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Cryopreservation of Channel Catfish Sperm: Storage in Cryoprotectants, Fertilization Trials, and Growth of Channel Catfish Produced with Cryopreserved Sperm

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Abstract.—We developed methods for cryopreserving sperm of channel catfish *Ictalurus punctatus* and evaluated the use of cryopreserved sperm for reproduction. Five cryoprotectants were evaluated: methanol, glycerol, dimethyl sulfoxide (DMSO), sucrose, and polyvinylpyrrolidone. We measured the motility of sperm that had been stored at 4°C in three concentrations of cryoprotectants (5%, 10%, 15%) dissolved in a modified Hank's balanced salt solution. All cryoprotectants reduced motility within 6 h; 5% methanol and 5% DMSO caused the smallest reduction. After sperm were frozen at −80°C and stored for 2 d at −196°C, motility was highest (5–10%) in samples cryopreserved with 5% and 10% solutions of methanol. Sperm cells cryopreserved in methanol solutions (5%, 10%, and 15%) were used to fertilize channel catfish eggs from three females. Fertilization ranged from 24% to 97%, and no difference in fertilization success was found between cryopreserved sperm and untreated sperm from the same males. Growth of channel catfish produced with cryopreserved sperm was not different from the growth of siblings produced with untreated sperm. Sperm cryopreservation offers utility as a routine method for gamete storage and genetic improvement of catfish.

Over the past 15 years aquaculture production of channel catfish *Ictalurus punctatus* has risen dramatically, and this species is now the most important fish cultured for food in the United States. Research on the biology of this species, however, has not kept pace with the economic growth, and many topics remain little studied. One such topic is the cryopreservation of gametes, a subject that has received considerable attention in connection with other fishes such as salmonids (e.g., Stoss 1983; Leung and Jamieson 1991). Cryopreservation of channel catfish sperm would aid genetic improvement through selective breeding programs, production of hybrids, and development of reference stocks. Cryopreservation is of particular value in this species because sperm cannot be stripped from channel catfish, and it is necessary to surgically remove the testis (Bart and Dunham 1990) or kill valuable brood stock to obtain sperm.

Freezing of channel catfish sperm was reported by Guest et al. (1976) but they did not attempt fertilization with cryopreserved sperm. The present study extends this earlier work on storage and cryopreservation of channel catfish sperm. Our purpose was to evaluate (1) the effect on sperm motility of five cryoprotectants (methanol, glycerol, dimethyl sulfoxide, sucrose, and polyvinylpyrrolidone) and (2) the utility of cryopreserved sperm for production of channel catfish. Our objectives were to study the effect of different cryoprotectants on motility of sperm stored at 4°C or cryopreserved at −196°C, to evaluate the viability of cryopreserved sperm for fertilization, and to document first-year growth of channel catfish produced with cryopreserved sperm. Also as part of this work, we evaluated a simple device for circulating liquid nitrogen vapor for freezing sperm.

Methods

Sperm preparation.—Two healthy, mature, 4-year-old male channel catfish (from a commercial supplier in Mississippi) were killed and their testes were removed. Adherent tissue was dissected away, and testes were blotted dry and weighed. Two grams of anterior testis (Sneed and Clemens 1963) from each fish was dissociated in a loose-fitting, glass-on-glass tissue grinder, and the spermatozoa were suspended in 10 mL of a modified Hanks' balanced salt solution (HBSS; Table 1) and refrigerated at 4°C (Tiersch and Tiersch 1993). Sperm density was estimated by use of a Coulter multisizer (Coulter Electronics, Hialeah, Florida) and was similar between males:

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2.5×10^9 sperm/g of testis (wet weight) for male 1, and 2.8×10^9 sperm/g for male 2.

Cryoprotectants were dissolved to 5, 10, or 15% in HBSS. We tested two groups of cryoprotectants that differ with respect to their permeability into cells (Doebbler 1966): (1) permeating solutes, represented by methanol, glycerol, and dimethyl sulfoxide (DMSO); and (2) nonpermeating solutes, represented by sucrose and polyvinylpyrrolidone (PVP). To allow direct comparison of results from this study with the results reported in other studies, the osmolality of cryoprotectant solutions was measured with a model 5500 vapor pressure osmometer (Wescor Inc., Logan, Utah) (Appendix).

Motility estimates.—Motility was determined for sperm refrigerated at 4°C with or without cryoprotectant and for sperm frozen and thawed with or without cryoprotectant. All samples (including thawed cryopreserved sperm) were refrigerated at 4°C, and motility was monitored periodically for 180 d. Motility estimates were made visually with dark-field illumination at 100× magnification. The addition of a 10- to 20-fold excess of well water was used to activate a 1–2-μL sperm suspension placed on a glass slide. Given the subjective nature of sperm motility estimates (Terner 1986), sperm were identified as motile-only if they displayed vigorous, sustained movement in a forward direction. Initial motility within the first 5 s was not recorded because motility of less than 5 s in duration was difficult to estimate reliably; swimming motions of sperm were not distinguishable from streaming or swirling due to bulk flow in this time period, and an assay including this period was difficult to standardize. Thus, values for percent motility reported in this study may be low in relation to the values reported in other studies.

Cryopreservation procedures.—Equal numbers of sperm (1×10^8 sperm/mL) from each male were used for cryopreservation experiments, without adjustment for differences in sperm motility. Sperm suspensions were diluted 1:4 in the various cryoprotectant solutions, drawn into 0.5-mL french straws (IMV International, Minneapolis, Minnesota), and allowed to equilibrate for 15 min at 10°C prior to freezing. Following equilibration, straws were placed on a stainless steel tray suspended above liquid nitrogen in a circular, insulated tank (Figure 1). Around the circumference of the bottom of the tank was a length of 6.4-mm (outer diameter) copper tubing pierced with 2-mm holes spaced at 5-cm intervals. Nitrogen gas was released from a pressurized tank through the tubing and bubbled through the liquid nitrogen. By regulating the flow of gas, circulation of cryogenic nitrogen vapor could be controlled and a temperature of −80°C maintained at the tray supporting the straws. After reaching a temperature of −75°C (about 4 min), the straws were plunged into liquid nitrogen and stored for 2 d at −196°C. For the motility estimates and fertilization trials, frozen sperm was thawed rapidly by plunging straws into a 40°C water bath for 7 s.

TABLE 1.—Ingredients of a modified Hanks' balanced salt solution used to dilute channel catfish sperm.

Ingredient	Concentration (g/L)
NaCl	8.0
KCl	0.4
$CaCl_2 \cdot 2H_2O$	0.16
$MgSO_4 \cdot 7H_2O$	0.20
$Na_2HPO_4 \cdot 7H_2O$	0.12
KH_2PO_4	0.06
$NaHCO_3$	0.35
$C_6H_{12}O_6$ (glucose)	1.00

Spawning and rearing of fish.—Fish were spawned in mid-June 1991 by standard procedures described by Goudie et al. (1993). Three healthy, mature female channel catfish (from a commercial supplier in Mississippi) were injected with human chorionic gonadotropin (1,100 IU/kg) and paired with channel catfish males in 80-L aquaria supplied with aerated flow-through well water. Fish were monitored for spawning behavior. When they began to release eggs, the females were removed from aquaria, anesthetized in 0.02% tricaine methanesulfonate, rinsed, and dried. Eggs were stripped by application of pressure along the abdomen.

Sperm frozen with methanol as the cryoprotectant displayed the highest motility, and methanol was chosen for use in fertilization trials. Eggs from each of three females were fertilized with sperm from two males. Sperm cryopreserved with 5%, 10%, or 15% methanol were stored frozen at −196°C for 2 d. Sperm collected from the same males, used as a fertilization control, were refrigerated at 4°C for 2 d and diluted to the same density as the cryopreserved sperm. Aliquots of 200–300 eggs were fertilized with cryopreserved sperm from single 0.5-mL straws or with 0.5 mL of control sperm. Sperm and eggs were activated by the addition of 50 mL of aerated well water.

Fertilized eggs were incubated in 8-L hatching troughs supplied with flow-through well water at 26°C. At 25 h after fertilization, eggs were inspected with a dissecting microscope for development

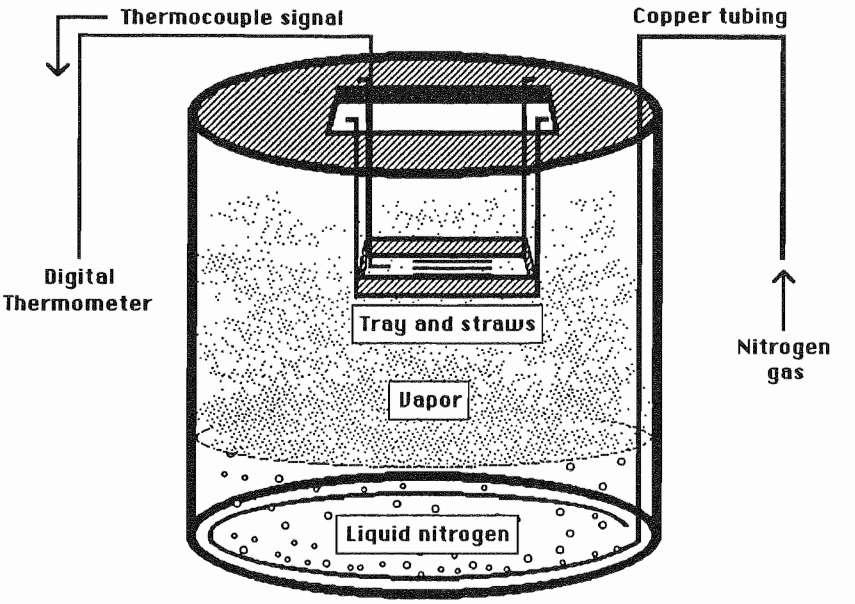

FIGURE 1.—Device used for freezing sperm in liquid nitrogen vapor. Nitrogen gas was bubbled through liquid nitrogen in the bottom of the tank to circulate the vapor. The tray used to hold the straws containing sperm was positioned in the vapor and maintained at −80°C by regulating the flow of nitrogen gas.

into the neurula stage. Neurulation was employed as the basis for identifying fertilization success because unfertilized eggs begin development but become arrested at gastrulation (Withler 1980). The embryos hatched after about 5 d, and fry were moved to 80-L tanks after yolk-sac absorption and the start of feeding. At this time, fish produced from different females were pooled, yielding four groups consisting of offspring produced either with frozen or untreated sperm from each of the two males. At 4 months of age, fish were moved to 160-L tanks; at 9 months they were moved to 800-L tanks. Fish were fed a series of suitably sized diets beginning with a 49% protein sinking diet and ending with a 35% protein floating diet. Individual weights of 25 fish from each group were recorded monthly.

Data analysis.—Fertilization success and fish growth were analyzed separately by two-way factorial analysis of variance (ANOVA) with sperm source (male) and sperm treatment (frozen or untreated) as the two factors. Analyses were performed with StatView 512+® software for the Apple Macintosh® (Abacus Concepts Inc., published by Brainpower Inc.). Separation of means was determined by Scheffe's F-test. For all tests, $P < 0.05$ was chosen as the level for statistical significance.

Results

Refrigerated storage of sperm in cryoprotectant solutions at 4°C reduced motility by as much as 100% compared with sperm stored in HBSS without cryoprotectant (Table 2). Reduction in motility was associated with increasing cryoprotectant concentration and with increasing time of storage. Some sperm stored as long as 180 d in methanol solutions retained motility.

The motility of sperm frozen and thawed in cryoprotectant solutions was reduced compared with sperm refrigerated in HBSS. No motility was observed among sperm frozen and thawed in HBSS without cryoprotectant (Table 3). Motile sperm were observed after thawing in every cryoprotectant, but 5% methanol yielded the largest percentage of motile sperm (5–10%). Some motility (<5%) was observed among sperm stored at 4°C in methanol solutions as long as 180 d after thawing.

Fertilization of channel catfish eggs by sperm frozen and thawed in 5%, 10%, or 15% methanol ranged from 24% to 97% and averaged 64% (N = 18) (Table 4). Fertilization of eggs by untreated sperm from the same males ranged from 62% to 100%, and averaged 82% (N = 6). There was no significant difference in fertilization success be-

TABLE 2.—Percent motility of channel catfish sperm, from two males, refrigerated at 4°C for as long as 180 d in Hanks' balanced salt solution (HBSS), or in HBSS containing cryoprotectants dissolved at different concentrations (PVP is polyvinylpyrrolidone).

Cryoprotectant	Concentration (%)	Percent motility (males denoted as 1 and 2) after: 6 h		20 h		30 h		14 d		180 d	
		1	2	1	2	1	2	1	2	1	2
HBSS		70	55	40	40	40	40	25	25	0	0
Methanol	5	60	30	50	20	50	20	0	30	0	<1
	10	50	20	50	20	50	30	20	30	0	<1
	15	35	5	30	5	50	30	20	30	0	<1
Glycerol	5	5	1	1	1	1	1	0	0	0	0
	10	5	1	1	<1	1	<1	1	<1	0	0
	15	1	1	1	<1	<1	<1	<1	<1	0	0
DMSO	5	60	25	50	25	30	30	<1	5	0	0
	10	5	5	5	5	5	5	<1	5	0	0
	15	5	1	1	<1	<1	<1	<1	<1	0	0
Sucrose	5	30	20	30	25	30	15	0	1	0	0
	10	15	5	5	1	<1	1	0	0	0	0
	15	5	1	0	<1	0	<1	0	<1	0	0
PVP	5	10	20	10	30	10	25	0	1	0	0
	10	20	5	15	5	15	5	0	1	0	0
	15	1	0	1	0	1	0	0	0	0	0

tween males ($P = 0.373$). There was no significant difference in fertilization success among untreated sperm and sperm cryopreserved in different concentrations of methanol ($P = 0.104$). Monthly weight through 1 year of age (Table 5) was not different between fish produced with frozen or untreated sperm ($P > 0.05$).

Discussion

To our knowledge, this is the first report of production of channel catfish by use of cryopreserved sperm. Previous studies of catfish sperm cryopreservation involved species of the families Clariidae and Pangasiidae. Sperm from the sharptooth cat-

TABLE 3.—Percent motility of channel catfish sperm stored frozen at −196°C for 2 d, then thawed and refrigerated at 4°C for as long as 180 d. Sperm were suspended in Hanks' balanced salt solution (HBSS), or in HBSS containing cryoprotectants dissolved at different concentrations (PVP is polyvinylpyrrolidone).

Cryoprotectant	Concentration (%)	Percent motility (males denoted as 1 and 2) after: 5 min		2 h		9 h		12 d		180 d	
		1	2	1	2	1	2	1	2	1	2
HBSS		0	0	0	0	0	0	0	0	0	0
Methanol	5	5	10	5	10	5	10	1	5	0	<5
	10	5	5	5	5	5	5	1	5	0	<5
	15	1	1	<1	<1	<1	<1	0	1	0	0
Glycerol	5	1	1	<1	0	0	0	0	0	0	0
	10	1	1	<1	0	<1	0	<1	0	0	0
	15	1	1	0	0	0	0	0	0	0	0
DMSO	5	5	1	5	1	1	1	<1	<1	0	0
	10	1	1	<1	<1	<1	<1	<1	<1	0	0
	15	0	1	<1	<1	<1	<1	0	<1	0	0
Sucrose	5	1	1	<1	<1	<1	<1	0	0	0	0
	10	1	1	<1	<1	<1	<1	0	0	0	0
	15	1	1	<1	<1	0	0	0	0	0	0
PVP	5	5	1	5	<1	0	<1	0	0	0	0
	10	1	1	<1	<1	<1	<1	0	0	0	0
	15	0	0	0	0	0	0	0	0	0	0

TABLE 4.—Percent fertilization (mean ± SD) of channel catfish eggs from three females by untreated sperm (control) and sperm cryopreserved with methanol at three concentrations. The sperm, from two males, were stored for 2 d at −196°C and thawed immediately prior to use. There was no significant difference in percent fertilization between males ($P = 0.373$) or among untreated sperm and sperm cryopreserved in different concentrations of methanol ($P = 0.104$).

Sperm source and measure	Cryopreserved in methanol at:			Untreated (control)
	5%	10%	15%	
Male 1				
Percent (actual)	72 ± 19	58 ± 29	46 ± 32	81 ± 19
Percent of control	89 ± 11	69 ± 19	53 ± 26	
Male 2				
Percent (actual)	71 ± 5	82 ± 17	52 ± 30	84 ± 5
Percent of control	85 ± 1	97 ± 24	64 ± 4	
Pooled values				
Percent (actual)	72 ± 12	70 ± 25	49 ± 28	82 ± 13
Percent of control	87 ± 7	83 ± 25	58 ± 31	

fish *Clarius gariepinus* were frozen in a computer-controlled freezer at a rate of −11°C/min (Steyn and Van Vuren 1987), and equal hatching percentage (51%) was observed for ova fertilized with cryopreserved and untreated sperm. Motility was not reported, but glycerol was more effective as a cryoprotectant than DMSO or methanol. In studies of the sharkfin catfish *Pangasius sutchi*, sperm were suspended above liquid nitrogen for 5–10 min (Withler 1982). Use of DMSO as cryoprotectant resulted in 1% motility and 1% fertilization of ova, compared with more than 90% fertilization by untreated sperm. In a study of the Mekong giant catfish *Pangasianodon gigas*, sperm were frozen in liquid nitrogen vapor with DMSO as the cryoprotectant (Mongkonpunya et al. 1992). Percent motility was not reported, but fertilization percentages with cryopreserved (68%) and untreated sperm (79%) were not significantly different.

Among ictalurid catfishes, sperm of channel catfish were frozen in double-layered, kapok-lined paper bags held above liquid nitrogen (Guest et al. 1976). Motility was assessed qualitatively. Although sperm frozen and thawed in DMSO outperformed sperm cryopreserved in glycerol or propylene glycol, few sperm were motile after thawing, whereas most sperm were motile in the untreated samples. The use of methanol was not reported in that study.

Other studies of fish have shown methanol to be an effective cryoprotectant for sperm cells (e.g., Harvey et al. 1982), although methanol was the least effective of three cryoprotectants tested with sharptooth catfish (Steyn and Van Vuren 1987). In our study, motility was reduced among sperm stored in each of the cryoprotectants at 4°C; however, the reduction was smallest for sperm stored in methanol solutions. Whether methanol exerted a superior cryoprotective action in relation to the

TABLE 5.—Monthly weights (means ± SD in grams) of offspring ($N = 25$/group) produced with cryopreserved sperm or untreated sperm from two channel catfish. No differences were observed at any age between offspring produced with cryopreserved or untreated sperm.

Age (months)	Male 1		Male 2	
	Cryopreserved sperm	Untreated sperm	Cryopreserved sperm	Untreated sperm
2	1.4 ± 0.5	1.4 ± 0.6	1.4 ± 0.6	1.4 ± 0.6
4[a]	9.4 ± 4.5	11.5 ± 5.3	7.8 ± 3.4	6.4 ± 2.8
5[a]	17.0 ± 10.2	15.7 ± 8.6	12.6 ± 7.7	12.3 ± 7.4
6	20.3 ± 11.0	18.2 ± 9.0	18.2 ± 9.6	16.7 ± 7.8
7	34.7 ± 18.2	26.9 ± 10.6	29.2 ± 16.3	31.9 ± 18.9
8	63.8 ± 30.3	51.4 ± 27.0	49.9 ± 22.0	48.7 ± 20.1
9	81.0 ± 37.1	75.3 ± 39.5	83.1 ± 44.3	81.9 ± 35.1
10	93.4 ± 44.9	88.1 ± 49.3	95.1 ± 48.0	85.5 ± 37.9
11	115.5 ± 53.3	101.7 ± 52.0	98.5 ± 44.2	104.0 ± 32.8
12	157.9 ± 86.0	114.6 ± 61.7	123.5 ± 59.4	127.1 ± 55.4

[a] Weights of offspring of male 1 were significantly greater than weights of offspring of male 2 ($P < 0.05$) at these ages.

other chemicals studied is unclear, because higher numbers of motile sperm prior to freezing could in part explain higher motility values following thawing. Longer time of equilibration before freezing (we used 15 min) could enhance the effectiveness of cryoprotectants that act more slowly than methanol.

Collection of channel catfish sperm involves destruction of the testis, so it is important to minimize the number of sperm used to fertilize eggs. In their study of sharptooth catfish, Steyn and Van Vuren (1987) emphasized the optimization of the milt–ova insemination ratio, and employed a ratio of about 49,000 live sperm for each egg. In our study, sperm were diluted to a concentration of about 5×10^7 per 0.5-mL straw. A motility of 1% would then yield about 5×10^5 motile sperm per straw and—given our use of aliquots of about 250 eggs—about 2,000 sperm with sustained motility for each egg. Accordingly, a 10-gram testis (providing 2.5×10^{10} sperm) would be sufficient to fertilize at least 100,000 eggs under those conditions. With improvement in cryopreservation procedures, a single male channel catfish could provide enough sperm to fertilize more than 1 million eggs.

Cryopreservation of sperm should be useful as a routine method of gamete storage and management for catfish. Thawed sperm stored at 4°C retained motility for days. This would allow refrigerated storage of large aliquots of thawed sperm and greater flexibility in the timing of induced spawning. Indeed, the presence of methanol in sperm solutions could provide antibacterial action and increased storage time. In addition, we have fertilized eggs with sperm cryopreserved in methanol (from this study) and stored for 13 months at −196°C prior to thawing (unpublished observation), suggesting that catfish sperm will remain viable after years of storage.

Sperm can be frozen in straws (Wheeler and Thorgaard 1991) or as pellets (Pursel and Johnson 1975) placed directly on dry ice (−80°C), a cheap and readily available alternative to liquid nitrogen. Because we froze sperm at −80°C prior to storage at −196°C, our findings should apply to use of dry ice as well as liquid nitrogen. The use of nitrogen gas to regulate the distribution of vapor from the liquid nitrogen allowed control of cryogenic conditions and provides an inexpensive method of freezing sperm at temperatures other than that of dry ice. Finally, channel catfish produced from frozen sperm grew at the same rate as siblings produced with untreated sperm. Thus, as was found for other fishes such as striped bass *Morone saxatilis* (Kerby et al. 1985), catfish produced with cryopreserved sperm should match the performance of catfish produced with untreated sperm.

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Appendix: Cryoprotectants and Solutions

Table A.1.—Chemical characteristics of cryoprotectants and solutions used for study of channel catfish sperm. All cryoprotectants were dissolved in Hanks' balanced salt solution (HBSS). The values for percent concentration and molarity were calculated; osmolality was measured for each solution.

Cryoprotectant	Molecular weight	Concentration (%)	Molarity	Osmolality (millios-mols/kg)
Methanol	32.04	5	1.56	268
		10	3.12	264
		15	4.69	256
Glycerol	92.09	5	0.54	987
		10	1.09	1,804
		15	1.63	2,570
Dimethyl sulfoxide	78.13	5	0.64	930
		10	1.28	1,568
		15	1.92	2,212
Sucrose[a]	342.3	5	0.15	432
		10	0.29	598
		15	0.44	776
Polyvinylpyrrolidone[a]	~10,000	5	0.005	331
		10	0.01	396
		15	0.015	483
HBSS				270

[a] Calculated as mass of cryoprotectant divided by volume of buffer.