Cryopreservation of Sperm of Asian Catfishes Including the Endangered Mekong Giant Catfish

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Introduction

In recent years aquaculture has become an important sector of fish production in the region of the Mekong River. Catfishes of the families Pangasiidae and Clariidae are commercially produced throughout the region. Indigenous species include the Chao Phraya catfish, or swai, *Pangasius hypophthalmus* (formerly *P. sutchi*) (Roberts and Vidthayanon 1991), the Mekong giant catfish, or pla buk, *Pangasius gigas* (formerly *Pangasianodon gigas*), the walking catfish *Clarias batrachus* and *Clarias macrocephalus*. The Mekong giant catfish is the world's largest freshwater catfish, often surpassing 250 Kg in weight (Figure 1). This fish is of considerable popularity in Thailand, Laos and Cambodia and has been the object of a traditional capture fishery for centuries. Abruptly declining harvests have indicated that the Mekong giant catfish is an endangered species. The Thai Fisheries Department initiated an artificial breeding program for wild-caught Mekong giant catfish in 1983 and produced 200,000 fry which were reported to have attained an average of 21 Kg in 4 yr in earthen ponds (Pholprasith 1996). Appropriate aquaculture techniques have been developed for *P. gigas* and hybrids (*P. hypophthalmus* x *P. gigas*). Moreover, aquaculture production of commercial scale is in the expansion stage.

Photograph by T. Tiersch

Figure 1. Transport of a 200-Kg Mekong giant catfish taken from the Mekong River in Northern Thailand.

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For *C. batrachus* and *C. macrocephalus* commercial culture has largely ceased following the introduction of the faster growing hybrid of *C. macrocephalus* x the African catfish *C. gariepinus* which is available in markets and is grown on a commercial basis. In addition, pollution in the Mekong region is more serious than ever. Thus, the indigenous clariids are also threatened with extinction from natural waters. Cryopreservation of fish sperm is one possible means of assisting culture and conservation (Table 1).

Table 1. Applications of sperm cryopreservation in cultured and endangered fishes.

1. Long-term storage for regular or future use.
2. Genetic improvement through selective breeding programs.
3. Production of reference stocks for culture or research.
4. Production of hybrids.
5. Reduction of the cost and labor of maintaining broodstocks.
6. Elimination of the need for precise synchronization of males and females.
7. Repeated spawning of specific males.
8. International shipment and use.
9. Germplasm conservation and development of germplasm repositories.
10. Use in conjunction with interspecific androgenesis to restore endangered or extinct species (e.g. Thongpan et al. 1997, Mongkonpunya et al. 1997).

Even though many species of shrimp and fish of the Mekong region are successfully cultured on a commercial basis, farming is not practiced with genetically improved seedstocks, and genetically improved catfish are virtually unavailable. The characteristics of an improved stock would include uniform size, fast growth, efficient food conversion, high dress-out percentage and disease resistance. Sperm preservation could be adopted for catfish culture in the region. With an effective sperm storage technique, catfish sperm could be transported to hatcheries to produce desirable hybrids or crosses. With adequate post-thaw motility and fertilization, cryopreserved sperm could provide hatcheries with improved seedstocks sufficient for commercial production.

We developed procedures for collection, handling, refrigerated storage (4 to 6 °C) and cryopreservation of sperm of pangasiid and clariid catfishes. Our objectives were to: 1) determine the physiochemical composition of the seminal plasma and blood serum; 2) examine the relationship of osmotic pressure and sperm activation to allow safe storage and to assure complete activation for fertilization; 3) evaluate the toxicity and effectiveness of different cryoprotectants, and 4) evaluate the success of different freezing rates. Motility estimates and tests of fertilizing capability were used to measure sperm viability. The techniques and equipment used in cryopreservation of sperm have been simplified to provide application or adaptation in other laboratories around the world (Monkonpunya et al., pp. 108-116, this volume).

Table 2. Inorganic electrolytes molarity, osmolality (mOsmol/Kg) and pH of blood serum and seminal plasma of *Pangasius gigas* and *P. hypophthalmus*. Sample sizes are indicated in parentheses.

	P. gigas		*P. hypophthalmus*	
	Blood serum	Seminal plasma	Blood serum	Seminal plasma
mOsmol/Kg	232 ± 17 (8)	267 ± 45 (5)	273 ± 1 (15)	264 ± 3 (15)
PH	7.5 ± 0.3 (5)	8.2 ± 0.8 (5)	7.1 ± 0.5 (10)	8.3 ± 0.6 (10)
Ca^{++} (M)	3.5 ± 3.2 (3)	0.78 ± 1.1 (2)	ND (8)	ND (8)
K^{+} (M)	ND (1)*	ND (2)	0.01 ± 0.01 (16)	0.01 ± 0.01 (8)
Cl^{-} (M)	0.22 (1)	0.76 ± 10.4 (2)	0.03 ± 0.03 (5)	0.93 ± 0.54 (10)
Na^{+} (%)	1.52 (1)	0.43 ± 0.06 (2)	1.67 ± 0.56 (10)	1.51 ± 0.56 (6)

*ND, not detected; below detection level by the ion-selective and pH electrodes (Orion, Boston, Massachusetts).

Sperm Density and Seminal Plasma Physiochemistry

Undiluted pangasiid semen contained tremendous numbers of spermatozoa ranging from 2 x 10^{10} to 4 x 10^{10} sperm per mL in *P. gigas* (Mongkonpunya et al. 1996) to 7.6 x 10^{10} sperm per mL in *P. hypophthalmus* (Hambananda and Mongkonpunya 1996a). The mean pH of blood serum (~7.3) was lower than the pH of seminal plasma (~8.2) in each species (Table 2). The higher pH of seminal plasma could indicate contamination of stripped semen with urine (which tends to be alkaline). The mean osmotic pressure of blood serum and seminal plasma (230 to 270 mOsmol/Kg) were not significantly different between the two species or within species. Potassium ion concentration in blood serum and seminal fluid was not detectable (below the sensitivity limit) in *P. gigas* and it was 0.01 M in *P. hypophthalmus*. In contrast, calcium levels in the body fluids of *P. gigas* were relatively high (to ~ 3.5 M), but calcium was not detectable in blood serum of *P. hypophthalmus*. The major cation in the blood serum of each species was Na^{+} (0.4 to 1.8%) while Cl^{-} was the major anion. Results from these studies indicated that osmolality of any extender solutions should be adjusted to about 300 mOsmol/Kg to avoid sperm activation due to reduced osmotic pressure (Mongkonpunya et al. 1996). The effects of Ca^{++} and K^{+} on sperm activation should also be given careful consideration.

Extenders and Refrigerated Storage

Extenders are generally designed to be compatible with the physiochemical composition of the seminal plasma of the candidate species to maintain the sperm in a non-motile but viable state (Stoss 1983). Motility and fertilizing capability of pangasiid sperm diluted (1:3) in 0.9% normal saline (NaCl) or calcium-free Hanks' balanced salt solution (C-F HBSS) (Tiersch et al. 1994) (Table 3) could be maintained for 6 d at 5 °C with reduced motility (10 to 60%) and about 30 to 40% fertilization (Table 4) which was comparable to that of fresh sperm (~40 to 50% fertilization). A refrigerated sample of

P. gigas sperm used in this study was also used by the Thai Fisheries Department for production of 50,000 *P. gigas* fry on day 7 of storage (~50% motility) when a ripe female was available without a male or fresh sperm (1997 Technical Report, Inland Fisheries Section, Thai Fisheries Department).

Table 3. Ingredients (g/L) and osmotic pressure (mOsmol/Kg) of Hanks' balanced salt solution (HBSS), calcium-free Hanks' balanced salt solution (C-F HBSS), bicarbonate buffer (BCB) and 0.9% NaCl used to dilute sperm of pangasiid and clariid catfishes. Each mL of the extender also contained 500 IU of penicillin and 1 mg of streptomycin.

Ingredient	HBSS	C-F HBSS	BCB	NaCl
$CaCl_2 \cdot H_20$	0.16	--	--	--
NaCl	8.00	8.89	--	9.00
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	--	--	40.71	--
Glutathione, reduced	--	--	3.00	--
mOsmol/Kg	286	320	356	300

Table 4. Percent motility and percent fertilization observed for pangasiid sperm stored at 4 to 6 °C in bicarbonate buffer (BCB), calcium-free Hanks' balanced salt solution (C-F HBSS) or 0.9% sodium chloride (NaCl) for 6 d. Aliquots of sperm (~1 uL) were activated with 20 uL of distilled water.

	Percent motility during storage (hr)							
	0.5*	24	48	72	96	120	144	Percent fertilization**
Pangasius gigas								
BCB	100	10	0	--***	--	--	--	0
C-F HBSS	100	100	100	100	90	60	20	34 ± 8
NaCl	100	100	100	100	100	90	60	44 ± 17
Fresh sperm	--	--	--	--	--	--	--	47 ± 7
P. hypophthalmus								
BCB	40	--	--	0	--	--	--	0
C-F HBSS	80	--	--	30	--	--	10	10 ± 5
NaCl	80	--	--	20	--	--	10	27 ± 8
Fresh sperm	--	--	--	--	--	--	--	50 ± 3

* Motility was examined ~30 min after dilution at 1:3 (sperm:extender).
** In fertilization trials (on day 6) the eggs of *P. hypophthalmus* were used with a ratio of 4.2×10^6 sperm per egg.
*** Non-motile samples were discarded.

Because these extenders (NaCl and C-F HBSS) were significantly different in ionic composition when adjusted to 300 mOsmol/Kg, it was evident that maintaining osmotic pressure at levels isotonic or hypertonic to sperm cells was the main factor in maintaining viability and fertilizing capability of pangasiid sperm. Moreover, reduction in osmotic pressure played a major role in activation of sperm motility of *P. gigas* and *P. hypophthalmus* (Mongkonpunya et al. 1996). However, detrimental effects of bicarbonate buffer (BCB) on sperm motility were observed at 300 mOsmol/Kg. Sperm viability could not be extended longer than 24 hr even with dilution in glutathione-free BCB and replacement of $KHCO_3$ with Tris or the zwitterionic buffer HEPES (Hambananda and Mongkonpunya 1996a). Sperm of *P. gigas* diluted (1:3) in a mixture of 25% BCB and 75% 0.9% NaCl could be stored for 5 d with 60% motility, comparable to sperm stored in C-F HBSS, but with reduced motility (30%) compared to sperm stored in 0.9% NaCl alone (Table 5).

Table 5. Percent motility of *Pangasius gigas* sperm stored for 5 d at 5 °C in bicarbonate buffer (BCB), 0.9% sodium chloride (NaCl) or calcium-free Hanks' balanced salt solution (C-F HBSS) or mixture of BCB with the other extenders. The dilution ratio was 1:3 (sperm:extender). Aliquots of sperm (~1 µL) were activated with 20 µL of distilled water.

Extender mixture ratio	Osmotic pressure (mOsmol/Kg)	Percent motility during storage (hr)					
		0.5*	24	48	72	96	120
BCB:C-F HBSS							
1:0	356	100	10	0**	--	--	--
0:1	286	100	100	100	100	90	60
1:2	307	100	80	10	10	0	--
1:4	296	100	100	80	60	60	10
BCB:NaCl							
1:0	356	100	5	10	0	--	--
0:1	300	100	100	100	100	100	90
1:2	320	100	90	50	50	10	0
1:4	316	100	100	100	100	100	60
Undiluted sperm	300	100	100	20	20	0	--

* Motility was examined about 30 min after dilution.
** Non-motile samples were discarded.

Given our current knowledge of refrigerated storage of pangasiid sperm, use of saline solution (0.9% NaCl at 300 mOsmol/Kg) is recommended because it is inexpensive and easy to prepare. Moreover, pangasiid sperm diluted 1:1 either in 0.9% NaCl or C-F HBSS, held at half-volume (2.5 mL) in 5-mL cryotubes and stored on ice could be transported overnight (~ 12 hr) from the field to the laboratory for cryopreservation. Motility of the transported sperm was equal to the motility at the time of collection, allowing cryopreservation to be performed under controlled conditions.

This is important for work with *P. gigas*, where the sperm is collected in the northern village of Cheng Kong which has no facilities for laboratory work. By transporting the samples ~800 km by train to Bangkok, we could utilize a controlled-rate freezer and laboratory available at Kasetsart University.

Standardization and Cryopreservation Protocols

Utilization of information in many published protocols for cryopreservation of fish sperm is difficult because the reports are incomplete, and given that only positive results are usually published, the true variability of results remains unknown. Because of these difficulties, development of reliable cryopreservation protocols for fish sperm is often practiced on a species-by-species basis. To assist in sharing knowledge, emphasis should be placed on standardization of each step in a procedure. For example, sperm procurement, dilution ratios, extenders and cryoprotectants used, equilibration times, freezing and thawing rates, and egg-to-sperm ratios in fertilization tests can be standardized. Before any protocol is adopted, methods for evaluation should be carried out for each step.

Extenders and Cryopreservation

An inexpensive and easy to prepare extender would be useful for the farmer, and it should be effective for refrigerated storage (described above) as well as for cryopreservation. Although HBSS of 280 to 300 mOsmol/Kg was an acceptable extender for sperm cryopreservation of channel catfish *Ictalurus punctatus* (Tiersch et al. 1994) and Mekong giant catfish (Mongkonpunya et al. 1995), we found that using 0.9% saline resulted in no difference in percent fertilization when compared to other extenders (Table 6).

Table 6. Percent fertilization based on number of eggs of *Clarias macrocephalus* inseminated with fresh semen of its own species (control) or with *Pangasius gigas* sperm cryopreserved in 10% dimethyl sulfoxide (DMSO) or propylene glycol (PG) in either 0.9% NaCl, bicarbonate buffer (BCB) or calcium-free Hanks' balanced salt solution (C-F HBSS) in 2-mL cryotubes and cooled at –10 °C per min using a controlled-rate freezer (Forma Scientific, New York). The cryopreserved sperm were thawed at 50 °C prior to use. The sperm to egg ratio was 4.2 x 10^6:1.

	Percent fertilization						
	10% DMSO			10% PG			
Female	NaCl	BCB	C-F HBSS	NaCl	BCB	C-F HBSS	Fresh sperm
1	84	66	70	65	67	78	74
2	19	18	41	15	45	23	23
3	4	7	2	7	3	2	5
4	8	9	3	7	2	1	27
Mean ± SD	29 ± 32	25 ± 24	29 ± 28	23 ± 24	29 ± 28	26 ± 31	27 ± 28

Toxicity of Cryoprotectants

Reductions in sperm motility and viability of pangasiid and clariid sperm were associated with increasing concentrations (5 to 14%) of dimethyl sulfoxide (DMSO), methanol or glycerol and increased exposure time (equilibration) before freezing (Mongkonpunya et al. 1995, Chairak and Mongkonpunya 1996). Among these chemicals, glycerol was more toxic to sperm than was DMSO, while methanol was the least toxic.

We used concentrations of 5 to 14% of each cryoprotectant, and held equilibration time at 15 min. Five percent DMSO in BCB or C-F HBSS was found to yield lower post-thaw motility than did 9% DMSO. However, at 14%, DMSO and methanol were too toxic for use in cryopreservation of pangasiid sperm, yielding 0% motility at 30 min equilibration (Mongkonpunya et al. 1995) (Table 7). Thus, to minimize the acute toxic effects, while maintaining the effectiveness of DMSO, we chose to use a concentration of 9% with a 15-min equilibration time as a general practice. Pre-freeze motility (at the start of the freezing process) of sperm exposed at this concentration was not different from the motility of sperm which were not exposed to the cryoprotectant.

Table 7. Percent motility of sperm of *Pangasius gigas* and *Clarias macrocephalus* stored at 5 °C for 30 min in extenders with various concentrations of cryoprotectants.

Species	Cryoprotectant in extender*	Percent motility	Reference
Clarias macrocephalus	8% glycerol in K-F CB	0	Chairak and Mongkonpunya 1996
	8% DMSO in K-F CB	43	Chairak and Mongkonpunya 1996
	8% Methanol in K-F CB	60	Chairak and Mongkonpunya 1996
Pangasius gigas	5% DMSO in C-F HBSS	100	Mongkonpunya et al. 1995
	5% MeOH in C-F HBSS	100	Mongkonpunya et al. 1995
	14% DMSO in C-F HBSS	0	Mongkonpunya et al. 1995
	14% MeOH in C-F HBSS	0	Mongkonpunya et al. 1995

*K-F CB, potassium-free modified Cortland's buffer; C-F HBSS, calcium-free Hanks' balanced salt solution.

Freezing and Thawing

Five or 10% DMSO, dimethyl acetamide (DMA) or glycerol, as well as 5% propylene glycol produced jelled samples with <1% post-thaw motility, while 10% propylene glycol yielded motility of <5%. We froze samples on aluminum canes in shipping dewars (Cryomed, Forma Scientific, Ohio). The average rate of freezing depended on the configuration of containers used (straws, tubes, etc.), the amount of liquid nitrogen (LN_2) and the position of the sample tubes on the canes. We found that the average rates of cooling were -40 °C per min for the lower position, -20 °C per min for the middle position, and -10 °C per min for the upper position. The post-thaw motility of sperm in the lower position was 0% for the four cryoprotectants studied at all concentrations. However, freezing in the middle and upper positions yielded ~5% motility after thawing.

In another study with Mekong giant catfish, sperm were frozen in LN_2 vapor with 8% DMSO in BCB using AI catheters (used for artificial insemination of cattle) as sperm containers. Prior to use, the sperm were thawed at 80 °C for 20 sec (Mongkonpunya et al. 1992). Fertilization percentages using eggs of *P. hypophthalmus* inseminated with cryopreserved (68%) and fresh sperm (79%) were not significantly different. In another study, *P. gigas* sperm in 5-mL cryotubes were frozen in LN_2 vapor with 9% DMSO in C-F HBSS or BCB at different freezing rates (Mongkonpunya et al. 1995). The cryopreserved sperm were thawed at 70 °C for 2 min prior to use. The sperm subjected to a cooling rate of -12 °C per min yielded the highest percent fertilization (66%), which was not significantly different from that of fresh sperm (74%). Although thawed sperm showed relatively high percent fertilization, few motile sperm were observed, whereas most sperm were motile in the untreated samples.

This might be surprising to those familiar with mammalian sperm where fertilizing capability is highly correlated with motility. From our experience, viability and fertilizing capability of pangasiid sperm were not directly correlated with motility. In many cases, sperm with no observable post-thaw motility yielded fertilization rates as high as those of fresh sperm with 80 to 100% motility. In fish, sperm penetration of the eggs is probably a function of the egg micropyle. Soon after the eggs are released from the female, both water and sperm could be drawn into the egg through the micropyle.

Overall, these studies represent more than a decade of research. Studies of *P. gigas* are especially difficult due to the high value and low numbers of fish captured, sometimes only allowing study of one or two males each year. These studies have also been hindered by limited facilities at the collection sites, and recently by the economic upheaval in Thailand. Despite these problems, workable techniques have been developed that are suitable for use with endangered species in developing countries.

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