewars. In each experiment, sperm samples had 80 to 95% initial motility and were held frozen for at least 24 hr.

In the first experiment, cryoprotectant (and concentration) influenced post-thaw motility. Motility of sperm cryopreserved with 10% MeOH was significantly higher (ANOVA, $P < 0.01$) than the motility of sperm cryopreserved with 5% MeOH, or either concentration of DMSO, DMA, or glycerol. Similarly, in the second experiment, motility of sperm cryopreserved with 10% MeOH (24 ± 2%) was significantly higher (ANOVA, $P < 0.01$) than the motility of sperm cryopreserved with 20% MeOH, or either

concentration of DMSO, propylene glycol, or ethylene glycol.

Fertilization of Eggs Using Cryopreserved Sperm

We cryopreserved sperm from three male razorback suckers with 10% MeOH (pre-freeze motility was 50% for each male). Sperm samples were aspirated into straws (0.5-mL) and placed into shipping dewars for freezing. Two sperm samples from each male were thawed after 24 hr and motility was estimated. These sperm samples were mixed immediately with 500 to 600 eggs from each of two females so that there were a total of six fertilization attempts (a 0.5-mL sperm sample from each male for eggs from each female). Additionally, we fertilized eggs from both females using refrigerated sperm from two males (90 and 95% motility) to serve as controls. After 96 hr, we determined the percentage of developing eggs. Refrigerated sperm yielded fertilizations of 50% (female one) and 33% (female two). Cryopreserved sperm yielded fertilizations of 35% (female one) and 18% (female two). The mean percent motility of thawed sperm samples was 18% (n = 6).

Incubation of Eggs

We used polyethylene storage bags (Ziplock® brand) for the fertilization of eggs and the incubation and hatching of embryos. We placed 200 to 2,000 eggs within each bag (although best results were obtained with less than 500 eggs). Eggs were fertilized by addition of either refrigerated or cryopreserved sperm and 50 mL of Colorado River water. After 30 sec, water volume was increased to 250 mL for water hardening of the eggs. Bottled oxygen was used to supplement the air within the bags. The water (23 °C) was exchanged twice daily, and fungal growth was controlled by manual removal of hyphae-infected eggs. Use of these bags enabled us to keep experimental groups of eggs separated and to replicate large number of trials. This was especially important when examining multiple crosses in large experiments. Additionally, these bags provided ease in transportation of embryos and fry, and the transparency of the bags permitted observations on embryo development and treatment effects.

Discussion and Conclusions

We were able to combine research on the collection, storage, and cryopreservation of sperm with an established sampling program for razorback suckers. Our studies demonstrated that collection of sperm was rapid and did not disrupt fish sampling procedures. Collection of sperm was performed in the boat during routine data collection and tagging, and was quickly mastered by sampling crews. Sperm of razorback suckers became active when diluted in river water and swam vigorously for 20 sec, losing all motility at about 70 sec after dilution. Given this relatively short time of maximal activity, care should be taken to ensure good, early mixing of gametes during artificial spawning of razorback suckers.

Refrigerated storage of sperm is an effective method for management of razorback sucker broodstock in that it allows flexibility in spawning of females. Calcium-free Hanks' balanced salt solution allowed refrigerated storage of razorback

sucker sperm for at least 7 d. Bacterial contamination may have caused degradation of sperm samples after that time. Potentially, sperm survival could be prolonged by addition of antibiotics to inhibit bacterial growth (Stoss et al. 1978, Stoss and Refstie 1983). Methanol appeared to be the most effective cryoprotectant for razorback sucker sperm. Sperm cryopreserved with 10% methanol had higher post-thaw motility than did sperm with other cryoprotectants. Although sperm motility was reduced because of the cryopreservation process, the sperm were useful for fertilization of eggs. Razorback suckers produced in 1996 at WBNFH with cryopreserved sperm have been reared and currently (as this volume goes to press) are maintained in ponds at the Dexter National Fish Hatchery and Technology Center.

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