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Cryopreservation of Sperm of the Endangered Razorback Sucker

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Abstract.—The endangered razorback sucker *Xyrauchen texanus* is endemic to the Colorado River system in western North America and is threatened with extinction because of limited recruitment. To assist in management and recovery efforts, we developed methods for the cryopreservation of sperm, evaluated the influence of various factors on motility of thawed sperm, and examined the effect on fertilization of cooling rate and the addition of caffeine. Sperm samples cryopreserved with 10% methanol (MeOH) had significantly higher postthaw motility than did samples preserved with 5% or 20% MeOH or with 5% or 10% dimethyl sulfoxide, *N,N*-dimethylacetamide, glycerol, propylene glycol, or ethylene glycol. Sperm samples cryopreserved in 0.5-mL and 2.5-mL straws had significantly higher postthaw motility than did samples cryopreserved in 0.25-mL straws. Exposure to 10% MeOH for up to 30 min did not significantly influence sperm motility before freezing or after thawing. Cooling rate (−21°C/min or −91°C/min) did not significantly influence sperm motility. Samples thawed in a water bath at 20°C, 30°C, or 40°C had significantly higher motility than did samples thawed on the laboratory bench (19°C). Refrigerated sperm had significantly higher motility after the addition of 0.005 M caffeine; however, caffeine did not increase the motility of thawed sperm. Fertilization percentage was 41 ± 31% for the egg quality control treatments (fresh sperm) in the freezing rate study. The freezing rate of −91°C/min yielded 66% fertilization relative to the control (actual value, 27 ± 26%), which was significantly higher than the 12% fertilization (actual, 5 ± 3%) yielded by the freezing rate of −21°C/min. Fertilization percentage was 25 ± 24% for the egg quality control treatments in the caffeine study. Caffeine-treated sperm yielded 60% fertilization relative to the control (actual, 15 ± 13%), which was significantly higher than the 16% fertilization (actual, 4 ± 4%) yielded by sperm without caffeine treatment.

The Colorado River system of the southwestern United States is at a critical period for the conservation of native fishes. Dam construction and the introduction of exotic species have extensively disrupted natural habitats (Naiman and Soltz 1981) and have resulted in major changes in the aquatic fauna (Vanicek et al. 1970). Razorback suckers *Xyrauchen texanus* were once abundant and widely distributed throughout the entire Colorado River and its tributaries (Minckley et al. 1991) but now are commonly found only in the middle Green River (near Jensen, Utah; Lanigan and Tyus 1989; Minckley et al. 1991; Modde et al. 1996) and Lake Mohave (above Davis Dam, Nevada–Arizona; Minckley 1983; Minckley et al. 1991). Razorback suckers are threatened with extinction (McAda and Wydoski 1980; Tyus 1987; USFWS 1987) because of limited recruitment into the adult size-classes (Minckley et al. 1991; USFWS 1991). Adult fish

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spawn in the spring, particularly in Lake Mohave, although juvenile and subadults have rarely been collected (Marsh and Minckley 1989; Mueller 1995). Marsh (1994) estimated that the Lake Mohave population has declined from 60,000 fish in the late 1980s to less than 25,000 fish and is projected to diminish rapidly unless steps are taken to augment populations. Because of the lack of successful natural recruitment, emphasis on artificial propagation and reintroduction is an essential component for conservation and management of these fish. Active management of razorback suckers has included producing and distributing larval fish in protected backwaters (Mueller 1995). Methods that augment the transfer of gametes from wild populations to hatchery broodstock are needed to aid this recovery program.

Cryopreservation of sperm is an effective management tool for conserving genetic resources of threatened and endangered populations. Sperm cryopreservation offers advantages in transferring genes from wild populations to hatchery populations (Cloud et al. 1990), provides greater control in breeding programs, and enables the storage of a large number of samples for long periods. Methods for sperm cryopreservation have been developed for more than 30 families of fishes (reviewed in Stoss 1983; Billard 1995; Rana 1995); however, no study has successfully examined methods for the cryopreservation of sperm from the razorback sucker or other catastomid fishes.

Razorback suckers are especially suited for the application of sperm cryopreservation technology. Males, which are easily collected at natural spawning sites, provide large volumes of sperm (as much as 25–50 mL), and sperm can be collected without harm to the fish (Tiersch et al. 1997). 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 (NFHTC) (Johnson and Jensen 1991) and the Willow Beach National Fish Hatchery (WBNFH).

This study developed techniques for the cryopreservation of razorback sucker sperm. Our goal was to examine the influence of various factors on postthaw motility of cryopreserved sperm. Specifically, we examined the effects of (1) cryoprotectants at different concentrations, (2) straw size, (3) duration of exposure to 10% methanol, (4) cooling rates, (5) thawing regime, and (6) the addition of a stimulant (caffeine). Additionally, we examined the effects of cryopreservation on fertilization in two experiments: (1) the influence of cooling rate, and (2) the addition of caffeine to thawed sperm.

Methods

Research was performed at the WBNFH, Arizona (28.2 river kilometers downstream from Hoover Dam, Lake Mohave). Sperm samples were collected and diluted 1:1 with calcium-free Hanks' balanced salt solution (C-F HBSS), as described in Tiersch et al. (1997), in conjunction with annual razorback sucker surveys by the USFWS in March of 1994, 1995, and 1996. Preliminary results were obtained in 1994, and data presented here represent work done in 1995 and 1996. In all experiments, we used high-quality sperm (80–95% initial motility) from four or five males. Samples were maintained as separate collections. No attempt was made to quantify sperm concentrations. Unless otherwise stated, we cryopreserved at least two sperm samples for each male in 0.5-mL French straws (IMV International, Minneapolis, Minnesota). Nitrogen vapor shipping dewars (Taylor–Wharton models CP-35 and CP-65, Theodore, Alabama) were used to freeze and store sperm samples below −105°C. Also unless otherwise stated, samples were thawed rapidly by plunging straws into a 40°C water bath for 7 s. In most cases, we estimated sperm motility (defined as the percentage of sperm swimming vigorously in a forward direction) to evaluate treatment effects. Percent motility data were arcsine-square-root-transformed before analyses.

For fertilization experiments, females were collected, transferred to the WBNFH, and injected in the dorsal musculature with 225 IU/kg human chorionic gonadotropin (Intervet, Inc., Millsboro, Delaware) at 24-h intervals over a 3-d period. We collected eggs 24–48 h after injection by applying gentle pressure along the abdomen. We fertilized the eggs within 1 h of collection and used plastic food storage bags (Ziploc®) for fertilization and incubation of eggs (Carmichael et al. 1996).

Cryoprotectant effect.—We examined the effect of six cryoprotectants on sperm motility: dimethyl sulfoxide (DMSO), *N,N*-dimethylacetamide (DMA), glycerol, methanol (MeOH), propylene glycol, and ethylene glycol. All cryoprotectants were reagent grade (Sigma Chemical Corp., St. Louis, Missouri). In experiment one (1995), we examined the effect of 5% and 10% DMSO, DMA, glycerol, and MeOH on the postthaw motility of sperm of four males (two replicates per male for a total of eight replicate straws for each concentration of cryoprotectant). Cryoprotectants were dissolved in C-F HBSS and added to

the samples to yield the appropriate final concentration. Sperm samples were exposed to the cryoprotectant for 30 min at room temperature (19°C) before freezing. We placed the straws into plastic goblets (10-mm diameter, 12-cm length; IMV International) attached to 28-cm aluminum canes (Southland Cryogenics, Inc., Carrollton, Texas) and placed them into the shipping dewars. In experiment two (1996), we examined the effect of 5% and 10% DMSO, propylene glycol, and ethylene glycol and 10 and 20% MeOH on the postthaw sperm motility of five males (two replicates per male for a total of ten replicate straws for each concentration of cryoprotectant). We followed the same procedures as above, except we were able to reduce cryoprotectant exposure of sperm samples to 8 min before freezing. In each experiment, sperm samples were held frozen for at least 24 h before thawing. We used one-way analysis of variance (ANOVA; SAS, version 6.08, SAS Institute, Cary, North Carolina) for each experiment to examine if cryoprotectant affected postthaw sperm motility.

Straw size.—In 1995, we froze sperm samples from four males (two replicates per male for a total of eight replicates) in three sizes of straws (2.5-mL, 0.5-mL, or 0.25-mL) with two cryoprotectants (10% DMSO or 10% MeOH) to examine how these factors influenced postthaw sperm motility. We produced 2.5-mL straws by cutting 5-mL macrotube straws (Minitube of America, Madison, Wisconsin) in half. All open ends of straws were sealed with glass balls obtained from the straw manufacturers. Sperm samples were exposed to cryoprotectants for 22 min before freezing, and samples were held frozen for 12 h before thawing. We analyzed this 3 × 2 experiment (containing eight replicate straws) by using two-way ANOVA to examine the main effects and interactions of straw size and cryoprotectant on postthaw sperm motility.

Duration of exposure to cryoprotectant.—Based on the previous experiments, in 1996, we studied the potential for improving the cryoprotective ability of 10% MeOH. Cryoprotectant exposure time (defined as the period between the addition of the cryoprotectant to the sperm sample and the initiation of freezing) was investigated with respect to sperm motility before freezing and after thawing. We added 10% MeOH to sperm samples from four males (two replicates per male for a total of eight replicate straws). At specific intervals (1.5, 5, 10, 15, and 30 min), we estimated sperm motility and froze two sperm samples from each male (a total of eight replicate straws per time period). We thawed the samples after 48 h and again estimated motility. Data were analyzed with one-way ANOVA to examine the effects of exposure time on sperm motility before freezing and after thawing.

Cooling rates.—In 1996, we used three methods for freezing sperm to examine the effect of cooling rate on postthaw sperm motility. Three cooling rates were obtained by placing straws containing sperm samples from four males (two replicates per male for a total of eight replicate straws, cryopreserved with 10% MeOH) into goblets attached to canes for freezing in dewars (slow cooling rate), into dewars without use of goblets and canes (medium cooling rate), or into goblets attached to canes before plunging into liquid nitrogen (fast cooling rate). Use of these methods allowed straws to cool at three rates (slow = −21 ± 2°C/min; medium = −91 ± 9°C/min, and fast = −350 ± 83°C/min). Cooling rates were recorded with a five-channel data logger (OM-550, Omega Engineering, Inc., Stamford, Connecticut) with inputs from type-T thermocouples (TMISS-040G-12, Omega) placed into 0.5-mL straws filled with C-F HBSS. Samples were thawed after 48 h and sperm motility was estimated. Data were analyzed with one-way ANOVA to examine the effect of cooling rate on sperm motility.

Thawing regime.—In 1996, we examined the importance of thawing regime on the sperm cryopreservation process by four thawing treatments: three in a water bath (20°C for 9 s; 30°C for 8 s, or 40°C for 7 s) and one on the bench top (room temperature = 19°C for 390 s). We cryopreserved sperm samples in 40 straws (eight straws from each of five males) using 10% MeOH. After 24 h, 10 replicate straws (two from each male) were thawed at each temperature. We used one-way ANOVA to examine whether sperm motility was significantly different among the thawing regimes.

Addition of caffeine.—In 1996, we investigated the effect of 0.005 M caffeine (1,3,7-trimethylxanthine, product C-0750, Sigma) dissolved in distilled water on sperm motility in three experiments. In the first experiment, we obtained motility estimates for refrigerated (4°C) sperm (<3 d old) from 17 males (motility range, 5–95%; mean ± SD = 47 ± 32%). We added 20 μL of the caffeine solution to 2 μL of sperm and estimated motility again for each male. In a second experiment, we repeated these procedures on refrigerated sperm (<3 d old) from 10 additional males (25–45%; 33 ± 8%). We repeated these procedures in a third experiment with thawed sperm cryopreserved with 10% MeOH (10 males; initial motility = 83 ± 7%;

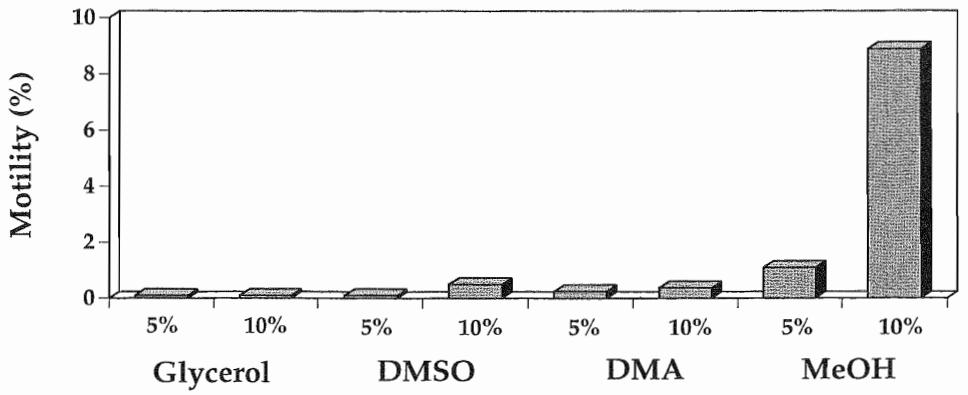

FIGURE 1.—Mean postthaw motility of razorback sucker sperm after exposure to two concentrations (5% or 10%) of four cryoprotectants: glycerol, dimethyl sulfoxide (DMSO), dimethylacetamide (DMA), or methanol (MeOH).

two 0.5-mL straws each) and cooled at −91°C/min. All motility estimates in these experiments were performed after samples had been allowed to reach room temperature (19°C). The samples were coded to prevent identification during motility estimations. We analyzed each experiment with a paired-sample, two-tailed *t*-test.

Fertilization trials.—In 1996, we fertilized razorback sucker eggs with thawed sperm in two experiments. In the first, we froze sperm samples from three razorback suckers at two cooling rates: slow (−21 ± 2°C/min) or medium (−91 ± 9°C/min). The samples were cryopreserved with 10% MeOH (0.5-mL straws) and an exposure time of 7.3 min. An equivalent volume (0.45 mL) of refrigerated sperm (<3 d old; 75–80% motility) from two males served as controls to assess the quality of the eggs. We obtained postthaw motility estimates for the sperm samples before mixing with egg samples (N = 584 ± 47 eggs/sample; range = 509–684) obtained from two females, such that sperm from each male (including controls) were mixed with eggs of each female according to procedures of Carmichael et al. (1996). After 4 d, unfertilized and fertilized eggs (as indicated by developing embryos) were counted with the aid of a microscope.

In the second experiment, we thawed two straws from each of 10 males and estimated postthaw motility. We mixed these sperm with eggs obtained from four females; eggs were pooled in pairs to yield two batches, each containing the eggs of two females. Sperm and eggs were activated with either 150 milliosmols (mosmol)/kg C-F HBSS or 150 mosmol/kg C-F HBSS containing 0.005 M caffeine, such that half of the sperm samples (paired samples with or without caffeine from each of the ten males) were used to fertilize the eggs from each pair of females. We also mixed an equivalent volume (0.45 mL) of refrigerated sperm (<3 d old; motility = 90%) from two males with each of the two batches of eggs to serve as fertilization controls. We used procedures of Carmichael et al. (1996) for all crosses (number of eggs per cross = 198 ± 18; range, 175–232). After 3 d, unfertilized and fertilized eggs were counted with the aid of a microscope.

In each experiment, the percent of fertilized eggs (100 × number of eggs fertilized divided by total number of eggs) was arcsine-square-root-transformed and these data were used for statistical analysis (Snedecor and Cochran 1980). We used one-way ANOVA to examine whether cooling rate or presence of caffeine influenced percent fertilization. We also tested for differences in fertilization rate among males and among females to evaluate potential confounding effects.

Results

Cryoprotectant Effect

Cryoprotectant type and concentration influenced postthaw motility in each experiment. In the first experiment, sperm samples containing 10% MeOH had significantly higher postthaw motility (ANOVA, $P < 0.01$) than did samples with 5% MeOH or either concentration (5% or 10%) of DMSO, DMA, or glycerol (Figure 1). In the second experiment, motility of samples cryopreserved with 10% MeOH was significantly higher (ANOVA, $P < 0.01$) than that of samples cryopreserved with 20% MeOH or either concentration (5% or 10%) of DMSO, propylene glycol, or ethylene glycol (Figure 2).

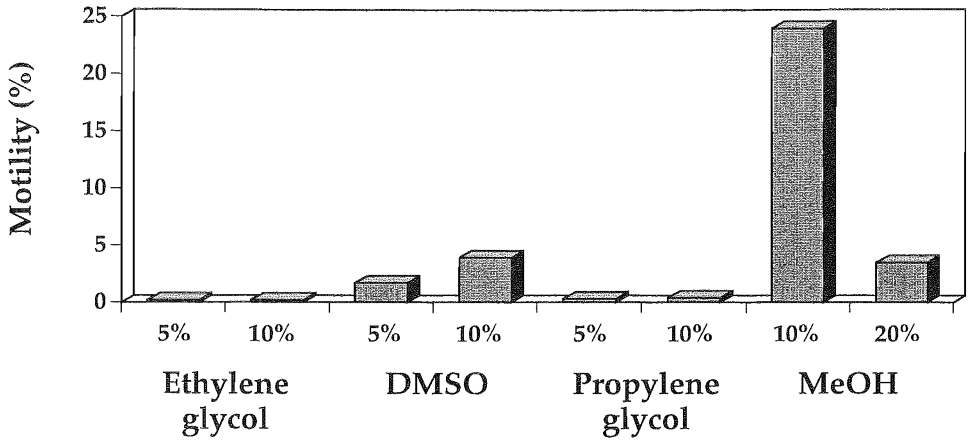

FIGURE 2.—Mean postthaw motility of razorback sucker sperm after exposure to two concentrations (5%, 10%, or 20%) of four cryoprotectants: ethylene glycol, dimethyl sulfoxide (DMSO), propylene glycol, or methanol (MeOH).

Straw Size

Straw size had a significant effect on postthaw sperm motility (Table 1). Sperm cryopreserved in 0.25-mL straws had significantly lower motility than did sperm cryopreserved in 0.5-mL or 2.5-mL straws (ANOVA, $P < 0.01$). Additionally, cryoprotectant had a significant effect on postthaw sperm motility (ANOVA, $P < 0.01$). Sperm cryopreserved with 10% MeOH had higher postthaw motility than did samples cryopreserved with 10% DMSO, independent of straw size. There was no significant difference in motility of sperm cryopreserved in 2.5-mL or 0.5-mL straws, nor were there interaction effects between straw size and cryoprotectant (Table 1).

Duration of Exposure to Cryoprotectant

There were no significant differences in motility of sperm among the five exposure times before freezing (ANOVA, $P = 0.97$). Sperm motility for the four males (initial motility = 84 ± 5%) did not change through time after the addition of 10% MeOH (time periods and mean ± SD: 1.5 min = 84 ± 8%; 5 min = 85 ± 6%; 10 min = 84 ± 5%; 15 min = 83 ± 7%; 30 min = 83 ± 5%). Additionally, these exposure times did not significantly influence motility of thawed sperm (ANOVA, $P = 0.78$; 1.5 min = 17 ± 8%; 5 min = 19 ± 4%; 10 min = 18 ± 8%; 15 min = 16 ± 7%; 30 min = 19 ± 8%).

TABLE 1.—Results of two-way analysis of variance for the effects of sperm volume (straw sizes of 2.5-mL, 0.5-mL, or 0.2-mL) and cryoprotectant (10% dimethyl sulfoxide or 10% methanol) on the percent of postthaw motility of razorback sucker sperm. Data were arcsine-square-root-transformed before analysis.

Source of variation	df	Sum of squares	*F*	*P*
Sperm volume	2	0.1001838	11.197	0.0001
Cryoprotectant	1	0.1338373	29.917	<0.0001
Volume × cryoprotectant	2	0.0150150	1.678	0.1993
Error	41	0.1834192		
Total	46			

Cooling Rates

Sperm samples from one of the four males became gelatinous before cooling, causing exclusion of this male from the experiment. Additionally, six of the straws plunged into liquid nitrogen (fast cooling rate) burst during thawing, causing exclusion of this treatment variable from analysis. Sperm samples cooled at the medium rate (i.e., unpackaged straws placed directly into dewars) had postthaw motility (18 ± 4%) double that of samples (9 ± 4%) cooled at the slow rate (packaged in goblets and canes in the dewars); however, these differences were not significant (ANOVA, $P = 0.09$).

Thawing Regime

Samples thawed on the bench top (room temperature = 19°C for 390 s; mean ± SD = 1 ± 0%) had significantly lower motility (ANOVA, $P < 0.01$) than did samples thawed at each of the three temperatures in the water bath (20°C for 9 s = 13 ± 2%; 30°C for 8 s = 12 ± 2%; or 40°C for 7 s = 14 ± 3%). Motilities of sperm samples

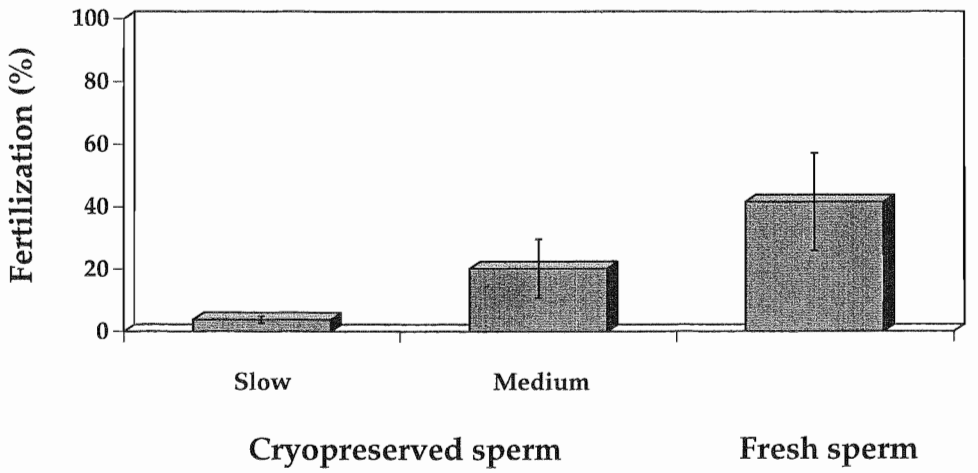

FIGURE 3.—Percent fertilization (mean ± SE) with cryopreserved razorback sucker sperm after freezing at two rates (slow = −21 ± 2°C/min; medium = −91 ± 9°C/min). Fresh sperm (from two males) refrigerated at 4°C for less than 3 d was used as a fertilization control treatment to estimate the quality of eggs.

thawed at the three water bath temperatures were not significantly different from each other (Tukey's test, $P > 0.05$).

Addition of Caffeine

In the first experiment, sperm samples had significantly higher motility after the addition of caffeine (61 ± 35%) than did the same samples before the addition of caffeine (47 ± 32%; paired t-test: $t = 2.64$, df = 16, $P < 0.02$, $N = 17$). In the second experiment, sperm samples had significantly higher motility after the addition of caffeine (56 ± 22%) than did the same sperm samples without caffeine (33 ± 8%; $t = 4.03$, df = 9, $P < 0.01$, $N = 10$). However, in the third experiment, there was no difference in motility of thawed sperm before (26 ± 13%) or after (25 ± 10%) the addition of caffeine ($t = 0.75$, df = 19, $P > 0.05$, $N = 20$).

Fertilization Trials

In the first experiment, there were no differences in fertilization success among males (ANOVA, $P = 0.13$) or between females (ANOVA, $P = 0.61$), allowing these factors to be used as replicates in further analysis. There was no significant difference in fertilization success between cooling rates (ANOVA, $P = 0.09$). Sperm samples frozen at the medium rate (−91°C/min) had a seven-fold greater fertilization success than did samples frozen at the slow rate (−21°C/min; Figure 3). Fresh sperm samples used as egg quality controls had double the fertilization success of cryopreserved sperm samples (Figure 3).

In the second experiment, there were no differences in fertilization success among males (ANOVA, $P = 0.44$), which allowed males to be used as replicates. There was a significant difference between the percentage of eggs fertilized with sperm containing or not containing caffeine (Table 2; Figure 4). Eggs mixed with caffeine-treated sperm had four times the percent fertilization of those with untreated sperm. Additionally, there was a significant difference in fertilization success between the pairs of females (Table 2; Figure 4). Percent fertilization for eggs of one pair was five times greater than that of the eggs of the other pair. There was a significant two-way-interaction effect between females and the presence of caffeine (Table 2). Fresh sperm used as egg quality controls had greater fertilization success than did cryopreserved sperm samples (Figure 4).

TABLE 2.—Results of two-way analysis of variance for the effects of caffeine and female on the percentage of razorback sucker fertilized eggs. Data were arcsine-square-root-transformed before analysis.

Source of variation	df	Sum of squares	F	P
Caffeine	1	0.1521684	9.479	0.0072
Female	1	0.1827115	11.381	0.0039
Caffeine × female	1	0.1078028	6.715	0.0197
Error	16	0.2568637		
Total	19			

Discussion

Development of sperm cryopreservation techniques with a variety of cryoprotectants has been

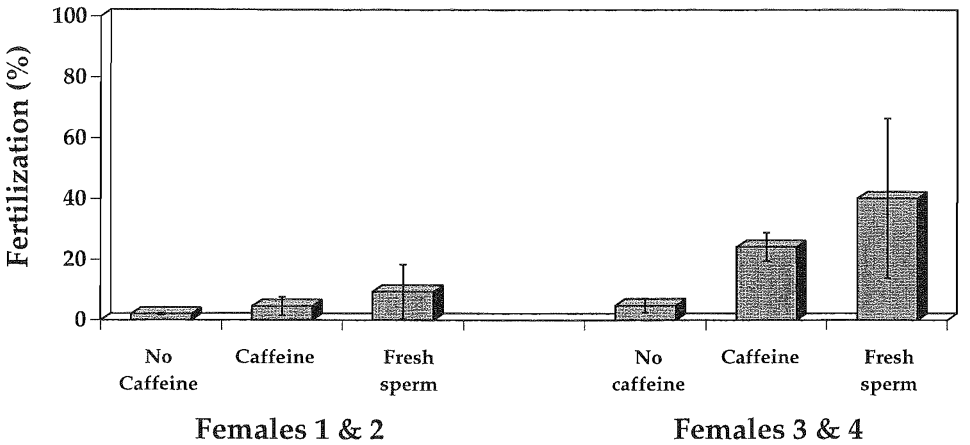

FIGURE 4.—Percent fertilization (mean ± SE) with or without the addition of caffeine to cryopreserved razorback sucker sperm used to fertilize eggs from two pairs of females. Fresh sperm (from two males) refrigerated at 4°C for less than 3 d was used as a fertilization control treatment to estimate the quality of eggs.

reported for over 80 fish species (Billard 1995; Rana 1995). Methanol, used infrequently in previous studies, resulted in higher postthaw sperm motility (as high as 70% in one male) in our experiments than did the more commonly used cryoprotectants (i.e., DMSO and glycerol). Furthermore, we found that 10% methanol did not influence prefreeze or postthaw sperm motility during 30 min of exposure before freezing. Previous studies indicate that refrigerated sperm of channel catfish *Ictalurus punctatus* retained motility significantly longer when stored with methanol (up to 16 d) than did sperm stored without methanol (Christensen and Tiersch 1996), and motility of thawed channel catfish sperm was not reduced after 5 d of refrigerated storage with 5% methanol before freezing (Christensen 1994). This suggests that methanol provides opportunities for storing and freezing razorback sucker sperm without loss of viability resulting from toxic effects. The cryoprotectants DMSO, DMA, glycerol, propylene glycol, and ethylene glycol (at the concentrations tested) could have been more toxic to razorback sucker sperm cells or cryoprotected less effectively than methanol. Methanol can enter and exit cells rapidly and thus may confer an advantage over other cryoprotectants (Ashwood-Smith 1980; Harvey and Ashwood-Smith 1982); however, experiments that further address these factors and their influence on razorback sucker sperm viability would be useful.

Sperm frozen at a rate of −91°C/min had higher postthaw motility and fertilized a greater percentage of eggs than did sperm frozen at −21°C/min. Mazur (1970) indicated that the optimal cooling rate is dependent on cryoprotectant type and concentration, and Steyn (1993) indicated that cooling rate depends on cell size and composition of the cell membrane. Because sperm size and membrane structure can potentially vary among species, among males of the same species, or within the same male during a breeding season (see Billard 1992), the optimal cooling rate is potentially a range of cooling tolerances. The ability to regulate cooling rates in field and hatchery conditions without the use of an expensive computer-controlled freezer would be valuable in addressing this factor.

The various thawing regimes in the water bath (20–40°C for 7–9 s) yielded similar postthaw sperm motilities. Only sperm thawed on the bench top (18°C for 390 s) had significantly reduced motility. Our results are similar to findings for red drum *Sciaenops ocellatus* in which sperm thawed on the bench top (21°C for 240 s; Wayman et al., in press) resulted in lower motility than that of sperm thawed in a water bath. Our thawing treatments in water were similar to those used successfully for sperm of the whitefish *Coregonus muksun* (Piironen 1987), channel catfish (Tiersch et al. 1994), and spotted seatrout *Cynoscion nebulosus* (Wayman et al. 1996).

This study is the first to report successful fertilization of razorback sucker eggs with cryopreserved sperm, and it is among the first to examine sperm cryopreservation techniques for catastomid fishes. Drokin et al. (1989) attempted to cryopreserve sperm of the bigmouth buffalo *Ictiobus cyprinellus* with 1.5 M ethylene glycol, but they did

not observe any motile sperm after thawing. Overall, fertilization rates for razorback sucker sperm cryopreserved in 10% methanol were as high as 58% of the control treatments used to estimate egg quality (fresh sperm). For an individual fish, fertilization was as high as 82% (95% of control). Fertilization success has varied considerably for cryopreserved sperm in other fish species. Direct comparisons among studies and treatments should be made with caution and only when sperm to egg ratios are known. Fertilization trials in this study were undertaken only to demonstrate that thawed sperm of razorback suckers could fertilize eggs. Further studies dealing with specific aspects of fertilization should be done under controlled conditions and should examine the significance of sperm volume and concentration.

Legendre and Billard (1980) showed that increased volumes of thawed sperm of rainbow trout *Oncorhynchus mykiss* yielded significantly higher fertilization rates, and Yamano et al. (1990) determined that an increase in sperm volume and concentration increased fertilization rates in masu salmon (=cherry salmon) *O. masou.* However, Stoss and Holtz (1981) determined that higher sperm density increased fertilization rates only to a certain level, after which increased sperm density decreased fertilization rates (perhaps due to sperm–sperm competition). Furthermore, an excess of dead sperm (>90%) has been shown to reduce fertilization success (Levanduski and Cloud 1988). Fertilization trials that use greater volumes of sperm (e.g., 2.5-mL straws) or higher sperm concentrations should be tested for razorback suckers.

Our experiments demonstrated that an activating agent, caffeine (a methylxanthine), increased the motility of stored sperm and increased fertilization rates of cryopreserved sperm. Similarly, Scheerer and Thorgaard (1989) found fertilizing capacity of cryopreserved rainbow trout sperm increased when eggs were fertilized in a buffered saline solution containing theophylline, another methylxanthine. The addition of theophylline has been shown to restore sperm motility and to prolong sperm activity in cobia *Rachycentron canadum* (Caylor et al. 1994). Given that there was no difference in percent motility of thawed sperm in response to the dose of caffeine that we tested, the increased fertilization rates we observed in response to caffeine are perhaps related to the prolongation or intensification of sperm motility (Benau and Terner 1980; Terner 1986), although the action of caffeine on razorback sucker eggs is unknown.

Because of the lack of successful natural recruitment in razorback suckers, an emphasis on reproductive physiology and artificial propagation can be an essential component for the conservation and management of these fish. Our results indicate that cryopreservation of sperm can be successfully applied in management programs of endangered fish populations and can perhaps be combined with existing propagation efforts such as the stocking of larval fish into protected backwaters (Mueller 1995). Use of this technique provides greater control in artificial spawning of fish and provides the ability to make specific crosses when desired. Cryopreserved sperm can be stored for extended periods (Ashwood-Smith 1980; Piironen 1993), ensures the survival of germplasm from specific stocks, and protects against loss of genetic resources due to changes in aquatic habitats.

Overall, our studies have involved collection, storage, cryopreservation, and hatchery use of sperm from razorback suckers (Carmichael et al. 1996; Tiersch et al. 1997). These efforts were undertaken in conjunction with established sampling programs, and they demonstrate the potential to successfully integrate gamete collection with ongoing projects, although cryopreserved sperm is not currently used for routine production of razorback suckers. Our results were obtained with the compromises associated with working in temporary facilities under field and hatchery conditions and were limited to 1-week periods each year during established sampling programs. Use of these procedures has yielded postthaw motilities of 95% in other species (e.g., Wayman 1996) under similar conditions. Cryopreservation will not replace protection and restoration of habitats and ecosystems but could augment transfer of genes from the wild to hatchery populations and provide for the long-term conservation and management of genetic material of razorback suckers or other endangered species.

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