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Storage, Transportation, and Fertility of Undiluted and Diluted Paddlefish Milt

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Abstract.—Milt samples from paddlefish (*Polyodon spathula*) were collected from specimens obtained from the Missouri River near Chamberland, South Dakota, in 1991 and the Opelika City Reservoir System near Auburn, Alabama, in 1992. Ice-chilled milt samples either were left undiluted or were diluted with one of three chilled extenders, transported overnight on ice, and analyzed for sperm concentration, extracellular pH, electron microscopical characters, sperm motility percentage, and duration of sperm motility. Paddlefish milt had relatively low sperm counts compared with milt of other fish species, averaging 1.8×10^9 spermatozoa/mL. Extracellular pH averaged 8.22. A sperm acrosome was demonstrated by electron microscopy. Dechlorinated tap water and 10% artificial sea water (ASW) activated and sustained sperm motility better than 25% ASW. Transportation of milt had no apparent negative effect on fertility. For storage 1–5 d after collection, undiluted milt provided an average fertilization rate (93%) that was as good as the rate with fresh milt and better than the rates for milt diluted with any of the three extenders. For longer storage, an extender containing only sodium chloride and antibiotics provided better fertilization rates (97% at day 14 and 61% at day 25 after collection) than other extended or undiluted milt samples.

The paddlefish (*Polyodon spathula*) is an important commercial species. Many federal and state hatcheries are propagating paddlefish for restoration and stock enhancement programs, and some private hatcheries are propagating paddlefish for use as food (Semmens and Shelton 1986). Advance collection and storage of milt may be a convenient method to attain good quality spermatozoa for fertilization of paddlefish ova, reduce crowding of broodstock in hatcheries (Mims 1991), and lower spawning cost by reducing the number of males induced with leuteinizing hormone releasing hormone analog (LHRH-A).

Successful storage and transportation of paddlefish milt would allow milt to be collected when males are available and held until ripe females can be obtained. Mims (1991) reported that a saline (NaCl) solution used as a carrier of antibiotics also extended to 8 d the time that chilled paddlefish spermatozoa could be stored and still be motile when activated. Our paper describes means of extending the period of chilled storage beyond 8 d and provides the techniques for storage and transportation of paddlefish milt.

Methods

Animals and milt collection.—Male paddlefish were caught at two sites. Three specimens were caught in the Missouri River near Chamberland, South Dakota, in May 1991 and transported to 2.5-m circular holding tanks (3,200 L) at the Gavins Point National Fish Hatchery, Yankton, South Dakota. Five specimens were caught in the Opelika City Water Reservoir near Auburn, Alabama, in March 1992 and transferred to 2.5-m circular holding tanks. In all cases, fish were given an intraperitoneal injection of LHRH-A (des-gly^{10}, D-ala^6-LHRH ethylamide; Sigma Chemical Co., St. Louis, Missouri) as a single dose of 0.1 mg/kg body weight.

For milt collection, a paddlefish was blotted dry around the genital opening. Tygon tubing (5 cm long) attached to a 10-mL plastic syringe was inserted into the urogenital pore. By using gentle abdominal pressure, approximately 20 mL of milt was collected from each fish. Each sample was examined for percentage motility, and those samples showing less than 75–100% motility were discarded. No more than 4 mL of extender-diluted or fresh milt was put into each 25-cm^2 polystyrene tissue culture flask (Corning) for storage and transportation. Samples from all locations were transported to Iowa State University or Kentucky State University for analyses. All samples were stored on ice or at 1°C in a refrigerated incubator; no aeration was used.

Milt and sperm characteristics.—Sperm concentrations in all milt samples were measured, within 24 h of collection, with a Bright Line hemacytom-

TABLE 1.—Composition of extenders for paddlefish milt.

Composition	Extender[a]		
	4H	7	13
$CaCl_2 \cdot 2H_2O$	0.205 g	0.205 g	
$MgCl_2 \cdot 6H_2O$	0.440 g	0.440 g	
$NaHCO_3$	0.470 g	0.470 g	
KCl	5.115 g	5.115 g	
NaCl	11.560 g	11.560 g	17.520 g
Glucose	20.0 g		
Pyruvate		12.0 g	
Citric acid	0.200 g	0.200 g	
HEPES buffer	4.76 g	4.76 g	
Double-distilled H_2O	2,000 mL	2,000 mL	2,000 mL
KOH (1.27 g/100 mL)	20 mL	20 mL	
Pen–strep[b]	20 mL	20 mL	20 mL
Osmolarity	310 mosmol	310 mosmol	310 mosmol
pH	7.6	7.6	7.6

[a] Extenders were mixed and pH was adjusted with KOH; then the extenders were used or frozen.
[b] Penicillin–streptomycin: Sigma, P0906; 5,000 units of pencillin and 5 mg streptomycin per milliliter of 0.9% NaCl.

eter according to directions for use of Levy and Levy-Hausser corpuscle-counting chambers. For each measurement, 25 μL of sample was diluted with 25 mL of extender 7. (Extenders are characterized in Table 1.) A drop was placed on the hemacytometer, and sperm counts were made with the aid of a 16-mm objective and 10× ocular mounted on an Olympus BH compound microscope. The extracellular pH of undiluted and diluted samples was measured with a Chemtrix pH meter type 60A.

Sperm motility was examined by placing a small drop (about 1.0 mm in diameter and 2.5 μL in volume) of undiluted or diluted milt on a coverslip with a Pasteur pipette. Under a dissecting microscope (6×), a large drop (about 0.05 mL) of dechlorinated tap water, 10% artificial sea water (ASW), or 25% ASW was allowed to fall directly on the milt. This caused a rapid dilution, and in high-quality samples, spermatozoa immediately became motile (75–100% motility). Determination of motility percentage was made within 15 s. The following indexing scores were used to estimate percentage motility: 5 (75–100% motility), 4 (25–75% motility), 3 (5–25% motility), 2 (<5% motility), 1 (<1% motility), and 0 (no motility). Motility was recognized as forward motion of sperm, not as vibratory motion. Duration of motility was measured by observing the percentage of motile spermatozoa in the entire drop.

Electron microscopy.—Undiluted milt used for electron microscopy was first centrifuged at 0°C to form a pellet. The pellet was fixed for 12 h in 3% glutaraldehyde in Millonig's buffer (2.26% monobasic sodium phosphate and 2.52% sodium hydroxide in 100 mL of double-distilled water, pH 7.3) with added 5% sucrose. The sample was post-fixed in 10% osmium tetroxide with buffer and 3% sucrose, then serially dehydrated with ethanol from 25% to absolute. Propylene oxide was added with two changes; then resin (Diamond 812) infiltration (50% propylene and 50% resin) was allowed to occur overnight. There were two changes (30 min each) of 100% resin with incubation at 60°C overnight. Five-micrometer-thick sections were cut first with a glass knife and then with a diamond knife. Sections were stained with Reynold's lead citrate and 2% uranyl acetate and examined with an JEOL electron microscope.

Extenders and storage and transportation of milt.—Three extenders (4H, 7, and 13) were used to examine milt characteristics during storage (Table 1). Extenders 4H and 7 were modified from an extender previously reported by Erdahl et al. (1984). Milt was diluted 1:1 with the prepared extenders immediately after it was collected. The extender was added slowly to the semen with constant but gentle agitation inside a 25-cm^2 tissue culture flask. All components were cooled on ice before being mixed. To aid in the prevention of anoxia, the depth of fluid in flasks was routinely maintained around 0.3 mm (4 mL). Extended and undiluted samples were transported in flasks, which were packed flat on ice and shipped by overnight carrier. Upon arrival, samples were evaluated for motility and stored in a Hotpack incubator at 1°C. Flasks were stored flat and were opened once a week to obtain aliquots for testing sperm motility and to measure extracellular pH. At this time, each

flask was gently agitated to resuspend spermatozoa.

Hatchability studies.—In 1991, milt samples were collected from two males and diluted at Gavins Point National Fish Hatchery in late May, transported to Iowa State University for motility evaluation, and then transported to Garrison Dam National Fish Hatchery, Riverdale, North Dakota, for a preliminary fertility study. The milt samples used for this study were stored for 14 d.

In 1992, milt samples were collected from three males on March 31 and diluted at the Fisheries Research Unit of the Alabama Agricultural Experiment Station, Auburn University, transported to Iowa State University for evaluation and storage, and then transported to Down on the Farm, Inc., Ashland, Kentucky, on April 4 (5-d storage) and April 24 (25-d storage) for fertility studies.

In both 1991 and 1992, a female fish was injected with LHRH-A (0.1 mg/kg body weight) in a two-injection series: 0.1 and 0.9 of total dosage, 12 h apart. Eggs were manually stripped from the female (Graham et al. 1986) and collected in a dry culture bowl (18 cm diameter). Two hundred to three hundred eggs were then placed into each of several Petri dishes. The milt sample in each flask (4 mL) was poured into a dish, 35 mL of dechlorinated tap water were added, and the mixture was stirred with a separate clean feather. Fertilized eggs were mixed with Fuller's earth for 10–15 min to coat the adhesive eggs, rinsed, and placed in culture jars (Graham et al. 1986). After 3 d, the fertilized eggs were placed in 3.79-L Ziploc freezer bags containing 3 L of dechlorinated tap water bubbled with oxygen and shipped overnight to either Iowa State University or Kentucky State University for hatchability studies. Upon arrival at Iowa State University, the eggs were placed in a running-water Heath incubator tray at 18.9°C and percentage hatch was determined. At Kentucky State University, the eggs were placed into culture dishes and randomly set onto a shaker table in water at 19.2 ± 0.5°C.

Five milt treatments were used to fertilize paddlefish eggs for all three time periods: fresh milt (control), stored undiluted milt, and stored milts diluted with extenders 4H, 7, or 13. There were two replications per male fish for all treatments on storage days 5 and 25. For storage day 14, there were one replicate per male fish for stored diluted samples and three replications for fresh and stored undiluted samples.

Data analysis.—All data were analyzed by the SAS general linear models procedure (SAS Institute 1988). The alpha level was 0.05 for all within-day comparisons by Fisher's least significant difference test. The fertilization model was a randomized block design: fertility percentage = $\mu + T_i + D_j + (TD)_{ij} + E_{ijk}$; μ is the grand mean percent hatch, T_i is a term for milt treatment, D_j is a term for days of storage, $(TD)_{ij}$ is an interaction term, and E_{ijk} is an error term. A term representing individual males could not be used due to confounding with treatment and data for day 14. Differences in means were determined by using the least square mean.

Results and Discussion

Milt and Sperm Characteristics

Sperm counts for eight paddlefish males averaged $1.8 \times 10^9 \pm 0.8 \times 10^9$ spermatozoa/mL (mean ± SD). This concentration is similar to those of many species in the sturgeon family (Acipenseridae), but only 10–20% as great as most salmonid and cyprinid sperm concentrations (Ginzberg 1968). The pH of the extracellular fluid averaged 8.22 and ranged from 7.80 to 8.44.

Initiation and duration of sperm motility were similar in dechlorinated tap water and 10% ASW. During a period of 4–5 min, sperm motility with both activators gradually decreased from 75–100% to less than 25%. Sperm motion in 25% ASW was vibratory and lasted less than 20 s. Mims (1991) found that dechlorinated water and 9% saline supported similar activations and durations of motility for paddlefish spermatozoa. Several studies have shown that addition of dilute saline solution to spermatozoa of freshwater fishes can lengthen the duration of motility (Ginzberg 1968; Holtz et al. 1977; Steyn et al. 1989). In the present study, because there was little difference in motility duration when 0.38% saline solution (10% ASW) or dechlorinated tap water was used as the activator, only motility percentages measured in 10% ASW are reported.

Electron Microscopy

The morphology of paddlefish sperm is similar to that of other primitive fish sperm (Afzelius 1978). Paddlefish sperm has a long cylindrical head, a short midpiece, and a flagellum (Figure 1). The shape of the sperm head is conducive to penetration through narrow micropylar canals and a dense, thick egg membrane (Ginzberg 1968). The head contains an acrosome and nucleus; the midpiece is connected to a sheath that surrounds the proximal portion of the flagellum. Two lateral fins (not demonstrated in the figure) are also found on

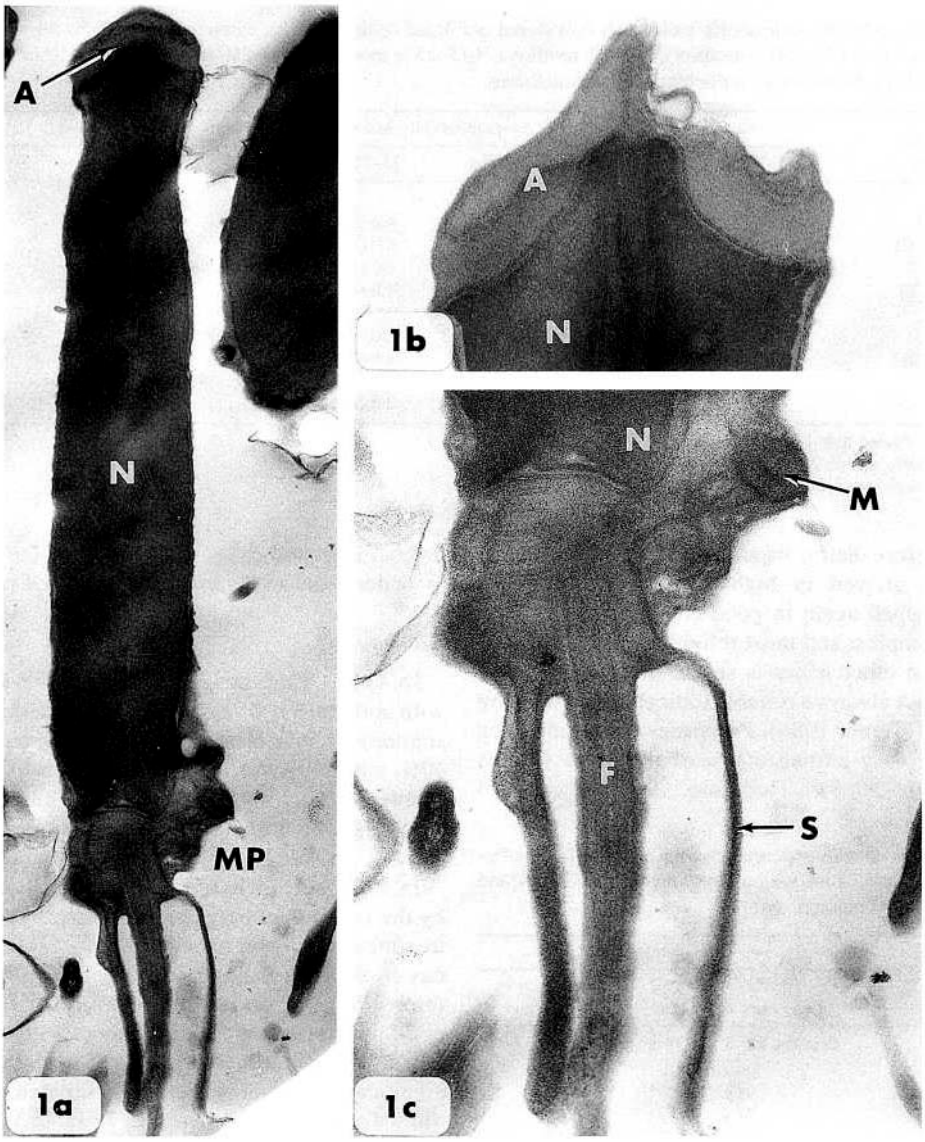

FIGURE 1.—Ultrastructure of paddlefish sperm. **(a)** Longitudinal section of a sperm demonstrating acrosome (A), nucleus (N), midpiece (MP), and flagellum (at bottom) (20,000 × magnification). **(b)** Longitudinal section through the anterior end of a sperm demonstrating the acrosome (40,000×). **(c)** Longitudinal section through a sperm midpiece demonstrating mitochondria (M), centrioles, cytoplasmic sheath (S), and flagellum (F) (40,000×).

the flagellum. The presence of an acrosome is an important consideration in the storage of paddlefish milt, because premature activation of the acrosome renders the sperm inviable (Dan 1956). The methods of storage used in this study apparently did not activate the sperm acrosome.

Extenders, Storage, and Transportation

In 1991, the three extenders scored equally well for sperm motility through 8 d of storage (Table 2). Because two samples of each extender treatment were shipped to North Dakota for fertilizing eggs, only one extended sample of each type was monitored after 8 d. By day 21 of storage, both undiluted milt and milt diluted with extender 4H had high motility scores, but milt diluted with extenders 7 and 13 had low scores. In 1992, all extenders had similar scores through 15 d of storage. However, extender 13 had the best scores through 56 d. In 1991 and 1992, milt samples were shipped

TABLE 2.—Motility indexes for paddlefish milt stored undiluted or diluted with extenders for various lengths of time. Motility index: 5 (75–100% motility), 4 (50% motility), 3 (5–25% motility), 2 (<5% motility), 1 (<1% motility), and 0 (no motility). Numbers of replicates are in parentheses.

Year and milt treatment[a]	Average motility index after storage for:						
	1–2 d	7–8 d	14–15 d	21–22 d	28–29 d	35–36 d	56 d
1991							
Undiluted	5.0 (6)	4.5 (6)	4.0 (6)	4.0 (3)	3.0 (2)	3.0 (2)	
Extender 4H	5.0 (3)	5.0 (3)[b]	5.0 (1)	4.0 (1)			
Extender 7	5.0 (3)	5.0 (3)[b]	4.0 (1)	1.0 (1)			
Extender 13	5.0 (3)	5.0 (3)[b]	5.0 (1)	1.0 (1)			
1992							
Undiluted	5.0 (6)	5.0 (6)	4.0 (6)	2.0 (6)[c]	1.0 (3)		
Extender 4H	5.0 (4)	5.0 (4)	4.0 (4)	4.0 (4)[c]	2.0 (2)	1.0 (1)	
Extender 7	5.0 (4)	5.0 (4)	4.0 (4)	3.5 (2)[c]	1.0 (1)		
Extender 13	5.0 (4)	5.0 (4)	4.0 (4)	4.0 (4)[c]	4.0 (2)	3.5 (2)	3.0 (1)

[a] Extenders diluted milt 1:1.
[b] Samples were sent to North Dakota for fertility studies.
[c] Samples were sent to Kentucky for fertility studies.

twice before being used in hatchability studies. Samples arrived in high-quality condition and were shipped again in good condition.

The simplest and most reliable indicator of milt quality in other fishes is sperm motility, but motility is not always a reliable indicator of fertilizing capacity (Terner 1986). Properties of spermatozoa also can vary among males of the same species (Ginzberg 1968). Therefore, fertilization and hatchability studies are needed before the value of extenders can be demonstrated conclusively.

TABLE 3.—Hatching percentages of paddlefish eggs fertilized with milt from various treatments after 5, 14, and 25 d of chilled storage.

Milt treatment	Percentage hatch	
	Actual[a,b]	Relative to control
Storage for 5 d (1992)		
Control[c]	93 z	100
Undiluted	93 z	100
Extender 4H	77 y	83
Extender 7	18 x	19
Extender 13	89 z	96
Storage for 14 d (1991)		
Control[c]	98 z	100
Undiluted	48 y	49
Extender 4H	47 y	48
Extender 7	0 x	0
Extender 13	97 z	99
Storage for 25 d (1992)		
Control[c]	83 z	100
Undiluted	0 w	0
Extender 4H	31 x	37
Extender 7	0 w	0
Extender 13	61 y	73

[a] Within a storage duration group, values followed by the same letter are not significantly different ($P > 0.05$).
[b] Pooled standard errors are 2.5% for 5-d storage, 5.0% for 14-d storage, and 3.3% for 25-d storage.
[c] Control milt was fresh (not stored).

Hatchability

In 1991, when paddlefish eggs were fertilized with milt that had been stored 14 d, diluted milt supported hatchabilities of 99% with extender 13, 47% with extender 4H, and 0% with extender 7 (Table 3). This range of fertility is much greater than might be implied by the motility indices (Table 2).

In 1992, percent hatch was significantly affected by the interaction between day of storage and milt treatment (analysis of variance $P < 0.0001$). On day 5, there were no significant differences in effective fertility among control (fresh) milt, stored undiluted milt, and stored milt diluted with extender 13, and milt in these three treatments gave significantly higher percentages of hatch than milt diluted with extenders 4H and 7 (Table 3). On day 25, none of the stored milt was as fertile as fresh milt, but milt diluted with extender 13 gave significantly higher percentage hatch than milt stored undiluted or diluted with extenders 7 and 4H.

Hatchabilities resulting from the three milt storage experiments (Table 3) were jointly analyzed by the general linear models procedure (Table 4). Extender 13, a saline solution with antibiotics, allowed stored sperm to remain as fertile as fresh sperm ($P > 0.05$). Stored milt diluted with extender 4H was no more fertile than stored undiluted milt; both these treatments were significantly less successful than extender 13 ($P < 0.05$), and both were significantly better than extender 7, which had little value at all.

Sodium chloride offers protection to sperma-

TABLE 4.—General linear models comparisons of paddlefish hatchability after fertilization of eggs with milt treated in several ways. The statistics summarize three experiments over which milt storage time varied from 5 to 25 d. For each comparison, the upper number is the t-value for the hypothesis that the least-square mean hatchabilities are equal for the two treatments, and the lower number is the probability of obtaining a larger t-value. The calculated overall least-square mean (LSM) fertilities by treatment are also shown.

Milt treatment	Milt treatment				LSM fertility (%)
	Undiluted	Extender 4H	Extender 7	Extender 13	
Control	7.4 0.0001	6.5 0.0001	13.8 0.0001	1.5 0.1543	91.3
Undiluted		−0.6 0.5307	6.7 0.0001	−5.6 0.0001	47.4
Extender 4H			7.0 0.0001	−4.8 0.0001	51.3
Extender 7				−11.8 0.0001	5.9
Extender 13					82.3

tozoa by decreasing the energy expenditure for osmotic activity during storage (Turdakov 1962). Results from the present study demonstrate that using a saline solution as an extender will increase the length of the chilled-storage period from 8 to 25 d, provide good-quality spermatozoa and relatively high percentage hatch, and permit milt to be transported without negative effect on its viability. We hope the results will lead to long-term preservation or cryopreservation of paddlefish milt. A suitable extender, such as reported in this paper, is the first step required in developing cryogenic procedures. Cryopreservation would not only provide milt samples for fertility and metabolic activity studies, but also could provide viable samples year-round for hatchery production of paddlefish at public and private facilities.

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