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SPERM CRYOPRESERVATION OF AFRICAN CATFISH, Clarias gariepinus: CRYOPROTECTANTS, FREEZING RATES AND SPERM:EGG DILUTION RATIO

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ABSTRACT

Methods for cryopreserving spermatozoa and optimizing sperm:egg dilution ratio in African catfish Clarias gariepinus were developed. Five percent to 25% DMSO and methanol were tested as cryoprotectants, by diluting semen in Ginzburg fish ringer and freezing in 1-milliliter cryovials in a programmable freezer. To avoid an excess of spermatozoa per egg, post-thaw semen was diluted 1:20, 1:200 or 1:2000 before fertilization. Highest hatching rates were obtained by spermatozoa frozen in 10% methanol and post-thaw diluted to 1:200. Then, slow freezing rates (-2, -5 or -10°C/min) to various endpoint temperatures (range -25 to -70°C) before fast freezing in liquid nitrogen (LN_2) were evaluated. Hatching rates equal to control ($P>0.05$) were obtained by spermatozoa frozen at -5°C/min to -45 to -50°C and at -10°C/min to -55°C. In 3-step freezing programs, at -5°C/min, the effect of holding spermatozoa for 0, 2 or 5 min at -30, -35 or -40°C before fast freezing in LN_2 was analyzed. Hatching rates equal to control ($P>0.05$) were produced by spermatozoa frozen to, and held at, -35°C for 5 min and at -40°C for 2 or 5 min. Finally, frozen spermatozoa (10% methanol, -5°C/min, 5-min hold at -40°C, LN_2, post-thaw diluted to 1:200) were tested in on-farm fertilization conditions. Again, no difference ($P>0.05$) in hatching rate was observed between frozen and fresh spermatozoa. Cryopreservation offers utility as a routine method of sperm storage and management for catfish.

Key words: fish, African catfish, cryopreservation, sperm, freezing rate

INTRODUCTION

Catfishes are an economically important group of fresh and brackish water fish worldwide. Several species have been successfully introduced in aquaculture (22), and the African catfish, Clarias gariepinus (formerly C. lazera; 5), is perhaps the most important

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one, not only in Africa but also in S-E Asia (e.g., Thailand) and in Europe (e.g., The Netherlands).

The availability of gametes throughout the year is important to ensure a constant supply of fish. In captivity (25°C; 12 h light per day), C. gariepinus gametogenesis is continuous once sexual maturity is reached (8). However, whereas females can be stripped of eggs after treatments with carp pituitary extracts (cPE; 7) or human Chorionic Gonadotropin (hCG; 4), spermiation and male reproductive behavior do not take place spontaneously (26), even after hormonal therapy. To obtain spermatozoa it is necessary to kill male brood fish (17) or surgically remove part of their testes (1). Storing batches of spermatozoa by cryopreservation would significantly improve the reproductive potential of male catfish.

Cryopreservation of fish spermatozoa has been the subject of many investigations, especially in salmonids (e.g., 19). Successful cryopreservation depends not only on the right choice of cryoprotectant and extender, but also on the freezing protocol used. Cryoprotectant and freezing rate together determine the damage to spermatozoa due to intracellular ice crystallization (12). Research on sperm cryopreservation of African catfish and related species is summarized in Table 1. African catfish spermatozoa were first successfully cryopreserved by Steyn et al. (18), who obtained 40% motility 24 h after storage in liquid nitrogen (LN_2). Glucose in combination with glycerol has been the most widely used cryoprotective solution. Recently, glucose in combination with DMSO was also shown to be effective (24). Freezing rates can be rapid (e.g., pellet freezing on dry ice or in LN_2 vapor; 9,13,14) or slow (e.g., at fixed rates in a programmable freezer; 16,25). However, in most cases, sperm quality was only evaluated in terms of motility after thawing. When fertilization was included in the evaluation, sperm:egg ratios were not optimized and were often excessive (14). Using excess spermatozoa for fertilization obviously masks the quality of cryopreserved spermatozoa, making comparison of protocols difficult.

In the present paper we evaluate different combinations of cryoprotectant and freezing protocols using defined sperm:egg fertilization ratios, with the aim of developing a reliable protocol for catfish sperm cryopreservation under both laboratory and on-farm conditions.

MATERIALS AND METHODS

Husbandry of Brood Stock

The brood stock of the African catfish, C. gariepinus, originated from the Republic of Central Africa (4.22°N, 18.35°E), has been bred for several generations in the Hatchery of the Fish Culture and Fisheries Group at Wageningen University (52°N, 5.5°E), The Netherlands. Mature males and females were kept together under constant temperature (25°C) and photoperiod (14 h of light per day) in 250-L rectangular tanks connected to a recirculating system. The flow rate was 8 to 12 L/min. Fish were fed trout pellets (Trouvit, The Netherlands) at a maintenance level of 0.8% of body weight daily.

Table 1. Literature review on semen cryoconservation of African catfish and related species. The data presented shows recommended treatments based on the current literature.

Catfish species	Extender	Cryoprotectant	Container	Freezing rates	Hatching rates (%)	Spermatozoa per egg	Reference
Clarias gariepinus	5% glucose	5% glycerol	straw or bio-freeze vial	-7°C/min to -65°C; LN_2	not measured	not measured	18
	5% glucose	11% glycerol	1-mL cryo tube	-11°C/min to -70°C; LN_2.	frozen: 51% control: 51%	245×10^3	17
	4% glucose	9% glycerol	1-mL cryo tube	-5 and -11°C/min to -70°C; LN_2.	not measured	not measured	16
	4% glucose	9% glycerol	1-mL cryo tube	-5°C/min to -70°C; LN_2.	not measured	not measured	25
	333 mmol L^{-1} fructose	10% DMSO	250-µL straw	-11°C/min to -80°C; LN_2	not measured	not measured	24
Clarias batrachus	0.6% NaCl	10% glycerol	1.5-mL tube	directly to -70°C; stored at -70°C	75% of control	13.6×10^6	14
Heterobranchus longifilis	Mounib solution [a]	5% DMSO + 5% glycerol + 10% egg yolk	5-mL straw	20 min at 3cm above LN_2 level; LN_2	frozen: 79% control: 81%	50×10^3	13

[a] Mounib solution: (g/L) reduced glutathione 2.0; $KHCO_3$ 10.0; sucrose 42.0

Sperm Cryopreservation

Males (1 per experiment) weighing 850 to 2300 g were anesthetized with 8 g tricaine methanesulfonate (TMS; Crescent Research Chemicals, Phoenix, Arizona, USA) dissolved in 10 L tap water and sacrificed by spinal transection. Testes were removed by dissection and perforated with a needle, and semen was gently squeezed out. Motility was determined subjectively by mixing 1 drop of fresh semen with 2 drops of tap water and observed under a microscope at magnification 200x. Only samples with more than 80% motile spermatozoa were frozen.

The mean sperm concentration of 10 randomly chosen catfish males was determined by counting spermatozoa diluted 1:10,000 in a Bürker cell counter (Marienfeld, Germany).

Ginzburg fish ringer (123.2 mM NaCl; 3.75 mM KCl; 3.0 mM $CaCl_2$; 2.65 mM $NaHCO_3$; pH 7.6; 244 mOsm) was used as extender and mixed with methanol or dimethylsulphoxide (DMSO; Merck, Germany) at various concentrations in a 10-milliliter (mL) tube. Semen was gently added at 1:10 (v/v) final concentration. Aliquots of 500 microliter (µL) of diluted spermatozoa (containing 50 µL semen) were immediately transferred to 1-mL cryovials (Greiner Labortechnik; Düsseldorf, Germany) and frozen. The equilibration time between mixing spermatozoa with cryoprotective solution and freezing was approximately 2 min at 5°C.

For each treatment (i.e., a specific combination of cryoprotective agent, cryoprotectant concentration, freezing rate, endpoint temperature in the first step of freezing and holding time at the endpoint), 6 cryovials (replicates) were frozen in a programmable freezer (Planer Kryo 10 series 3 Controlled Rate Freezer; Middlesex, UK) and stored in LN_2 at -195°C for 1 to 3 wk before being tested in fertilization trials.

Artificial Insemination and Hatching of Eggs

Female catfish, 1 per experiment, were injected with 4 mg cPE/kg body weight and stripped 12 h later (25°C). Eggs were kept at room temperature (23°C) while being used and for a maximum of 2 h. Three cryovials per treatment were thawed in a water bath at 27°C for 5 min. The remaining 3 cryovials were kept as backup samples. After thawing, spermatozoa from each cryovial (previously diluted 1:10) were diluted again with fresh fish ringer to a final concentration of 1:20 (in Experiment 1A and 1C), 1:200 (in all experiments except 1A) or 1:2000 (in Experiment 1C).

From each sample of post-thaw diluted spermatozoa, 2 aliquots of 100 µL were mixed with 2 portions of 0.2 g of fresh eggs (approximately 150 eggs) in a plastic Petri dish. Fertilization was initiated by adding 1 mL tap water and mixing for 40 sec. The eggs were then transferred to a 10-cm diameter PVC basket with a 0.5-mm mesh bottom and incubated in shallow trays at 30°C, connected to a recirculating system. Every 30 min and at the end of each fertilization trial (that lasted for a maximum of 2 h), control egg batches were inseminated with fresh spermatozoa, diluted in fish ringer to the same ratio as the

frozen-thawed spermatozoa (i.e., 1:20, 1:200 or 1:2000, depending on the experiment) to check for changes in egg quality.

Cryoprotectant Agent, Concentration and Final Sperm Dilution Ratio

In this section, 3 experiments were conducted. In the first experiment (1A), spermatozoa was frozen in 5, 10, 15, 20 or 25% methanol or DMSO, and diluted post-thaw to 1:20 (v/v final concentration). Based on the results of this experiment, the same design, except for the cryoprotectant concentration of 25%, was repeated in Experiment 1B. However, spermatozoa were diluted post-thaw to 1:200. In Experiment 1C, spermatozoa were frozen only in 10 or 15% methanol or DMSO and diluted post-thaw to 1:20, 1:200 or 1:2000. In all 3 experiments, spermatozoa were frozen at -5°C/min from +5 to -50°C, stored in LN_2, and tested in fertilization trials.

Freezing Rates and Endpoint Temperatures in 2-Step Freezing Programs

Four 2-step freezing programs were designed to define the best freezing rate and endpoint temperature. After reaching the target endpoint (first step), sperm samples were directly transferred to LN_2 (second step). In Experiment 2A, spermatozoa were frozen at -5°C/min (the same rate used in the first series of experiments) from +5 to -35, -50 or -70°C. Based on these results, in Experiment 2B the same freezing rate was used, but more endpoints were tested: -40, -45, -50, -55, -60 or -65°C. In Experiments 2C and 2D, a slower and a faster rate (-2 and -10°C/min, respectively) were used, and spermatozoa were frozen from +5 to -25, -30, -35, -40, -45, -50, -55 or -60°C. All sperm samples were frozen in 10% methanol and diluted post-thaw to 1:200 (v/v final concentration).

Holding Spermatozoa at Supraoptimal Endpoints in 3-Step Freezing Programs

Based on results of the previous experiment, we have hypothesized that holding sperm samples at endpoint temperatures higher than the optimal ones (supraoptimal temperatures) for a few minutes before fast freezing in LN_2 would increase sperm survival. To Test this hypothesis, spermatozoa was frozen at -5°C/min from +5 to -30, -35 or -40°C and either transferred immediately to LN_2 (0-min hold) or held at the endpoint temperature for 2 or 5 min (second step) before being transferred to LN_2 (third step). All sperm samples were frozen in 10% methanol and diluted post-thaw to 1:200 (v/v final concentration).

Cryopreserved Spermatozoa in On-Farm Conditions

Semen was obtained after surgical removal of a part of the testis from 3 adult male catfish (4-5 years old and about 10 kg) from farm broodstock (Ir. Fleuren, Someren, The Netherlands). Spermatozoa were frozen according to one of our best protocols (10% methanol, at -5°C/min from +5 to -40°C and held for 5 min before storing in LN_2) and diluted post-thaw 1:200 (v/v final concentration). Ten mL diluted post-thaw spermatozoa (50 µL semen, 1 cryovial) were mixed with 20 g eggs (3 cryovials per male). After fertilization, 2 aliquots of 0.2 g eggs from each sample of 20 g were incubated separately in 2 mesh-bottom baskets suspended in an aquarium to determine the hatching rate. The

remaining 19.6 g were incubated in another tank, but in the same recirculating system as the other samples, at 30°C.

Statistical Analysis

The number of hatched larvae, as a percentage of total eggs exposed to spermatozoa, was calculated 24 h after fertilization. For cryopreserved spermatozoa, data from 6 replicates per treatment (2 samples of 0.2 g egg per vial and 3 vials per treatment) were pooled to calculate mean and standard deviation. For fresh spermatozoa, hatching rate data from fertilization every 30 min and at the end of each trial were pooled to calculate mean and standard deviation. All statistical analyses were done using the SAS 6.11 package (SAS Institute Inc., 1990). Hatching rates from fresh and cryopreserved spermatozoa per fertilization trial were tested for significant differences by ANOVA using the parametric General Linear Model procedure, followed by Duncan's Multiple Range Test. The residues from the different ANOVA models were tested for normal distribution using the univariate procedure. P-values <0.05 were considered to be significant.

RESULTS

The mean sperm concentration, counted in 10 randomly chosen males, was 3.4×10^9 (SD 2.0×10^9; range 1.8 to 7.2×10^9) spermatozoa per mL. From this value, sperm concentration per vial was estimated as 17×10^7 spermatozoa in 0.5 mL solution and the insemination ratio, as 113×10^3 at 1:20, 11.3×10^3 at 1:200, and 1.13×10^3 spermatozoa per egg at 1:2000 final sperm dilution.

Hatching rates of control groups, in the laboratory conditions, ranged from 55.7 to 94.3%. The hatching rate in on-farm conditions was 49.6%.

Cryoprotectant Agent, Concentration and Final Sperm Dilution Ratio

In Experiment 1A, all samples, except those frozen in 25% methanol, protected by either methanol or DMSO and diluted post-thaw to 1:20 v/v final dilution, produced hatching rates not significantly different ($P>0.05$) from the control. However, when spermatozoa was diluted post-thaw to 1:200 v/v final dilution (Experiment 1B), only samples frozen in 10% methanol produced hatching rates (77.8%) not significantly different from control (85.2%). A significant ($P<0.05$) decrease in hatching rate was observed in samples frozen in 5, 15 or 20% methanol and in 5 to 20% DMSO (Table 2).

When spermatozoa were diluted post-thaw to 1:20 v/v final dilution in Experiment 1C, different results from Experiment 1A were obtained. Only samples frozen in 10% methanol produced hatching rates (60.0%) similar to control spermatozoa (65.9%). Samples frozen in 15% methanol and 10 and 15% DMSO produced lower hatching rates ($P<0.05$) compared with control (Table 3). At 1:200 post-thaw sperm dilution ratio, 10% methanol was again the most effective in protecting spermatozoa against freezing, maintaining the same ($P>0.05$) hatching rate (58.3%) as control (55.7%). At 1:2000 v/v final dilution, none of the samples tested produced satisfactory results (Table 3).

Table 2. Hatching rates (mean ± SD, n=6) as percentage of total eggs in contact with spermatozoa frozen in DMSO or methanol at different concentrations and diluted post-thaw to 1:20 (Experiment 1A) or 1:200 v/v final dilution (Experiment 1B).

Cryoprotectant	concentration (%)	sperm dilution ratio (v/v) 1:20	1:200
Methanol	5	81.5 ± 6.1 [a]	40.8 ± 14.6 [bc]
	10	82.3 ± 6.2 [a]	77.8 ± 10.5 [a]
	15	81.3 ± 5.1 [a]	38.9 ± 19.8 [c]
	20	75.0 ± 8.0 [a]	6.2 ± 7.1 [e]
	25	0 [b]	
DMSO	5	82.1 ± 3.7 [a]	55.5 ± 22.1 [b]
	10	73.9 ± 3.9 [a]	38.1 ± 28.4 [c]
	15	85.7 ± 5.1 [a]	24.1 ± 14.0 [d]
	20	81.6 ± 8.7 [a]	28.6 ± 21.6 [cd]
	25	81.4 ± 6.8 [a]	
Control		77.4 ± 3.2 [a]	85.2 ± 12.6 [a]

[a-d]Means within the same column with different superscripts are significantly different ($P<0.05$).

Table 3. Hatching rates (mean ± SD, n=6) as percentage of total eggs in contact with spermatozoa frozen in 10 or 15% methanol or DMSO and diluted post-thaw to 1:20, 1:200 or 1:2000 (final dilution; Experiment 1C).

Cryoprotectant	concentration (%)	Final sperm dilution ratio (v/v) 1:20	1:200	1:2000
Methanol	10	60.0 ± 8.8 [ab]	58.3 ± 6.6 [a]	29.3 ± 22.4 [b]
	15	52.0 ± 2.5 [bc]	43.6 ± 12.9 [b]	17.5 ± 9.0 [b]
DMSO	10	48.3 ± 6.0 [c]	9.5 ± 5.1 [c]	1.5 ± 1.1 [c]
	15	38.1 ± 5.3 [d]	15.8 ± 3.7 [c]	0.3 ± 0.4 [c]
Control		65.9 ± 14.7 [a]	55.7 ± 12.8 [a]	58.1 ± 16.3 [a]

[a-d] Means within the same column with different superscripts are significantly different ($P<0.05$).

Freezing Rates and Endpoint Temperatures in 2-Step Freezing Programs

In Experiment 2A, when a freezing rate of -5°C/min was used, spermatozoa frozen to -35°C (first step endpoint) then plunged into LN_2 produced a hatching rate of 0%. However, when samples were frozen to -50°C, the hatching rates (95.6%) were similar

(P>0.05) to control (94.3%). A deeper endpoint temperature, -70°C, had a negative effect on hatching, producing rates (43%) significantly lower (P<0.05) than the control (Table 4).

Table 4. Hatching rates (mean ± SD, n=6) as percentage of total eggs in contact with spermatozoa frozen at -2, -5 or -10°C/min to different endpoints in the first step of freezing and stored in liquid nitrogen (second step) for 1 to 3 wk (Experiments 2A to 2D).

First step endpoints (°C)	Freezing rates (°C/min)			
	-2	-5	-5	-10
-25	0 ^{d}	N	N	0 ^{d}
-30	0 ^{d}	N	N	0 ^{d}
-35	2.7 ± 1.1 ^{d}	0 ^{c}	N	0 ^{d}
-40	40.7 ± 4.8 ^{b}	N	3.9 ± 2.0 ^{d}	0 ^{d}
-45	20.6 ± 10.3 ^{c}	N	77.2 ± 3.3 ^{a}	1.5 ± 2.2 ^{d}
-50	11.2 ± 5.3 ^{cd}	95.6 ± 3.9 ^{a}	66.2 ± 12.1 ^{a}	28.7 ± 28.5 ^{c}
-55	8.3 ± 3.4 ^{cd}	N	41.1 ± 14.4 ^{b}	51.8 ± 9.7 ^{ab}
-60	5.6 ± 3.5 ^{cd}	N	25.5 ± 18.1 ^{bc}	38.6 ± 16.1 ^{bc}
-65	N	N	10.3 ± 1.7 ^{c}	N
-70	N	43.0 ± 18.0 ^{b}	N	N
Control	72.5 ± 7.1 ^{a}	94.3 ± 6.4 ^{a}	79.9 ± 15.5 ^{a}	59.8 ± 10.7 ^{a}

N = endpoint not tested within a given freezing rate.
$^{a-d}$Means within the same column with different superscripts are significantly different (P<0.05).

In Experiment 2B, when more endpoint temperatures were tested with the same freezing rate of -5°C/min, spermatozoa frozen to -40°C and then plunged into LN_2 produced a very low hatching rate of only 3.9% (Table 4). However, samples frozen to either -45 or -50°C produced hatching rates equal (P>0.05) to control (77.2 and 66.2% vs. 79.9% of control). Endpoint temperatures of -55°C or lower had a negative effect on hatching rate.

At a rate of -2°C/min (Experiment 2C), spermatozoa frozen to -25 or -30°C (first step endpoint) and then plunged into LN_2 produced a hatching rate of 0%. When spermatozoa were frozen to -35°C, less than 3% of eggs hatched (Table 4). Maximum hatching rates were observed when samples were frozen to -40°C (40.7%), although this was significantly (P<0.05) lower than control (72.5%). Endpoint temperatures of -45°C or lower had a negative effect on hatching rate.

At a faster freezing rate of -10°C/min (Experiment 2D), spermatozoa frozen to -25, -30, -35 or -40°C and then plunged into LN_2 produced a hatching rate of 0%. When spermatozoa were frozen to -45°C, only 1.5% of eggs hatched (Table 4). Hatching rates

equal (P>0.05) to control (59.8%) were observed only when spermatozoa were frozen to -55°C (51.8%). Endpoint temperatures of -60°C or lower had a negative effect on hatching rate.

Holding Spermatozoa at Supraoptimal Endpoints in 3-Step Freezing Programs

No egg hatched when fertilized with spermatozoa frozen at -5°C/min to either -30°C at all holding times tested or to -35°C with 0-min holding time (Table 5). However, hatching rates as high (P>0.05) as control (85.9%) were produced with spermatozoa frozen to, and held at, -35°C for 5 min (85.1%) and -40°C for either 2 min (78.4%) or 5 min (86.8%).

Table 5. Hatching rates (mean ± SD, n=6) as percentage of total eggs in contact with spermatozoa frozen at -5°C/min to supraoptimal endpoint temperatures in the first step of freezing, with varying holding times (second step) and stored in liquid nitrogen (third step) for 1 to 3 wk (Experiment 3).

First step endpoints	Holding time, in min (second step)		
(°C)	0	2	5
- 30	0^{b}	0^{b}	0^{b}
- 35	0^{b}	4.3 ± 4.3^{b}	85.1 ± 0.9^{a}
- 40	0.8 ± 1.2^{b}	78.4 ± 2.5^{a}	86.8 ± 3.6^{a}
Control	85.9 ± 3.5^{a}		

[a,b]Means with different superscripts are significantly different (P<0.05).

Table 6. Hatching rates (mean ± SD, n=6) as percentage of total eggs in contact with spermatozoa frozen in 10% methanol at -5°C/min to, and held at, -40°C for 5 min, stored in liquid nitrogen for 1 to 3 wk and tested in on-farm fertilization conditions (Experiment 4).

Spermatozoa		Hatching rates (%)
Frozen	Male # 1	57.7 ± 2.9^{a}
	Male # 2	53.9 ± 5.0^{ab}
	Male # 3	51.8 ± 3.1^{ab}
	Average	54.6 ± 4.3^{ab}
Control		49.6 ± 1.6^{b}

[a,b]Means with different superscripts are significantly different (P<0.05).

Cryopreserved Spermatozoa in On-Farm Conditions

No significant difference in hatching rate was observed when eggs were fertilized with either fresh or frozen-thawed spermatozoa tested on a commercial scale (Table 6). However, when data from frozen spermatozoa were separated by male, significantly higher ($P<0.05$) hatching rates (57.7%) compared with control (49.6%) were produced by male # 1.

DISCUSSION

Sperm Dilution Ratio

To avoid such high sperm:egg ratios that even frozen-thawed spermatozoa with low numbers of live cells could yield [illegible] to hatching rates, the maximum sperm dilution ratio to achieve hatching rates s[illegible] to control was investigated (Experiment 1C). Spermatozoa could be frozen and diluted as much as 200 times without losing fertilization ability. However, at 1:2000 the hatching [illegible] produced with frozen spermatozoa were lower than the control (Table 3). These dilution ratios are higher than those reported in the literature. African catfish, Heterobranchus longifilis, spermatozoa were diluted 1:3 before freezing and 1:10 after thawing, and had the same fertilization ability (78.9%) as the control (81.1%; 13). In Cyprinus carpio, however, no spermatozoa survived when diluted higher than 1:5 before or after freezing (10).

Sperm:Egg Ratio

As sperm collection in African catfish, as well as in channel and Asian catfish, involves testis destruction or male death, it is important to maximize the use of a single male by optimization of sperm:egg insemination ratio. For fresh spermatozoa, the effective insemination ratio was estimated as 15×10^3 spermatozoa per egg in C. gariepinus (15) and as 4 to 8×10^3 spermatozoa per egg in C. macrocephalus (21). Because a percentage of spermatozoa die during freezing and thawing processes, the effective insemination ratio for frozen spermatozoa should be higher. In channel catfish, 50×10^6 frozen-thawed spermatozoa per 0.5-mL straw enabled fertilization of 250 eggs (200×10^3 spermatozoa per egg; 23). In blue catfish I. furcatus, a minimum of $13{,}000 \times 10^3$ frozen-thawed spermatozoa per egg were needed to achieve 54% of control fertilization (2). In C. gariepinus (17), 49×10^3 live frozen-thawed spermatozoa per egg achieved a hatching rate (51.2%) equal to the control (51%). In our experiments, mean fresh sperm concentration was 3.4×10^9 per mL, while only a small aliquot of 100 µL of frozen-thawed spermatozoa diluted to 1:200 ($1{,}700 \times 10^3$ spermatozoa) enabled fertilization of about 150 eggs. In our field trial, 10 mL of post-thaw diluted (1:200) spermatozoa (50 µL semen) were able to fertilize 20 g (15,000) eggs. The insemination ratio in both situations was within the range 6 to 24×10^3 spermatozoa per egg, which is comparable to the ratio tested by Steyn and van Vuren (17). Based on the present data, 5 mL of semen from a single male, frozen according to our protocol, should be enough to fertilize 1,500,000 eggs (2 kg) and produce 750,000 larvae.

Cryoprotectants

At 1:20 final sperm dilution (Experiment 1A; Table 2), it was possible to determine neither the best cryoprotectant nor the most effective cryoprotectant concentration to protecting spermatozoa against freezing. All treatments, except 25% methanol, reached hatching rates comparable with control. However, when this dilution ratio was used again in Experiment 1C, only spermatozoa frozen in 10% methanol produced high hatching rates (Table 3). It is possible that the explanation for these contrasting results reflects differences in sperm quality (better in Experiment 1A than in Experiment 1C) and not in egg quality, because fertilization with fresh spermatozoa produced high hatching rates in both experiments.

Using 1:200 as the final sperm dilution, 10% methanol was the most effective cryoprotectant for C. gariepinus spermatozoa compared with methanol at other concentrations and DMSO at all concentrations tested, in both Experiment 1B and 1C. Methanol also proved effective for freezing zebra fish spermatozoa (6) and was a better cryoprotectant for channel catfish, Ictalurus punctatus, spermatozoa than DMSO, glycerol, sucrose, polyvinylpyrrolidone (23) and n,n-dimethyl acetamide (3). In contrast, it was less effective (18.9% hatching rate) than glycerol (51.2%) and DMSO (40.5%) for protecting C. gariepinus spermatozoa against freezing (17). Glycerol has been the most widely used cryoprotectant for African catfish spermatozoa (Table 1). Ten percent glycerol also proved effective for freezing Asian catfish, Heteropneustes fossilis and C. batrachus, spermatozoa, yielding 69 to 84% of control hatching rates (14). However, glycerol was toxic to salmonid spermatozoa, whereas DMSO could be used for cryopreservation (20).

According to Tiersch et al. (23), a longer time of equilibration before freezing can enhance the effectiveness of cryoprotectants that act more slowly than methanol. This could explain why DMSO was less effective than methanol in that the equilibration time used in the present experiments was only 2 min.

Freezing Rates and Endpoints

According to Mazur (11), when any kind of cell is subjected to subzero temperatures, it initially supercools while ice forms in the external medium. The manner in which cells regain equilibrium with medium depends chiefly on the rate at which they are cooled and on their permeability to water. If cells are cooled slowly or if their permeability to water is high, cells will equilibrate by transferring intracellular water to external ice (dehydration and shrinkage). On the other hand, if cells are cooled rapidly, or if their permeability to water is low, or even if cells are stored in LN_2 before nucleation is completed, cells will equilibrate, at least in part, by intracellular freezing.

In our experiments, sperm survival was variable among different freezing rates, even within the same endpoint. For instance, when the endpoint was -50°C, hatching rates similar ($P>0.05$) to control were produced only by spermatozoa frozen at -5°C/min (Experiment 2A and 2B; Table 4); at -2 (Experiment 2C) and -10°C/min (Experiment 2D), hatching rates were lower. However, it is easy to distinguish, for each freezing rate, an

optimal temperature endpoint to which spermatozoa must be frozen slowly, before fast freezing in LN_2 as that where highest sperm survival are obtained. At -2°C/min, this optimal endpoint was -40°C; at -5°C/min, both -45 and -50°C and at -10°C/min, -55°C. When sperm samples were plunged into LN_2 before these optimal endpoints and used to fertilize eggs, the hatching rates produced were, in most cases, very low (Table 4). Based on Mazur's conclusion (11), we assume that dehydration was not completed and intracellular ice was formed in those samples. The ice formed inside the cells as a result of rapid cooling is likely to grow by recrystallization during warming, especially if warming is slow, as in our experiments. However, at -5°C/min, the optimal endpoint could be increased to supraoptimal temperatures like either -35 or -40°C when a 5-min holding time was included before fast freezing (Experiment 3; Table 5). Perhaps, during the holding time, sperm cells could dehydrate enough and not form intracellular ice, since hatching rates produced by spermatozoa frozen as described were similar ($P>0.05$) to those of fresh spermatozoa. However, this hypothesis requires further testing.

Regardless of whether cells equilibrate by dehydration or by intracellular freezing, they are subjected to a second class of physical-chemical events associated with the removal of liquid water and its conversion to ice. As temperature decreases, the amount of cell water decreases, extracellular and intracellular solutes concentrate, solutes precipitate as their solubilities are exceeded (thus changing pH), and all solutes precipitate below the eutectic point (the temperature at which the solution starts freezing) of the system. These physical-chemical events are referred to as "solution effects" (11,27). Using an optimal freezing rate, once intracellular nucleation temperature has been passed, cells can be safely stored in LN_2 at -196°C. If not, cells will be exposed for too long to the "solution effects." This phenomenon could explain our results in the second series of experiments, when spermatozoa frozen further than the optimal endpoint (-40°C a -2°C/min; -45/50°C at -5°C/min; -55°C at -10°C/min) before fast freezing in LN_2 produced decreasing hatching rates (Table 4). As far as we know, several freezing rates have been tested in African catfish spermatozoa, but no other endpoint in the first step of freezing but -65, -70 and -80°C were evaluated (Table 1). It is possible that the nucleation point had been surpassed and better hatching rates could have been obtained at other temperatures.

Solution effects are responsible for injury when cooling is slower than optimal, and intracellular freezing is responsible for injury when cooling is faster than optimal. The optimal rate, then, is slow enough to prevent production of intracellular ice and yet rapid enough to minimize the length of time cells are exposed to solution effects (11). The critical rate varies among different types of cells. Yeast, for example, will contain intracellular ice when cooled faster than -10°C/min, whereas red blood cells will contain intracellular ice only when cooled faster than -5000°C/min (11). In our experiments with catfish spermatozoa, 3 slow rates were tested in the first step of freezing (-2, -5 and -10°C/min), followed by plunging samples in LN_2 (second step of freezing). Because better results were obtained at -5°C/min (Table 4), we suggest it as the best freezing rate for Clarias gariepinus spermatozoa. Our results agree with Steyn (16), who tested several freezing rates between -2 and -17°C/min and found better motility rates at -5°C/min.

Cryopreservation of spermatozoa should be useful as a routine method of gamete storage and management for catfish. African catfish, C. gariepinus, spermatozoa stored at -196°C retained good fertility for 6 wk in our experiments (unpublished data) and for 16 mo in the experiments of Steyn and van Vuren (17). Frozen African catfish, H. longifilis, spermatozoa had the same fertilizing capacity as fresh spermatozoa after 8 mo of storage (13) and channel catfish spermatozoa, after 13 mo of storage (23). These data suggest that catfish spermatozoa will remain viable for years of storage and will allow greater flexibility in timing of induced spawning.

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