COLLECTION, STORAGE AND CRYOPRESERVATION OF SPERM FROM ENDANGERED RAZORBACK SUCKERS

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Introduction

The razorback sucker *Xyrauchen texanus* offers a novel opportunity for the application of laboratory and hatchery-based techniques for fish reproductive physiology in field conditions. An endemic fish of the Colorado River system, the razorback sucker is nearing extinction because of limited juvenile recruitment into the population. Recommendations identified by a multi-agency recovery effort include stocking up to 100,000 fish to replace the aging population. To assist in this effort, we developed methods for the collection, short-term storage, and cryopreservation of sperm. Additionally, we developed techniques using food storage bags for the storage, fertilization and incubation of eggs.

Storage and cryopreservation of gametes is an effective management tool for conserving genetic resources of threatened and endangered fishes in that it provides genes from wild populations for hatchery broodstock, permits greater control in breeding programs, and provides the capability to keep large number of valuable genes for extended periods. Gamete storage is an effective way of solving hatchery-related problems of non-coincident maturation of broodstock by allowing flexibility in spawning time. The objectives of this study 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 using cryopreserved sperm, and 6) investigate methods for incubation of eggs. Our purpose was to improve and integrate gamete collection, storage, and cryopreservation for enhancement of recovery efforts for endangered razorback suckers.

Collection of Gametes

We collected fish from a 17.6-km section of the Colorado River between Willow Beach National Fish Hatchery (WBNFH) and Hoover Dam on upper Lake Mohave during the 1994-1996 spawning seasons. We integrated our procedures with standard sampling protocols established by the USFWS: fish were weighed, measured (TL), tagged with a passive integrated transponders and general condition of fish was recorded. Sperm were collected immediately after USFWS procedures were complete or fish were kept in live-wells and hatchery tanks until collection. Males were held head down with ventral surface up, and were wiped to remove excess water and debris. To collect sperm,

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. Unless kept undiluted for use in other studies, sperm were diluted with calcium-free Hanks' balanced salt solution (C-F HBSS) (Tiersch et al. 1994) 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, did not require additional personnel, and did not interrupt the established sampling protocols. The application of pressure for semen collection was limited to the area behind the pectoral fins, which minimized handling of fish and the contamination of semen with feces and urine often associated with application of pressure posteriorly along the belly toward the vent.

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). The percentage of sperm swimming actively in a forward direction was estimated using dark-field microscopy at 200X magnification. The duration of motility was divided into 3 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.

Sperm became motile and initiated rapid swimming when diluted in water. The time required to reach maximum motility was 3 s after the addition of water (the minimum time at which accurate estimates of motility could be made was 2 s). Maximum motility was maintained for 16 ± 8 s, and the time until complete cessation of motility was 70 ± 32 s after the addition of water.

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 retention of undiluted sperm and sperm diluted with an equal volume of C-F HBSS. In the second, we compared motility retention of sperm stored in three dilutions of C-F HBSS: 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 h, and aliquots were diluted beyond 1:1 at WBNFH. We chose six high quality samples 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 experiment one, there was a significant difference in sperm motility within 24 h 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 h. About half (45%) of the diluted sperm samples retained at least 60% motility for 5 d. In experiment two, dilution of sperm with different proportions of C-F HBSS did not affect sperm motility on day 3 (ANOVA $F_{2,14} = 1.974$, $P = 0.1815$), or day 8 of the experiment (ANOVA $F_{2,14} = 0.540$, $P = 0.5958$). Overall, mean sperm motility was highest on day 1 (73.0 ± 10.0%) and decreased through time to day 8 (2.1 ± 5.6%). Sperm samples retained on average greater than 15% motility for 6 d, however, 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. Briefly, cryoprotectants minimize damage to sperm cells during the freezing and thawing process (Jamieson 1991), however cryoprotectants can have toxic effects on sperm as well. In experiment one, we examined the effects of 5 and 10% DMSO, 5 and 10% MeOH, 5 and 10% DMA, and 5 and 10% glycerol on post-thaw sperm motility of four males. Cryoprotectants were dissolved to the appropriate concentration and added to sperm samples. We allowed samples to equilibrate for approximately 90 min at room temperature before placing straws (0.5-ml) into goblets and then into

portable shipping dewars for freezing. In experiment two, we examined the effects of 5 and 10% DMSO, 10 and 20% MeOH, 5 and 10% ethylene glycol, and 5 and 10% propylene glycol 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 and straws (0.5-ml) were placed directly into dewars. In both experiments, sperm samples had 80-95% initially motility and were frozen for 24 h.

In experiment one, cryoprotectant (and concentration) influenced post-thaw motility (Table 1). Motility of sperm cryopreserved with 10% MeOH was significantly higher compared to motility of sperm cryopreserved with 5% MeOH, or either concentration of DMSO, DMA, or glycerol. Similarly, in experiment two, motility of sperm cryopreserved with 10% MeOH was significantly higher ($x = 24 \pm 2\%$) compared to motility of sperm cryopreserved with 20% MeOH, or either concentration of DMSO, propylene glycol, or ethylene glycol (Table 1).

Source of variation	df	SS	*F*	*P*
Experiment 1				
Cryoprotectant	7	0.5077625	22.658	< 0.0001
Error	52	0.1664751		
Total	59			
Experiment 2				
Cryoprotectant	7	1.8680136	43.376	< 0.0001
Error	71	0.4368124		
Total	78			

Table 1. Univariate analysis of variance for the effects of cryoprotectants on the percent of post-thaw sperm motility. Data were arcsine square-root transformed before analysis.

Fertilization of Eggs Using Cryopreserved Sperm

We cryopreserved sperm from 3 male razorback suckers using 10% MeOH (pre-freeze motility = 50% for each male). We froze sperm samples by aspirating into straws (0.5-ml) and placing these directly into portable shipping dewars. Two sperm samples from each male were thawed after 24 h and motility 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 fertilizing 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 h, we obtained the percent of developing eggs.

The percent of eggs fertilized with refrigerated sperm were 50.3% and 32.7% respectively for females one and two. Using cryopreserved sperm, 35.3% of eggs from female one and 18.2% of eggs from female two were fertilized. Mean percent motility of thawed sperm samples were 18.3% (n = 6).

Incubation of Eggs

We used polyethylene storage bags for the fertilization of eggs and the incubation and hatching of embryos. We collected eggs and placed 200 to 2000 eggs within bags. Eggs were fertilized by adding either refrigerated or cryopreserved sperm and 50 ml of Colorado River water. After 30 s, water volume was increased to 250 ml for water-hardening of the eggs. Bottled oxygen was used to supplement air in bags. Water (23 C) in bags was exchanged twice daily to maintain water quality, and fungus control was kept by manual removal of hyphae-infected eggs.

Use of these bags enabled us to keep group of eggs separated and allowed us to replicate large number of trials. This is especially important when examining multiple crosses in large experiments. Additionally, these bags provided ease in transportation of embryos and hatchling, and the bag transparency 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 already established sampling program for razorback suckers. Our studies demonstrate that collection of sperm can be rapid and does not disrupt normal sampling procedures. Collection of sperm could be performed in the sampling 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 s, losing all motility at about 70 s 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 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 adding antibiotics to inhibit bacterial growth (Stoss et al. 1978; Stoss and Refstie 1983). Methanol appears to be the most effective cryoprotectant for razorback sucker sperm. Sperm mixed with 10% methanol had higher post-thaw motility compared to sperm mixed with other cryoprotectants. Although sperm motility was reduced because of the freezing-thawing process, we were able to use sperm cryopreserved with 10% methanol to fertilize eggs.

Because of the lack of successful natural recruitment in razorback suckers, emphasis on reproductive physiology and artificial propagation is an essential component for the conservation and management of these fish. Cryopreservation methods that augment the transfer of gametes from wild populations to hatchery broodstock should aid in this recovery program and provide for the long-term conservation of genetic material of razorback suckers.

Literature Cited

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