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Cryopreservation of Mekong Giant Catfish Sperm

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Abstract

Sperm of Mekong giant catfish (*Pangasius gigas*; formerly *Pangasianodon gigas*) were cryopreserved in 5 or 9% dimethyl sulfoxide (DMSO) in bicarbonate buffer (BCB) or calcium-free Hanks' balanced salt solution (C-F HBSS) at different cooling rates (-5, -12, -22 or -120 °C·min^{-1}) and stored in liquid nitrogen for 3-4 months. Pooled ova of three female Gunther's walking catfish (*Clarias macrocephalus*) were inseminated with cryopreserved semen of Mekong giant catfish and fresh semen of the walking catfish (control). There was no significant difference in fertility of sperm cryopreserved in the two extenders. Best results were obtained when sperm were cryopreserved in 9% DMSO in either extender in 5 ml cryotubes and frozen at -12 °C·min^{-1}. The percentage of fertilization was 65-66% (actual) and 73-74% (control).

Introduction

Catfishes of the family Pangasiidae are important to commercial fisheries and aquaculture throughout Asia. Taxonomy of this family has been reorganized recently into two genera comprising 21 species (Roberts and Vidthayanon 1991). The Mekong giant catfish, or pla buk (*Pangasius gigas*; formerly *Pangasianodon gigas*), is of considerable cultural importance in Thailand and Lao PDR, and has been the object of a traditional commercial fishery. Declining harvests of pla buk suggested that the species might be in danger of extinction, and the Thai Fisheries Department initiated an artificial breeding program of this species in 1983.

Cryopreservation of sperm has been used to enchance the artificial breeding of *P. gigas*, and was used to produce hybrids with striped catfish (*P. hypophthalmus*) by Mongkonpunya et al. (1992). Fertilization success using cryopreserved fish semen is highly varied (Stoss 1983; Mongkonpunya et al. 1992) and until more consistent procedures are developed, cryopreservation is unlikely to be accepted for routine application by production hatcheries.

After exposure to cryoprotectant molecules in an extender solution, and freezing and thawing, the performance of sperm can be affected significantly by a series of factors including: 1) osmotic pressure; 2) toxicity of the cryoprotectant, which depends on equilibration time and concentration; 3) rates of freezing and thawing; and 4) post-thawing application.

Published methods for cryopreservation of fish sperm are similar, although not standardized: the semen is diluted with an extender, and a cryoprotectant is added to protect against freezing and thawing damage. Freezing methods differ to an unknown extent, because semen is frozen in a variety of containers such as vials or plastic straws which differ in configuration. Rapid thawing techniques are generally used before addition of semen to eggs.

The objectives of the present paper were to examine the effect of: 1) concentration of the cryoprotectants, dimethyl sulfoxide (DMSO) and methanol; 2) two different extenders; and 3) four different freezing rates (and two tube sizes) on fertilizing capability of cryopreserved sperm of Mekong giant catfish.

Materials and Methods

Experimental Animals

The Mekong giant catfish is one of the largest catfishes in the world (~200 kg live weight) and is considered a rare animal species. Gametes of this species are difficult to obtain, and are available only one month (in May) each year. In the 1994 spawning season, sperm from two males of *P. gigas* (designated as P_1 and P_2) became available, and 0.5-1.0 ml of semen from each fish was aspirated into plastic tubing via the genital pore. These samples were used for study of sperm activation (Mongkonpunya et al., unpubl. data), and chilled storage in this study. Another male (designated as P_3) was killed by fishers, and a testicular sample (~200 g) was taken and used in the cryopreservation study.

Gunther's walking catfish, *Clarias macrocephalus*, of a domesticated pond-raised stock, provided eggs and fresh sperm used in fertilization tests.

Preparation of Cryopreserved Sperm

About an hour elapsed between collection of sperm and the last step of the cryopreservation procedure (i.e., storage in liquid nitrogen). Although *P. gigas* semen can be collected by abdominal stripping, the procedure requires five to six persons and is difficult because of the large size of the fish. This was our first experience in using sperm samples derived from testicular tissue.

The steps used in preparing the sperm for cryopreservation were as follows:

1. The distal testicular lobules were removed, placed into separate plastic bags, weighed and labelled according to the extenders used.
2. The testicular samples were crushed in either Hanks' balanced salt solution (HBSS); calcium-free HBSS (C-F HBSS) or bicarbonate buffer (BCB) (Table 1). The ratio of the testis to the extender was 1:1 ($g \cdot ml^{-1}$) at this step.
3. The homogenized samples were filtered through nylon netting of 0.5-mm mesh.
4. For addition of cryoprotectant, the filtrates were diluted with two equal portions of the extender to bring the final ratio of testis to extender to 1:10 ($g \cdot ml^{-1}$): one portion of the extender contained no DMSO, while the other contained sufficient DMSO to produce a final concentration of either 5 or 9% (V/V) in each of the extenders.
5. The diluted samples with cell density of 4.2×10^9 $sperm \cdot ml^{-1}$ were mixed thoroughly and placed into cryotubes of 2- or 5-ml capacity. Each tube was filled to about 90% capacity.
6. The equilibration time, between addition of DMSO and the initiation of freezing, was set at 10-15 minutes to maximize the cryoprotection of DMSO while minimizing toxic effects on sperm.
7. Following equilibration, the cryotubes were placed on canes in a canister suspended in a liquid nitrogen storage tank (35 HC, Taylor Wharton, Indianapolis, USA) (Fig. 1). The lower tube was in contact with the surface of the liquid nitrogen; the upper tube was in liquid nitrogen vapor only. The average freezing rates (Fig. 2) were measured with type-T thermocouple (positioned at the middle of the tube) and a digital thermometer (Atkins Technical Inc., Gainesville, Florida, USA) (Fig. 3). The freezing rate observed for the upper 2-ml tube was slower than the rate observed for the upper 5-ml tube (despite the smaller volume), because the 2-ml tube occupied a higher position on the cane. After the temperature in the upper tube (the slow freezing rate) reached -80°C, the canister was plunged into liquid nitrogen and stored until utilization in fertilization tests.

Table 1. Ingredients (G/l) of full-strength Hanks' balanced salt solution (HBSS) and calcium-free Hanks' balanced salt solution (C-F HBSS), and bicarbonate buffer (BCB) used to dilute sperm of *Pangasius gigas*. Each ml of the extender also contained 500 IU penicillin and 1 mg streptomycin.

Ingredient	HBSS	C-F HBSS	BCB
$CaCl_2 \cdot H_2O$	0.16	-	-
NaCl	8.00	8.89	-
KCl	0.40	0.44	-
$MgSO_4 \cdot 7H_2O$	0.20	0.22	-
$Na_2HPO_4 \cdot 7H_2O$	0.12	0.13	-
KH_2PO_4	0.06	0.07	-
$NaHCO_3$	0.35	0.39	-
Glucose	1.00	1.11	-
$KHCO_3$	-	-	12.50
Sucrose	-	-	85.50
Glutathione, reduced	-	-	3.00
Osmotic pressure ($mOsm \cdot kg^{-1}$)	286	320	560

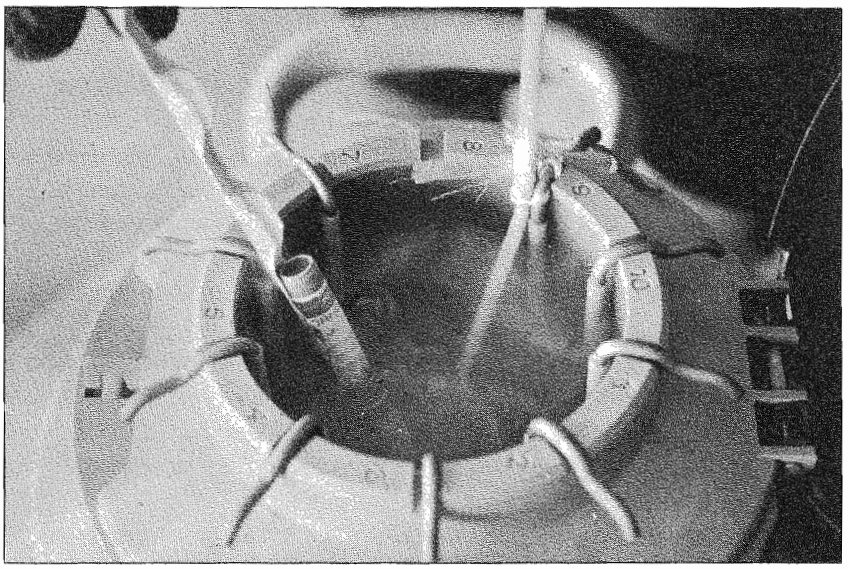

Fig. 1. Freezing procedure for cryopreservation of *P. gigas* sperm. Extended sperm was pipetted into cryotubes attached to aluminum canes. For freezing, the canes were placed into a canister suspended above the liquid nitrogen. Tubes in the lowest position on the cane were in contact with the surface of the liquid nitrogen and frozen at the fastest rate. After reaching -80°C, the canister was plunged into liquid nitrogen for storage.

Thawing Procedures

Frozen sperm was thawed rapidly by plunging the cryotubes into a 4-l water bath at 50°C for 2 minutes (2-ml cryotubes) or 3 minutes (5-ml cryotubes).

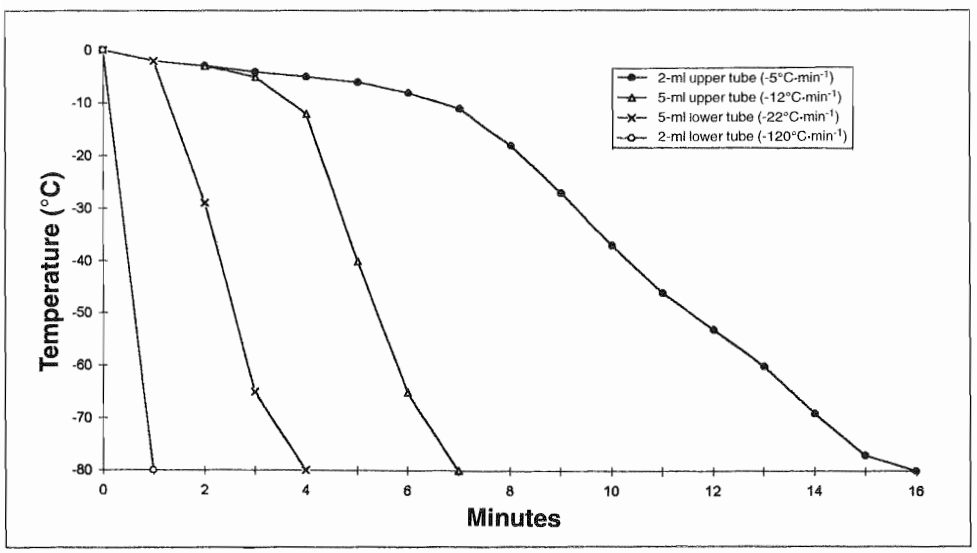

Fig. 2. Freezing rates measured with a type-T thermocouple and a digital thermometer.

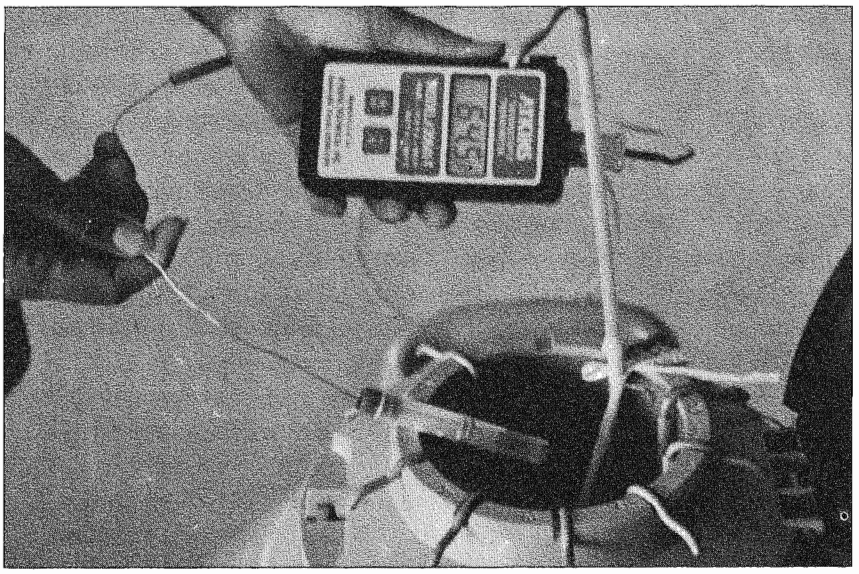

Fig. 3. To record freezing rates, a type-T thermocouple was inserted through the cap of a cryotube (2 or 5 ml) filled with extender (Hank's balanced salt solution). The cryotube was attached to specific positions on an aluminum cane and placed in a suspended canister for freezing. Temperature was monitored with a digital thermometer.

Sperm Density and Motility Trials

Sperm density was estimated by using a conventional hemocytometer counting chamber. Sperm motility was determined for undiluted semen, sperm refrigerated at 5-6°C with or without cryoprotectant, and for sperm frozen and thawed for fertilization tests. Motility estimates were made visually with brightfield microscopy at 100 x magnification. The addition of a 20-fold excess of distilled water was used to activate a 1-2 µl sperm sample on a glass slide. Motility was estimated within 5-10 seconds of activation and was recorded in two categories: 1) mass movement in swirling fashion was classified as: fast, medium, slow or none; and 2) percent motile sperm, as characterized by vigorous swimming motions in a forward direction (Mongkonpunya et al., unpubl. data).

Acute Toxicity Trials

Fresh semen of *P. gigas* was diluted at the ratio of 1:10 (V/V) in C-F HBSS containing 5 or 14% DMSO or methanol, and motility of the diluted spermatozoa was estimated as described before at various time intervals (20 minutes to 144 h).

Fertilization Tests

Because fresh eggs of *P. gigas* were not available, we used fresh eggs of Gunther's walking catfish, *C. macrocephalus*, which can be fertilized with *P. gigas* sperm (Mongkonpunya, unpubl. data). Embryos produced by this method develop at least to hatching stage.

Three female *C. macrocephalus* were induced to spawn by injection of 30 $\mu g \cdot kg^{-1}$ of leuteinizing hormone-releasing hormone analogue (LHRHa) (Suprefact Holshct AG, Main, Germany) and 10 $mg \cdot kg^{-1}$ of domperidone (Motilium, Olic, Bangkok, Thailand). Eggs were collected by abdominal stripping 12 h after injection. Pooled ova were used across the treatment groups. A 0.5-ml aliquot (~200 eggs) was placed into each of three 2-l plastic bowls per treatment. Each aliquot of eggs was fertilized by addition of 200 µl thawed sperm (calculated to be equivalent to the number of sperm in 20 µg testis, and sperm : egg ratio of 4.2×10^6 : 1). After the eggs and semen were mixed thoroughly, 20-25 ml water was added, and after 1-2 minutes, the eggs were rinsed and incubated at 25-26°C in 1.5 l of dechlorinated water with continuous aeration.

Three determinations of control fertilization were carried out in the same fashion to assess the quality of the eggs by use of fresh sperm (4.2×10^6 sperm · egg^{-1}) of Gunther's walking catfish. The fresh sperm demonstrated a fast swirling with ≥80% motility. Fertilization success was assessed as the percentage of embryos (neurular) observed at 20-24 h after insemination.

Trial 1: This trial involved *P. gigas* sperm cryopreserved in 5 or 9% DMSO in BCB and subjected to a freezing rate of $-5°C \cdot min^{-1}$ for the upper 2-ml cryotube, or $-120°C \cdot min^{-1}$ for the lower tube.

Trial 2: This was identical to trial 1, but C-F HBSS was used as the extender instead of BCB.

Trial 3: The sperm was cryopreserved with 9% DMSO in either C-F HBSS or BCB, in 5-ml cryotubes. Freezing rates were $-12°C \cdot min^{-1}$ for the upper tube, and $-22°C \cdot min^{-1}$ for the lower tube.

Analysis of Data

The basic design for the experiments involved two factors considered important in the outcome of fertilization tests with cryopreserved sperm. First, the concentration of DMSO or methanol in the extender, and second, the freezing rate. These two factors were controlled within the experiment, and therefore, were analyzed as fixed factors. Because the cryopreserved semen came from only one *P. gigas* male, the analysis was based on two-way anova without replication of fixed treatments (Sokal and Rohlf 1969). Where factors were found to have significant effects ($P<0.05$), comparison of means employed the Duncan's multiple range test.

Results

Sperm Density and Motility

The undiluted semen of *P. gigas* showed tremendous numbers of spermatozoa, ranging from 20×10^9 to 40×10^9 $sperm \cdot ml^{-1}$. Motility of the undiluted semen and sperm diluted in BCB, stored in the refrigerator at 5-6°C was maintained for 1-2 d, while motility of sperm diluted in C-F HBSS or HBSS was maintained for 5-6 d (Table 2).

In trial 1 and 2, using 2-ml cryotubes, all samples of sperm cryopreserved in 5% DMSO or either concentration of methanol and subjected to either freezing rate (-5 or -120°C·min^{-1}) gelled when thawed and the few remaining sperm found in the liquified portion exhibited <5% motility. Thawed sperm samples cryopreserved in 9% DMSO remained liquid and possessed 5-10% motility. Sperm cryopreserved in any concentration of the cryoprotectant, and subjected to -120°C·min^{-1} freezing rate yielded no post-thaw motility. In trial 3 (using 5-ml cryotubes), thawed samples in C-F HBSS or BCB were liquid and possessed about 1% motility for the lower tubes, and 5-10% for the upper tubes.

Acute Toxicity Studies

Chilled storage of the same samples of *P. gigas* sperm in C-F HBSS containing 14% DMSO or methanol resulted in decreased motility of as much as 100% within 20 minutes (Table 2). Reduction in motility was associated with increased cryoprotectant concentration (5-14% of either DMSO or methanol) and increased storage time. Sperm stored in C-F HBSS containing 5% DMSO exhibited ~50% motility as compared to 5% motility of sperm stored in 5% methanol for the same length of time (72 h) (Table 2).

Table 2. Swirling rate[1] and percent motility of *Pangasius gigas* sperm stored at 5°C.

Fish no.	% DMSO in extender	Storage time (h)							
		0.3	2	24	48	72	96	120	144
P_1	0 (undiluted)	-	F/100	N/90	N/10	N/0	—	—	—
	0 HBSS	-	F/100	M/100	M/100	M/100	S/80	N/20	—
	0 C-F HBSS	-	F/100	M/100	M/100	M/100	S/80	N/100	N/30
	0 BCB	-	M/100	N/90	N/0	—	—	—	—
P_2	5% DMSO in C-F HBSS	F/100	F/100	S/80	S/50	N/50	N/0	—	—
	5% MeOH in C-F HBSS	F/100	F/100	S/80	N/70	N/5	N/0	—	—
P1	14% DMSO in C-F HBSS	N/0	—	—	—	—	—	—	—
	14% MeOH in C-F HBSS	N/0	—	—	—	—	—	—	—

[1]Swirling rate : F = fast, M = medium, S = slow, N = none.

Fertilizing Capability of Cryopreserved Sperm

Trial 1: There were highly significant differences ($P<0.01$) between the fertilizing capabilities of *P. gigas* sperm cryopreserved in 5 and 9% DMSO in BCB, and also between the two freezing rates. The cryopreserved sperm which had been subjected to the -120°C·min^{-1} freezing rate were nonviable, yielding 0% fertilization for both 5 and 9% DMSO (Table 3). The sperm cryopreserved in 5 and 9% DMSO, but subjected to a -5°C·min^{-1} freezing rate, resulted in 7 and 15% fertilization, respectively. Thus, the sperm cryopreserved in 9% DMSO and frozen at -5°C·min^{-1} had significantly higher fertilizing ability than sperm cryopreserved in 5% DMSO at the same freezing rate.

Trial 2: The cryopreservation method used in this trial was the same as in trial 1, except that C-F HBSS was used instead of BCB, and the results followed the same trends observed for trial 1 (Table 3). *P. gigas* sperm cryopreserved in 9% DMSO in C-F HBSS and frozen at -5°C·min^{-1} yielded the highest fertilization rate (2.9%). However, there was no significant difference in fertilization rate due to DMSO concentration.

Trial 3: BCB and C-F HBSS were used in this trial with 9% DMSO, in 5-ml cryotubes and freezing at either -12° or -22°C·min^{-1}. There was no significant difference ($P>0.05$) between sperm cryopreserved in BCB and C-F HBSS. There was, however, a significant difference ($P<0.01$) between fertilization rates of sperm subjected to different freezing rates. Sperm frozen at a rate of -12°C·min^{-1} yielded a higher fertilization rate than sperm frozen at -22°C·min^{-1} (Table 4). The sperm : egg ratio was 4.2 x 10^6 : 1 across the three trials.

Table 3. Mean (±SE) percent fertilization (n=3) of *Clarias macrocephalus* eggs inseminated with fresh semen of its own species (control) or with *Pangasius gigas* sperm cryopreserved in 5 or 9% DMSO in bicarbonate buffer (BCB) or calcium-free Hanks' balanced salt solution (C-F HBSS) in 2-ml cryotubes and subjected to -5 or -120°C·min^{-1} freezing rate. The cryopreserved sperm was thawed at 50°C prior to use. The sperm : egg ratio was 4.2 x 10^6 : 1.

Freezing rate (°C·min^{-1})		Fertilization rate (%)			
		5% DMSO		9% DMSO	
		BCB (trial 1)	C-F HBSS (trial 2)	BCB (trial 1)	C-F HBSS (trial 2)
-5	Actual	6.6±1.5	0.6±0.6	15.1±5.0	2.9±0.5
	% control	24.5±5.5	1.5±1.5	56.5±19.6	7.9±1.4
-120	Actual	0	0	0	0
	% control	0	0	0	0
Control		26.7±4.9	36.1±4.6	26.7±4.9	36.1±4.6

Table 4. Mean (±SE) percent fertilization (n=3) of *Clarias macrocephalus* eggs inseminated with fresh sperm of its own species (control), or with *P. gigas* sperm cryopreserved in 9% DMSO in either calcium-free Hanks' balanced salt solution (C-F HBSS) or bicarbonate buffer (BCB) in 5-ml cryotubes and subjected to -12 or -22°C·min^{-1} and thawed at 50°C prior to use. The sperm : egg ratio was 4.2 x 10^6 : 1.

Freezing rate (°C·min^{-1})		Fertilization rate (%)	
		9% DMSO in C-F HBSS	9% DMSO in BCB
-12	Actual	66.0±6.5	65.0±5.5
	% control	73.7±7.2	72.5±6.2
-22	Actual	19.2±9.2	6.1±4.2
	% control	21.4±10.4	6.8±4.6
	Control		89.2±3.3

The Hybrid

No significant difference in the stages of embryo development was observed between the hybrid (*P. gigas* sperm x *C. macrocephalus* eggs) and the purebred *C. macrocephalus* maternal half-siblings. Both hatched within 36 h after insemination. The hybrid fry were, however, 99.9% morphologically abnormal, e.g., with short and curling or twisting body (Fig. 4) and died within 7 d.

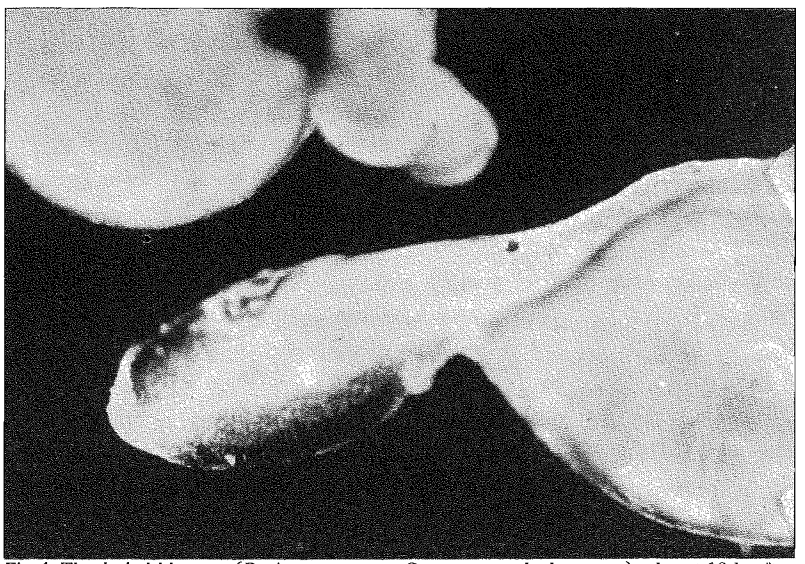

Fig.4. The hybrid larvae (*P. gigas* sperm x *C. macrocephalus* eggs), about 12 h after hatching.

Discussion

Because spawning *P. gigas* is not cultured and is a rare animal species, the eggs and semen, if available, are seriously needed for hatchery production. During our field study period it was not possible to obtain *P. gigas* eggs for intraspecific fertilization trials. Interspecific or intergeneric fertilization, using eggs of other catfishes (i.e., *P. hypophthalmus* and *C. macrocephalus*) were used for testing the fertilizing capability of cryopreserved sperm of *P. gigas*. In a previous report (Mongkonpunya et al. 1992) *P. gigas* sperm were frozen in nitrogen vapor with 8% DMSO as the cryoprotectant. The interspecific fertilization percentage of the cryopreserved sperm was 26-45% (*P. gigas* cryopreserved sperm x *P. hypophthalmus* fresh eggs) which was comparable to interspecific fertilization using fresh semen (42-95%). In addition, the hybrid of this cross (called "big swai" or "buk-swai" in Thai), produced with frozen sperm, appeared normal and grew at a faster rate than purebred *P. hypophthalmus* maternal half-siblings (unpubl. data). Similarly, *P. gigas* sperm demonstrated fertilizing capability with *C. macrocephalus* eggs, although in this case the hybrid fry were generally abnormal and died within 7 d after hatching (unpubl. data).

It is well known that the toxicity of cryoprotective agents is a factor affecting the success of cryopreservation of sensitive cells such as spermatozoa (Tiersch et al. 1994). For each experimental species, therefore, the effect on prefreezing motility of cryoprotectants at various concentrations should be evaluated to determine appropriate concentrations and equilibration times. Thus, in this study we began with toxicity testing of various concentrations of DMSO and methanol. The results suggest that for 5% DMSO or methanol, equilibration should be less than 2 h, and thus DMSO and methanol at concentrations of 5-10% would probably be best, although at higher concentrations (~10%), equilibration time should be less than 20 minutes.

The motility of stored sperm was extended in C-F HBSS or HBSS for 5-6 d. The motility of undiluted sperm, and the sperm diluted in BCB lasted 24-48 h. These differences in motility could be due to a variety of factors including bacterial contamination, concentration of specific ions, or osmotic pressure. The osmolalities of BCB, C-F HBSS and HBSS measured by vapor pressure osmometer (model 5500, Wescor, Logan, Utah) were 560, 320 and 286 $mOsm \cdot kg^{-1}$ (n=2). Mongkonpunya et al. (unpubl. data) reported that osmotic pressure of threshold activation (<25% motility of sperm cells in a sample) was 240 $\pm$ 10 $mOsm \cdot kg^{-1}$ for fresh sperm of *P. gigas*, and with full-strength HBSS, low levels of activation (~10% motility) were observed. No motility of sperm was elicited after dilution with BCB or C-F HBSS. The results suggest that further refinement of chilled storage techniques, e.g., adjusting the osmotic pressure of the extender could extend storage time.

Final DMSO concentrations of 5-10% have been used with varied success for cryoprotection of fish sperm. For example, optimal concentrations were 10.8% (Ott and Horton 1971a) in the extended sperm of steelhead trout, *Oncorhynchus mykiss* and 6.4% (Ott and Horton 1971b) in coho salmon, *O. kisutch*. Equilibration time was minimized for sperm of steelhead trout, and was 2 h for sperm of coho salmon. In our present study with *P. gigas* sperm, we found that 10 and 14% of DMSO or methanol were toxic to *P. gigas* sperm. Thus, we used 5 and 9% of each, and kept equilibration time at 10-15 minutes. Five percent DMSO in the extenders (BCB and C-F HBSS) was found to be less effective than 9% DMSO; however, 9% DMSO was probably toxic to *P. gigas* sperm. Monkonpunya et al. (1992) reported that *P. gigas* sperm cryopreserved in 8% DMSO in BCB provided 68% intraspecific fertilization that was not significantly different from untreated sperm (79%). However, the lower fertility rates observed in the present study could be due to lower viability of eggs. The fertility of untreated sperm in trials 1 and 2 in this study was only 27% and 36%, respectively. When egg quality (the control) was increased (trial 3), the fertility of cryopreserved sperm was comparable to that of previously reported data.

Though the fertility of sperm cryopreserved in BCB was slightly higher than that of sperm in C-F HBSS, there was no significant difference between fertility of sperm cryopreserved in 9% DMSO in BCB (66%) or C-F HBSS (65%) at a freezing rate of $-12°C \cdot min^{-1}$ in 5-ml cryotubes. Increasing the freezing rate from -5 to $-12°C \cdot min^{-1}$, but not to $-22°C \cdot min^{-1}$ or beyond, and increasing the capacity

of the tube increased fertility; although differences in survival or fertility of sperm due to differences in thawing rates between the two tube sizes could not be excluded.

Considering the results of all three trials, we suggest that a slow freezing rate (-5 to -22°C·min^{-1}) is better than a fast freezing rate (-120°C·min^{-1}) for cryopreserving *P. gigas* sperm in 9% DMSO in either BCB or C-F HBSS. The techniques described provide some insight to improve cryopreservation success of *P. gigas* sperm, and provide help in actual practice. Although low, the present rates of post-thaw fertility are satisfactory for gene-banking purposes in a rare animal species like *P. gigas*.

Acknowledgments

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