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Field Collection, Handling, and Storage of Sperm of the Endangered Razorback Sucker

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Abstract.—We developed methods for the collection, handling, and refrigerated storage of sperm from the endangered razorback sucker *Xyrauchen texanus*. Sperm samples were obtained from 56 of 78 male razorback suckers collected from Lake Mohave (Colorado River) during the 1994 and 1995 spawning seasons. There were significant differences among the osmolalities of blood plasma, ovarian fluid, and seminal plasma. Maximum sperm motility was observed 3 s after dilution with river water and was maintained for 16 ± 8 s (mean ± SD). Motility ceased 70 ± 32 s after the addition of water. Given the short time that sperm were motile, care should be taken to ensure complete and early mixing of gametes during artificial spawning of razorback suckers. Refrigerated sperm (4°C) retained more than 90% motility when stored at 290 milliosmols/kg in calcium-free Hanks' balanced salt solution (C-F HBSS). Sperm diluted 1:1 with C-F HBSS had significantly higher motility compared with motility of undiluted sperm at the end of 24 h. Motility of sperm diluted with three proportions of C-F HBSS decreased at similar rates during 8 d of storage. Gamete collection and storage offers potential for enhancing management efforts for razorback suckers by allowing increased control of artificial spawning. In this regard, sperm may be collected by sampling crews and stored for days at the hatchery for production of fry.

The native distribution of razorback suckers *Xyrauchen texanus* encompassed the entire Colorado River and its large tributaries, the Green, San Juan, and Gila rivers (Minckley et al. 1991). Razorback suckers were especially abundant in the lower Colorado River downstream from the confluence of the Gila River and in the lower Gila river drainage (Minckley et al. 1991). The decline of this species in areas of former abundance followed the construction of dams throughout the Colorado River basin in the first half of the Twentieth Century. This decline led to a proposed listing of the species as endangered in 1978 and a formal listing in 1991 under the Endangered Species Act (U.S. Fish and Wildlife Service 1993a, 1993b). A small population of razorback suckers persists in the upper Green River between the Yampa and White rivers in Utah (Tyus 1987; Lanigan and Tyus 1989), but the largest remaining wild population is found in Lake Mohave (Nevada–Arizona), which is downstream of Hoover Dam. The Lake Mohave population represents a critical genetic resource for recovery and management of razorback suckers, although natural recruitment has not been apparent since fish were trapped in the reservoir with the final closure of Davis Dam in 1954 (McCarthy and Minckley 1987; Minckley et al. 1991). This aging population has declined from 60,000 to fewer than 25,000 adults since the late 1980s, and it is estimated to decline rapidly within the next decade (Minckley et al. 1991; Mueller 1995). With the senescence of the largest natural population of razorback suckers, there is a continued and irreplaceable loss of genetic resources in this species.

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Active management of this population has resulted in concerted efforts by organizations such as the Native Fish Work Group to rear larval razorback suckers in protected areas for release of young adults into Lake Mohave (Mueller 1995).

Collection, storage, and cryopreservation of sperm provide management tools for conserving genetic resources of threatened and endangered fishes. These techniques enable transfer of genes from wild populations to hatchery broodstocks (Cloud et al. 1990), permit greater control in breeding programs, and provide the capability to safeguard genetic resources for extended periods. Additionally, gamete storage is an effective way of solving hatchery-related problems of noncoincident maturation of broodstock by allowing flexibility in spawning schedules.

Methods for collection, handling, refrigerated storage, and cryopreservation of sperm have been developed for fishes including salmonids (Scott and Baynes 1980; Stoss 1983; Cloud et al. 1990), cyprinids (Kurokura et al. 1984), and ictalurids (Guest et al. 1976; Tiersch et al. 1994). There are no reports, however, regarding such methods for sperm of catastomids (Jamieson 1991). We developed techniques for the collection, handling, and refrigerated storage of sperm of razorback suckers. Our studies on cryopreservation of razorback sucker sperm will be reported elsewhere (Tiersch and coworkers, unpublished). The present study combined work in the field, hatchery, and laboratory. Our goals were to formulate a buffer for prolonging refrigerated storage of razorback sucker sperm and to develop methods for sperm collection that could be integrated with established sampling programs. Our objectives were to (1) determine osmotic pressures of body fluids; (2) characterize sperm motility and duration; (3) establish the relationship between sperm motility and osmotic pressure, and (4) evaluate dilution of sperm for extension of refrigerated storage time.

Methods

Development of buffer for sperm extension.—We performed preliminary studies during March 1994 at Louisiana State University (LSU) with razorback sucker sperm shipped by overnight delivery from the Dexter National Fish Hatchery and Technology Center. Based on studies in other fish species, sperm samples were initially diluted in unmodified Hanks' balanced salt solution (HBSS) prepared at 290 milliosmols (mosmol)/kg (Tiersch et al. 1994). The samples became gelatinous within 24 h and were nonmotile when diluted with water. Subsequent samples were stored in calcium-free HBSS (C-F HBSS) prepared at 290 mosmol/kg. These remained liquid and retained high levels of motility (>90%) for several days.

Collection of razorback suckers.—Annual collections of razorback suckers have been made from Lake Mohave during the spawning season (February–April) for the last several decades (see Tyus 1987; Minckley et al. 1991). The U.S. Fish and Wildlife Service (USFWS) has conducted a sampling operation on Lake Mohave for razorback suckers every year since 1987, and the Willow Beach National Fish Hatchery (Willow Beach) on Lake Mohave provided a base of operations for fish sampling in the upper reservoir.

We collected fish by electroshocking in a 17.6-km section of the Colorado River between Willow Beach and Hoover Dam in upper Lake Mohave (35°15′–36°00′N, 114°35′–114°45′W) in March of 1994 and 1995. This portion of the reservoir is riverine. Debris flows from small side canyons provide gravel bars that are used by razorback suckers as congregation and spawning sites (see Minckley et al. 1991 for a more complete description). We integrated our studies with standard sampling protocols established by the USFWS: fish were weighed, measured (total length, TL), and tagged with 400-kHz passive integrated transponders (PIT; Biomark, Inc., or Destron/IDI, Inc.), and general condition was recorded. We handled the fish immediately after USFWS procedures were completed or we retained fish in live wells and hatchery tanks until use (<30 min in live wells; <12 h in the hatchery).

Collection of sperm.—We developed methods to allow integration of sperm collection with routine processing of sampled fish. 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. Semen was allowed to flow freely for several seconds before it was collected in sterile 50-mL screw-cap tubes (Fisher Scientific, number 05-539-8). Sperm were diluted immediately with an equal volume of C-F HBSS and stored on ice for transport to Willow Beach (unless experimental design required undiluted samples). We stored refrigerated (4°C) sperm samples in loosely capped tubes. We kept sperm volume at less than 25 mL per tube to ensure adequate aeration of samples during storage.

Sperm collection was completed within 1 min and

did not require additional personnel or interrupt the sampling protocols of the USFWS. 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. We collected sperm from 56 of 78 males (72%) during the 2 years of study and typically obtained about 30 mL of sperm from each male.

Osmotic pressure of body fluids.—During March 1995, we determined the osmotic pressure of blood plasma, ovarian fluid, and seminal plasma to aid in development of storage procedures for gametes of the razorback sucker. Blood (0.5–1.0 mL) was collected by syringe from caudal vessels of fish, placed into labelled 3-mL vacutainers (Becton-Dickinson, number 6397), and allowed to clot. Plasma samples were obtained by pipeting and blood cells were frozen for DNA analysis. We obtained seminal plasma by centrifugation (7,000 revolutions/min for 10 min) of 50 μL of undiluted semen in microhematocrit tubes. Tubes were broken to remove the plasma fraction. We obtained ovarian fluid by pipeting liquid from a small sample of eggs collected during stripping of females. We stored all samples of body fluids at −20°C or below until analysis by vapor pressure osmometer (model 5500, Wescor Corp., Logan, Utah).

Characterization of sperm motility and duration.—In freshwater fish, activation of sperm and initiation of motility are typically associated with reduction of osmotic pressure (Morisawa et al. 1983; Bates et al. 1996). We used sperm motility as an indicator of sperm quality. To estimate 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 with dark-field microscopy at 200× 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.

Relationship of sperm motility and osmotic pressure.—In March 1994, we exposed high-quality sperm (motility, >90%) from five razorback suckers to eight step-wise dilutions of C-F HBSS ranging in osmotic pressure from 176 to 316 mosmol/kg to investigate the relationship between sperm motility and osmotic pressure. 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 a vapor pressure osmometer. We defined the level of threshold activation as the osmotic pressure that elicited 10% motility. We defined the level of complete activation as the lowest osmotic pressure that elicited the maximal motility observed for that sample. We used mean values of motility and osmotic pressure to identify the levels of threshold and complete activation. Significant differences ($P < 0.05$) between these levels were tested with a paired-comparison Student's *t*-test (Microsoft Excel 5.0, Microsoft Corp.).

TABLE 1.—Osmolality of body fluids of razorback suckers collected during March 1994 and 1995 from Lake Mohave, Arizona.

Sample	*N*	Osmotic pressure (milliosmols/kg) Minimum	Maximum	Mean ± SD
Blood plasma	71	176	364	282 ± 27
Ovarian fluid	18	152	274	223 ± 32
Seminal plasma	33	82	297	198 ± 55

Evaluation of sperm dilution for refrigerated storage.—During March 1995, 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, transported within 1.5 h, and diluted beyond 1:1 at Willow Beach. We stored six, high quality samples, upright in loosely-capped 15-mL tubes (Fisher, #05-539-5). We estimated motility immediately after final dilution, and daily for 3 d until samples were shipped by commercial airline to LSU, where daily estimates were continued until all samples became non-motile. We compared treatments in Experiment 1 by a paired-comparison Student *t*-test. We analyzed the effects of dilution on sperm motility using one-way analysis of variance (ANOVA; SAS 6.08, SAS Institute Inc., Cary, North Carolina).

Results

There were significant differences among the osmolalities of the three body fluids (ANOVA: $F = 64$; df = 2, 119; $P < 0.0001$; Table 1). The osmolality of blood plasma (mean ± SD: 282 ± 27 mosmol/kg; $N = 71$) was significantly higher than that of

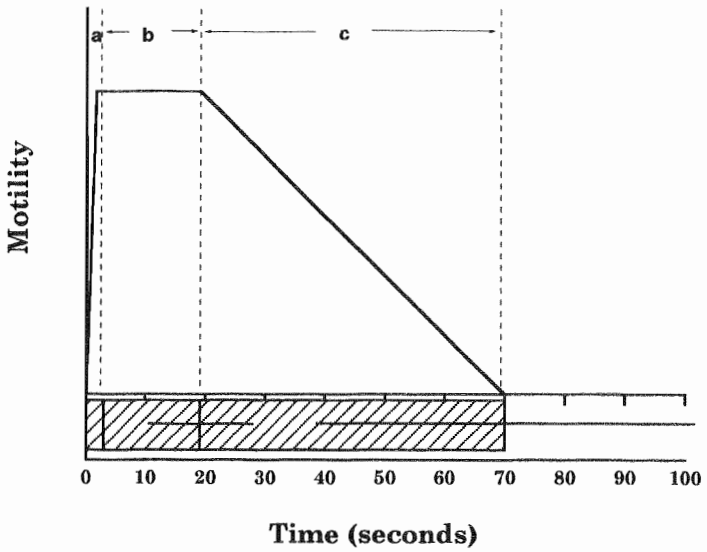

FIGURE 1.—Diagrammatic characterization of motility of razorback sucker sperm activated by the addition of a 20-fold excess of water from Lake Mohave. Motility was divided into three periods: (a) time until complete motility (activation); (b) duration of complete motility; and (c) time until cessation of motility. Shaded area shows mean motilities (solid vertical lines) and one standard deviation from those means (solid horizontal lines) for sperm samples from five fish.

ovarian fluid (223 ± 32 mosmol/kg; $N = 18$), which was significantly higher than the osmolarity of seminal plasma (198 ± 55 mosmol/kg; $N = 33$).

Sperm became motile and began rapid swimming when diluted in water collected from Lake Mohave. Duration of motility was classified into three periods (Figure 1). 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 (mean ± SD), and the time until complete cessation of motility was 70 ± 32 s after the addition of water.

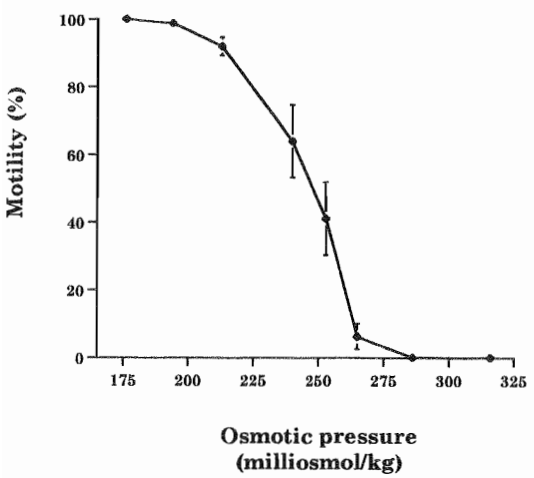

FIGURE 2.—Motility of razorback sucker sperm exposed to calcium-free Hanks' balanced salt solution prepared at various osmotic pressures. Aliquots of sperm (2 μL) were diluted with 20 μL of test solution, and motility was estimated with dark-field microscopy at 200× magnification. The mark at 264 mosmols/kg designates threshold activation. Blood osmolality was 282 mosmols/kg. Each point represents the mean value (±1 SD) of sperm from 5 fish.

Reduction in osmotic pressure of C-F HBSS to below that of blood plasma was associated with the activation of razorback sucker sperm (Figure 2). Sperm were observed vibrating in place or swimming slowly at osmotic pressures as high as 280 mosmol/kg. The threshold activation level (10% motility) was 264 mosmol/kg, and the level of complete activation was 176 mosmol/kg. The levels of threshold and complete activation were significantly different (*t*-test: $t = 34.204$, df = 4, $P < 0.0001$).

In experiment 1, a significant difference in motility occurred within 24 h between sperm diluted 1:1 with C-F HBSS and undiluted sperm (Figure 3). All undiluted sperm samples became nonmotile within 72 h. About half (45%) of the diluted sperm samples retained at least 60% motility for 5 d.

In experiment 2, sperm motility was not different on day 3 among the three treatments containing different proportions of C-F HBSS or on day 8 of the experiment (Figure 4). Overall, mean sperm

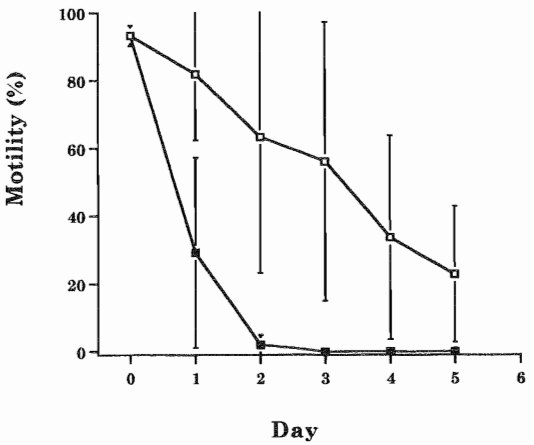

FIGURE 3.—Motility of razorback sucker sperm stored at 4°C undiluted (black squares) or diluted 1:1 (open squares) with calcium-free Hanks' balanced salt solution. Each point represents the mean value (±1 SD) for sperm of 11 fish. Differences between undiluted and diluted sperm motility were significant within 24 h ($t = -6.45$, $P < 0.0001$).

motility was highest on day 1 (73 ± 10%) and decreased through time to day 8 (2 ± 5%, Figure 4). Sperm samples retained on average greater than 15% motility for 6 d, although 9 of the 15 samples appeared degraded and were nonmotile after 3 d.

Discussion

Collection, handling, and storage of sperm has received attention for fish species that include carps (Hulata and Rothbard 1979; Jayaprakas and Bimal Lal 1996), salmonids (reviewed by Scott and Baynes 1980; Billard 1995), channel catfish *Ictalurus punctatus* (Christensen and Tiersch 1996), paddlefish *Polyodon spathula* (Mims 1991), and walleye *Stizostedion vitreum* (Moore 1987). However, this study is the first to address gamete collection and storage in catastomid fishes (Jamieson 1991). Application of the procedures described herein provided sperm for the field propagation of razorback suckers (Carmichael et al. 1996) and produced more than 100,000 razorback sucker fry in 1996 for use at Willow Beach National Fish Hatchery. These procedures could aid established management programs for razorback suckers (Mueller 1995), and they demonstrate the potential for integrating gamete collection with existing programs.

The osmolality of seminal plasma taken directly from sperm samples (198 mosmol/kg) was lower than the osmolality of the blood plasma (282 mosmol/kg) and the level for threshold activation of sperm (264 mosmol/kg). If we assume that the osmotic environment encountered by sperm in the testis

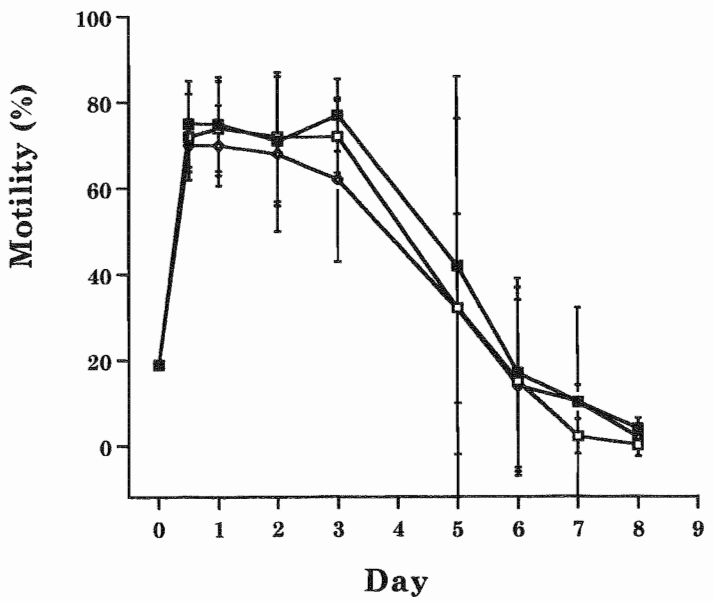

FIGURE 4.—Motility of razorback sucker sperm stored at 4°C in three dilutions (1:1, 1:3, or 1:7) prepared with calcium-free Hanks' balanced salt solution. Each point represents the mean value (±1 SD) for sperm of five fish. Values were not obtained during transport by commercial airline (day 4). Motility values of sperm were not significantly different on day 3 (ANOVA $F = 1.974$, df = 2, 14, $P = 0.18$) or day 8 of the experiment (ANOVA $F = 0.540$, df = 2, 14, $P = 0.60$). Black squares = 1:1, open squares = 1:3, and open diamonds = 1:7 dilution.

is isotonic with that of blood, semen apparently was diluted during collection. Because care was taken to avoid contamination of semen with water or feces, it is possible that the razorback sucker sperm samples were diluted with urine during collection. Seminal plasma displayed the largest range of osmolality values (215 mosmol/kg) of the three fluids studied and the lowest absolute value (82 mosmol/kg) encountered. Freshwater fishes typically excrete hypotonic urine (Bond 1979), and it is likely that variable amounts of urine were collected with the semen in our study. It would be difficult to collect urine-free sperm without extensive modification of field collection methods. To address this factor, we chose to mix sperm immediately with C-F HBSS at the time of collection, thereby preventing sperm activation. Because ovarian fluid was also lower in osmotic pressure than blood plasma, urine could also be mixing with eggs during stripping. The addition of C-F HBSS to eggs could extend the time that fertilization is possible by minimizing premature activation.

We found that the duration of sperm motility (70 s) and maximal sperm activity (20 s) in razorback suckers was brief, indicating that care should be taken to ensure good, early mixing of gametes during artificial spawning of razorback suckers. Maximal motility of sperm samples required dilution to at least 176 mosmol/kg, which suggests that estimation of motility for assessment of sperm quality for razorback suckers requires a minimum dilution of at least 100% to reduce osmotic pressure by one-half. This would also be the case for ensuring sufficient dilution for maximizing fertilization rates (Bates et al. 1996).

The dilution of sperm with buffer solutions has been well studied for mammals and is becoming increasingly studied in fishes (Stoss 1983; Gwo et al. 1991; Gallant et al. 1993), often by application of mammalian sperm extenders to use in fish. Hanks' balanced salt solution has been used as an extender for sperm of the eastern oyster *Crassostrea virginica* (Zell et al. 1979), channel catfish (Tiersch et al. 1994) and spotted seatrout *Cynoscion nebulosus* (Wayman et al. 1996). Additionally, we have used this buffer for storage of sperm of freshwater drum *Aplodinotus grunniens,* black drum *Pogonias cromis,* common carp *Cyprinus carpio,* humpback chub *Gila cypha,* bonytail *Gila elegans,* and Colorado squawfish *Ptychocheilus lucius,* although osmolality of the buffer was adjusted for each species (unpublished data). When HBSS was modified by the removal of calcium chloride, this extender allowed refrigerated storage of razorback sucker sperm for at least 7 d. Bacterial contamination likely resulted in the degradation of sperm of razorback suckers after that time. Sperm survival may be prolonged by addition of antibiotics to inhibit bacterial growth (Stoss et al. 1978; Stoss and Refstie 1983).

Our study demonstrates that collection of sperm can be rapid and does not disrupt normal sampling procedures. Sperm, once collected, can be used for breeding purposes, and provides a source of DNA that can be collected without damage to the fish. Our procedures were performed in sampling boats during routine data collection and tagging and were mastered quickly by sampling crews. Gamete collection and short-term storage offers potential for enhancement of management efforts for razorback suckers. In combination with cryopreservation of sperm (Tiersch and coworkers, unpublished), these techniques provide flexibility in spawning, allow storage of genetic material for extended periods, and enable transfer of genes from wild populations to hatchery broodstock (Cloud et al. 1990). Similar procedures could be applicable to other endangered species.

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