Cryopreservation of Bagrid Catfish *Hemibagrus wyckioides* Spermatozoa

Wisanuporn Ratanatrivong[a], Wara Taparhudee[b], Ruangvit Yoonpundh[b] and Prapansak Srisapoome[b]

ABSTRACT

In order to establish a protocol for the cryopreservation of Bagrid catfish (*Hemibagrus wyckioides*) spermatozoa, semen was collected from male Bagrid catfish through surgical testis removal technique. The research objectives were 1) to determine the effect of three cryoprotectants on sperm motility, 2) to establish cryopreservation procedures by comparing various extenders and cooling rates, and 3) to estimate the spermatozoa fertilization potential by comparing artificial fertilization between cryosperm and fresh sperm. The cryosperm was stored for 30 and 365 days, and thawed for the second and third experiments, respectively. Methanol had the least toxic effect to the sperm whereas DMSO had the most toxic effect. The use of 6% fructose and 5% glucose as extenders, 10% (v/v) methanol as cryoprotectant, and cooling rates of 2 and 5°C/min showed higher thawed sperm motility, compared with using GFR as an extender, 10% methanol and 10°C/min cooling rate. The percentage of eyed stage egg between thawed and fresh sperms was not different. The results also suggested that Bagrid catfish semen could be diluted to 15 folds dilution before mixing with eggs during artificial fertilization.

Keywords: Cryopreservation, spermatozoa, *Hemibagrus wyckioides*

INTRODUCTION

Bagrid catfish *Hemibagrus wyckioides* Chaux and Fang (1949) is considered one of the important candidate catfish species for aquaculture in Thailand. Traditionally, production of Bagrid catfish mainly depended on fingerlings in natural water resources, resulting in unpredictable catfish production. Mass production of catfish fry by artificial breeding from domesticated broodstock in Thailand succeeded for the first time at Pitsanulok Inland Fisheries Research and Development Center (Ratanatrivong *et al*., 1994). Unfortunately, Bagrid catfish is one of the catfish species wherein the testis is located deeply inside the body cavity, thus the male brood fish must be killed in order to collect semen for fertilization during artificial breeding. Therefore, the research

[a] Corresponding author: Uttaradit Fisheries Test and Research Center, 34 M.10 Banmai Pattana, Wang Dang, Tron, Uttaradit, 53140, Tel. 66 0 5549 1002, E–mail: wisanupornr@gmail.com
[b] Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand 10900

on semen preservation technique is an important key for the development of breeding in this and other catfish species. Currently, preserving fish semen for long periods could be achieved by cryopreservation. Cryopreservation is a technique whereby biological material is frozen at very low temperatures, usually at –196 °C, using liquid nitrogen. At this temperature, the cellular viability can be stored in stable form for a long period (McAndrew *et al*., 1995). The general protocol for fish-sperm cryopreservation includes: 1) collection of semen or milt from fish, 2) addition of extender solution and cryoprotectant, 3) loading into cryovials or straw tubes, 4) freezing samples at specific cooling rate, and 5) plunging the samples into liquid nitrogen. The success in cryopreservation depends on the types of extenders, cryoprotectants and freezing protocol used. Generally, cryoprotectants and the cooling rate applied determine the degree of damage on spermatozoa due to intracellular ice crystallization (Mazur, 1977). There have been a few reports on cryopreserved testicular spermatozoa of catfish. Chairak (1996) successfully conducted cryopreservation in walking catfish (*Clarias macrocephalus*) sperm by using a modified Cortland and 8–12% DMSO as cryodiluent with cooling rates of 5 and 10 °C/min. Steyn *et al*. (1985) reported successful cryopreservation in sharptooth catfish (*C. gariepinus*) by using 5% glucose and 5% glycerol as cryodiluent combined with multi–steps freezing rates. Steyn and Van Vuren (1987) reported the fertilization capability of a 16-month cryopreserved spermatozoa, wherein no significant difference in fertilization rates between fresh and thawed sperm was found. It is evident that cryopreservation is species specific; however, no successful cryopreservation of Bagrid catfish spermatozoa has yet been reported. Therefore, the research on cryopreservation in Bagrid catfish spermatozoa is very crucial for artificial breeding. The objectives of this research were (1) to evaluate the toxicity and effectiveness of different cryoprotectants, (2) to determine the effects of various extenders and freezing rates on post thawed sperm motility, and, (3) to examine the fertilization potential of thawed sperm.

MATERIALS AND METHODS

Semen collection

The research was conducted at Uttaradit Fisheries Test and Research Center, Uttaradit province, Thailand in June 2007. Semen was collected from male catfish strain F_2 selected line by mass selection. Testes were collected following the surgical removal of testis technique, i.e. male fish were intramuscularly injected with luteinizing hormone releasing hormone analog ($LHRH_a$) at 15 ug/kg body weight. After 12 hours post injection, the catfish were anesthetized in 100 ppm MS–222 and sacrificed by spinal transection. Testes were removed by dissection, followed by the removal of connective tissue and blood. Testes were then cut, stored in plastic bags, weighed and labeled accordingly. The semen was extracted by gently squeezing the testes then filtered through a 0.5 mm mesh nylon netting. Semen samples were then kept in 50 ml vials, at 4°C for the experiments.

Estimation of semen quality

The quality of semen was determined based on sperm motility (%). Sperm motility was examined by mixing 2 µl of semen with 100 µl of fertilization solution (68.45 mM NaCl, 50 mM Urea, pH 7.0) then observed under a microscope at 100× magnification. Only sperms actively swimming with progressive movement were counted to calculate sperm motility. Fresh semen samples showing more than 75% sperm motility were used in the experiment. Sperm concentration was estimated by haemacytometer method (Mongkolpunya, 1993), wherein the semen was diluted with 0.9% NaCl at 800-fold dilution, and then 20 µl of semen solution was placed onto the haemacytometer field under a microscope at 100× magnification. The total sperm cells were counted from five counting fields. Spermatozoa density was calculated as follows:

Spermatozoa density = Average number of sperm counted from 5 fields x Dilution rate x 10,000

Effect of cryoprotectants on sperm motility

The effect of cryoprotectants on sperm motility was determined by screening various types of cryoprotectants. Pooled semen from six males was separated into 10 ml vials. Ginsberg fish's Ringer (GFR) (180.12 mM NaCl, 3.74 mM KCl, 2.55 mM $CaCl_2.2H_2O$, 2.64 mM $NaHCO_3$, pH 7.6 (Viveiros *et al*., 2000)) was used as an extender. Each sample was mixed with three cryoprotectants including methanol, dimethyl sulfoxide (DMSO) and glycerol at 2, 5, 10, 15, 20 and 25% (v/v) final concentration. Sperm motility was evaluated at 0, 30 and 120 minutes post exposure of sperm to cryoprotective agents. The optimal cryoprotectant with the lowest toxicity to sperm was selected for further steps.

Cryopreservation procedures

To examine the effects of extenders and cooling rate on thawed sperm, three extenders of GFR, 0.6% Fructose and 5% Glucose were used, at three cooling rates of 2, 5 and 10°C/min. Semen was diluted with three extenders and mixed with the cryoprotectant, which was selected based on its toxicity to sperm in the previous experiment, at 10% (v/v) final concentration. The dilution ratio (semen:cryodiluent) was 1:4. Aliquot of 0.5 ml of semen solution was drawn into 0.5 ml plastic straws, and was labeled accordingly based on the extender and the cooling rate used. Straws were sealed and placed on a plastic rack. Treated samples allowed the cryoprotectant to completely diffuse into sperm cell (equilibration) at 4°C for 15 minutes. Samples were then transferred into a 16×29×15 cm styrofoam box which contained 930 ml of liquid nitrogen. Samples were placed on a plastic rack at three vertical positions to achieve three cooling rates (2, 5 and 10°C/min). After a 20-minute exposure to liquid nitrogen vapor, samples were transferred into the liquid nitrogen storage dewar. After storage in liquid nitrogen for 30 days, cryosperm were thawed at 37°C and sperm motility was immediately determined.

Estimation of spermatozoa fertilization potential

To determine the fertilization potential of cryopreserved sperm after thawing, a comparison of fresh and thawed sperm was conducted under similar condition of cryodiluent and dilution. Then, sperm quality and artificial breeding were evaluated. The cryopreserved sperm kept for 365 days was thawed at 37°C before the experiment. Artificial breeding by hormone induction was performed according to the method of Ratanatrivong *et al*. (1994). Eggs were stripped and pooled from 3 females. Fertilization was conducted in plastic dishes, and 50 g of eggs (approximately 25,000 eggs) was placed into each dish. Batches of eggs were inseminated with thawed and fresh semen by modified dry method (Na–Nakorn, 1992). Each batch of fertilized eggs was then transferred to an incubation tray with a 0.5 mm mesh bottom, connected to a recirculating system, with water temperature controlled at 28–30°C. The fertilization potential of the fresh and cryopreserved semen was determined by the percentage of eyed stage egg at 12 hours post fertilization.

Statistical analysis

Sperm motility and percentage of eyed stage egg were arcsine transformed before statistical evaluation. All data were pooled to calculate mean and standard deviation. One–way analysis of variance followed by Student–Newman–Keuls (SNK) test was employed to determine differences among treatments at 95% confidential level (α=0.05). Statistical analysis was conducted using SPSS 12.0 software.

RESULTS

Effect of cryoprotectants to sperm motility

Table 1 summarizes the results of the trial on sperm motility. Exposure of sperm to cryoprotectants at six concentrations strongly affected sperm motility ($p<0.05$). Reduction of sperm motility was associated with increasing concentrations of DMSO, methanol and glycerol and increasing exposure time. The trials showed that using 25% Methanol and 25% glycerol for 120 minutes, 25% DMSO for 30 minutes, and 15 and 20% DMSO for 120 minutes caused 0.00 % motility. It can be concluded that, among cryoprotectants, methanol has the least toxic effect on Bagrid catfish sperm whereas DMSO had the most toxic effect. Therefore, methanol was chosen to be used in further tests in this study.

Table 1. Sperm motility (%) at 0, 30 and 120 minutes post exposure to 3 cryoprotectants.

Cryoprotectant	Concentration (%)	Sperm motility (%)		
		0 minute	30 minutes	120 minutes
None	0	92.67	91.83	91.04
Methanol	2	78.60 ± 2.73^{Aa}	54.21 ± 3.92^{Aa}	54.15 ± 0.23^{Ab}
	5	76.75 ± 1.58^{Ab}	61.75 ± 0.79^{Aa}	57.34 ± 2.04^{Aa}
	10	71.62 ± 2.23^{Ac}	72.56 ± 6.14^{Aa}	64.23 ± 4.41^{Ab}
	15	56.82 ± 1.87^{Ad}	41.89 ± 1.72^{Ab}	25.52 ± 1.98^{Ac}
	20	57.79 ± 6.10^{Ae}	14.54 ± 4.59^{Ac}	3.84 ± 2.08^{Ad}
	25	44.59 ± 8.58^{Af}	5.45 ± 1.86^{Ad}	0.00 ± 0.00^{Ad}
DMSO	2	68.61 ± 4.54^{Ba}	34.65 ± 5.00^{Ca}	35.81 ± 8.13^{Bb}
	5	66.78 ± 0.54^{Bb}	42.72 ± 3.11^{Ca}	40.55 ± 5.64^{Ba}
	10	43.49 ± 4.65^{Bc}	32.95 ± 1.50^{Ca}	12.09 ± 4.12^{Bb}
	15	31.90 ± 3.85^{Bd}	2.80 ± 1.34^{Cb}	0.00 ± 0.00^{Bc}
	20	31.90 ± 3.63^{Be}	2.15 ± 0.58^{Cc}	0.00 ± 0.00^{Bd}
	25	11.11 ± 1.55^{Bf}	0.00 ± 0.00^{Cd}	0.00 ± 0.00^{Bd}
Glycerol	2	78.39 ± 3.22^{Ca}	40.31 ± 9.67^{Ba}	10.52 ± 2.46^{Bb}
	5	57.48 ± 3.71^{Cb}	27.85 ± 4.84^{Ba}	25.58 ± 4.70^{Ba}
	10	26.53 ± 0.96^{Cc}	28.77 ± 3.51^{Ba}	25.54 ± 5.44^{Bb}
	15	21.82 ± 2.91^{Cd}	25.12 ± 5.16^{Bb}	21.26 ± 3.20^{Bc}
	20	12.80 ± 3.19^{Ce}	12.34 ± 1.60^{Bc}	6.27 ± 2.26^{Bd}
	25	4.50 ± 1.82^{Cf}	3.99 ± 2.50^{Bd}	0.00 ± 0.00^{Bd}

Note: Vertical difference of capital and normal superscript letters mean statistical difference ($p<0.05$) regarding to the type of cryoprotectant and concentration, respectively.

Cryopreservation procedure

Cryopreservation tests were evaluated by comparing various extenders and freezing rates. Results on sperm motility are summarized in Table 2 and Figure 1. Methanol, selected from the previous experiment due to its low toxicity, was added at 10% (v/v) in each experimental condition as a cryoprotectant. The pre–freeze (equilibrated) sperm motility in 6% fructose, 5% glucose and GFR with 10% methanol were 93.26±2.73, 94.37±3.54 and 94.04±3.13%, respectively. There was no statistical difference among these three cryodilutents ($p>0.05$). Results from post–thawed semen showed that both extender types and cooling rates strongly affected the post–thawed sperm motility ($p<0.05$). Thawed semen from conditions using 6% fructose and 5% glucose with cooling rates of 2 and 5° C/min resulted in a higher sperm motility than that of GFR with cooling rate of 10 °C/min.

Table 2. Sperm motility (%) of thawed semen in three extenders and at three cooling rates.

Cooling rate (^{0}C/min)	Extender	Sperm motility (%)	
		Pre freeze	Thawed
2	6% fructose	93.26±2.73	89.58±1.14^{Aa}
	5% glucose	94.37±3.54	88.96±0.37^{Aa}
	Ginsberg Fish's Ringer	94.04±3.13	85.83±2.41^{Ab}
5	6% fructose	93.26±2.73	88.88±2.51^{Aa}
	5% glucose	94.37±3.54	90.21±1.18^{Aa}
	Ginsberg Fish's Ringer	94.04±3.13	88.35±3.62^{Ab}
10	6% fructose	93.26±2.73	88.59±2.86^{Ba}
	5% glucose	94.37±3.54	93.69±0.89^{Ba}
	Ginsberg Fish's Ringer	94.04±3.13	63.68±4.38^{Bb}

Note: Vertical difference of capital and normal superscript letters mean statistical difference ($p<0.05$) re: cooling rate and type of extender, respectively.

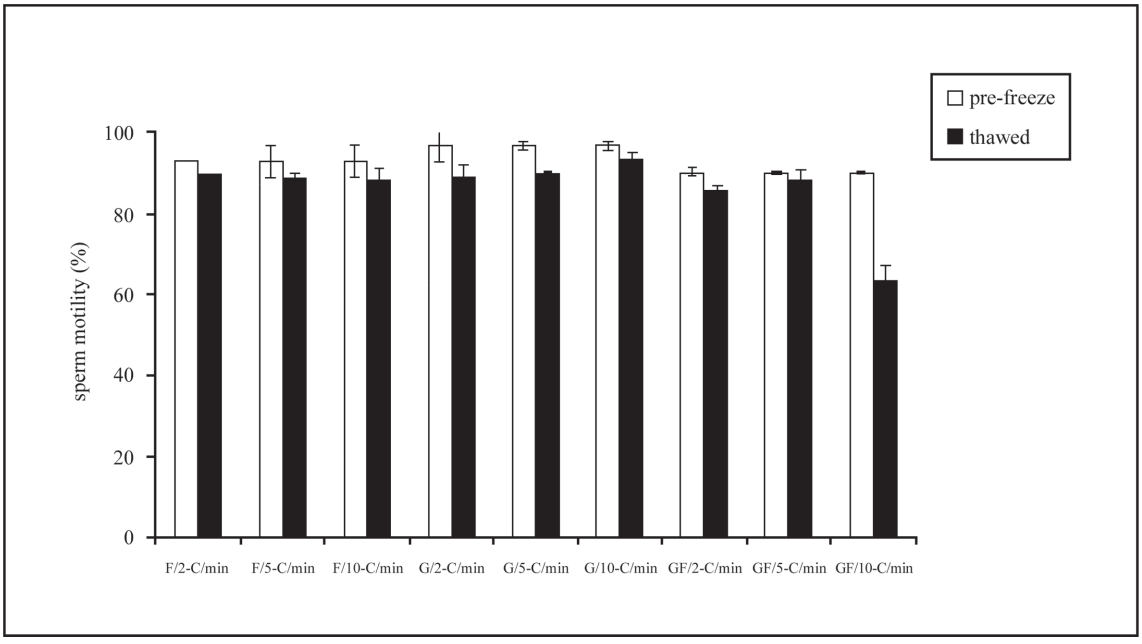

Figure 1. Effect of extender F (6% fructose), G (5% glucose) and Ginsburg fish's Ringer (GF) and cooling rate (2, 5 and 10°C/min) on sperm motility (%) at the end of equilibration period (pre-freeze) and after freezing (thawed).

Estimation of sperm fertilization potential

The sperm fertilization potential was determined and results are summarized in Table 3 and Figure 2. Cryosperm, using 6% fructose and 10% methanol as cryodiluent at a cooling rate of 5°C/min, was stored for 365 days and thawed at 37°C for 8 seconds before using. The result was compared with that of fresh sperm prepared from similar condition of extender and dilution ratio. Sperm motility and live sperm density of thawed sperm were 43.90 ±7.06% and $0.59\pm0.11\times10^9$ cell/ml, respectively, whereas those of fresh sperm were 83.32±4.23% and $1.12\pm0.05\times10^9$ cell/ml, respectively.

Thawed and fresh sperm were inseminated with eggs at 133.33 gm/ml or 35,000 egg/ml. Sperm motility and live sperm density of thawed semen were lower than that of the fresh semen. However, the percentage of eyed stage egg of fresh and thawed sperm was not statistically different ($p>0.05$). Percentage of eyed stage egg of fresh and thawed semen were 72.16±10.32 and 73.43±13.93 %, respectively.

Table 3. Sperm motility (%), total and live spermatozoa density (cell/ml), number of live spermatozoa/egg and percentage of eyed stage egg of Bagrid catfish's egg inseminated with fresh and thawed semen

	Fresh semen	**Thawed semen**
Sperm motility	83.32 ± 4.23^{a}	43.90 ± 7.06^{b}
Total spermatozoa density	$1.34 \pm 0.02 \times 10^{9a}$	$1.34 \pm 0.03 \times 10^{9a}$
Live spermatozoa density	$1.12 \pm 0.05 \times 10^{9a}$	$0.59 \pm 0.11 \times 10^{9b}$
No. of live spermatozoa/egg	$2.15 \pm 0.09 \times 10^{5a}$	$1.13 \pm 0.20 \times 10^{5b}$
Percentage of eyed stage egg	72.16 ± 10.32^{a}	73.43 ± 13.39^{a}

Note: Horizontal difference of capital and normal superscript letters mean statistical differences ($p<0.05$) between fresh and thawed semen.

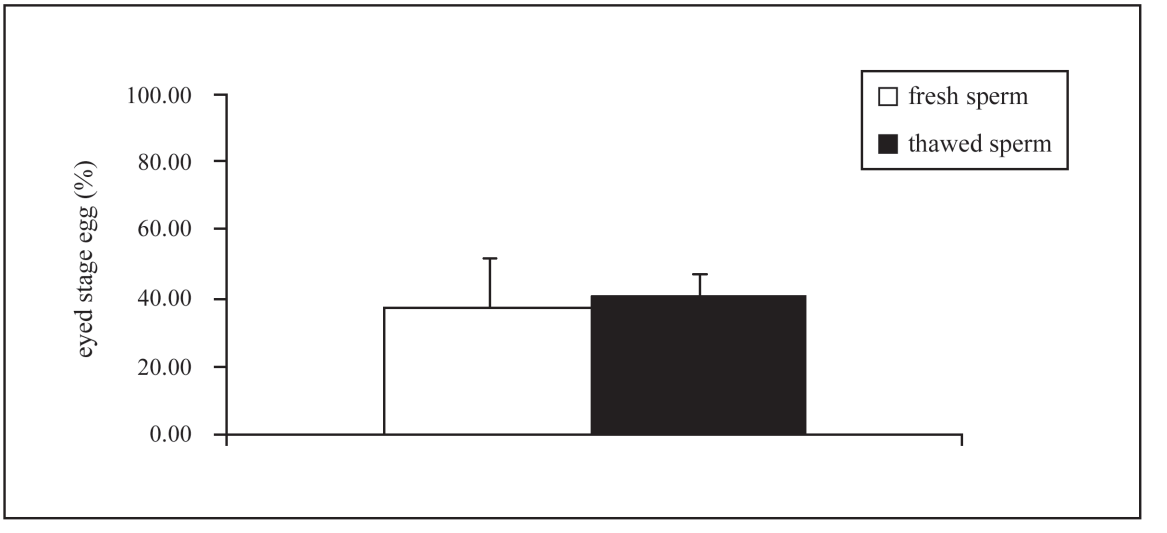

Figure 2. Percentage of eyed stage egg between thawed and fresh sperm

DISCUSSION

Results from the cryoprotectants study demonstrated the toxic effect of cryoprotectants on Bagrid catfish's sperm. Decreasing sperm motility was associated with increasing cryoprotectant concentration at 3 periods post exposure. However, addition of cryoprotectant at optimal concentration to fish semen is very essential for cryopreservation success. Methanol, DMSO and glycerol are commonly used as permeating cryoprotectants in many fish species at 5–15 % concentration (McAndrew *et al*., 1995; Zhang, 2004). Damage of sperm cell caused by permeating cryoprotectants was observed, which was due to sperm dehydration when the concentration equilibration was maintained (Leung, 1991; Denniston *et al*., 2000). From this research, it was concluded that methanol was the least toxic cryoprotectant to Bagrid catfish's sperm. The superiority of methanol over DMSO and glycerol may be related to the property of small-molecular-weight cyroprotectant which could easily penetrate into cell faster than the high-molecular-weight cryoprotectant (Denniston *et al*., 2000). Thus, methanol (CH_3OH, 32.04 g/mol) penetrated into sperm cell faster than DMSO ($(CH_3)_2SO$, 78.13 g/mol) and glycerol ($C_3H_5(OH)_3$, 92.09 g/mol). Methanol also required a shorter equilibration period than for DMSO and glycerol. According to Table 1, the toxicity of cryoprotectants is highly related to exposure time. This may help explain the fact why methanol was the least toxic to Bagrid catfish's sperm. Low toxicity of methanol was also reported by Mongkonpunya *et al*. (2000), in which it have been reported that methanol had the lowest toxicity to striped catfish (*Pangasius hypopthalmus*), walking catfish (*Clarias macrocephalus*) and Mekong giant catfish (*Pangasianodon gigas*) sperm. In addition, methanol was also found superior to other cryoprotectants in cryopreservation of sperm in Cichlid (*Sarotherodon mossambicus*), Zebrafish (*Danio rerio*) and Channel catfish (*Ictalurus punctatus*) (Harvey, 1983; Tiersch *et al*., 1994).

Cryopreservation procedure was determined by using three extenders, combined with 10% methanol and at three cooling rates. Methanol at 10% concentration was chosen based on the toxicity result from the first experiment. Methanol had the lowest toxicity, and 10% concentration of methanol was considered as commonly used in general fish species (McAndrew *et al*., 1995). The concentration of 10% methanol was sufficient to suppress cell dehydration because of the solute effect, which occurred during cryopreservation progress at –20°C (Leung, 1991). Results from the cryopreservation trial showed the interaction effect between extenders and cooling rates. The conditions using 6% fructose or 5% glucose as extender and cooling rates at 2 and 5°C/min showed higher thawed sperm motility than from the treatment using GFR at 10°C/min cooling rate. The use of a sugar solution, such as glucose and fructose, has been applied in Formosan landlocked salmon (*Oncorhynchus masou formosanus*) (Gwo *et al*., 1999), and in sharptooth catfish (*Clarias gariepinus*) (Steyn *et al*., 1985; Urbanyi *et al*., 2000), where it was found that glucose and fructose solution had high potential for cryopreservation. Generally, cooling rate is considered as a critical factor in cryopreservation success. Theoretically, cooling rate depends on cell volume, and it is species specific (Steyn

et al., 1985). Using cooling rates of 2 and 5°C/min obtained higher thawed sperm motility than 10°C/min which indicated that Bagrid catfish sperm preferred a lower cooling rate for cryopreservation. Interestingly, the superiority of a sugar solution above seminal plasma mimicking solution such as GFR may be due to the non–permeating cryoprotection property of fructose and glucose (Leung, 1991), and these sugars may associate with methanol, the main permeating cryoprotectant. This may lead to cryoprotectant synergism, causing higher thawed sperm motility than with GFR.

Result from the artificial breeding demonstrated that the fertilization potential of fresh and cryosperm (which had been stored for 365 days) was not significantly different. Although sperm quality including sperm motility, live sperm density and number of live sperm/egg of fresh semen was higher than that of the cryopreserved semen. This result is similar with that of a previous research in sharptooth catfish (*C. gariepinus*), another catfish species with similar method of semen collection from testes. In sharptooth catfish experiment, the result found that thawed semen from treatment condition using 5% glucose as extender, 5% glycerol as cryoprotectant, cooling rate of 6.5 and 11°C/min and with 480 days storage gave a 51% hatching rate. This result was not significantly different from that of fresh semen as well (Steyn and Van Vuren, 1987). Interestingly, the number of surviving sperm/egg from thawed and fresh sperm was $1.13\pm0.20\times10^5$ and $2.15\pm0.09\times10^5$ cells, respectively. Although fresh sperm had almost twice the value than thawed sperm, the percentage of eyed stage egg between thawed and fresh sperm was not different. Fresh and thawed sperm in this experiment were diluted at 5-folds. This indicated that fresh semen could be diluted $3\times5 = 15$ folds dilution in order to inseminate with eggs. This is a very important suggestion for artificial breeding of Bagrid catfish because the more dilution of semen will result in a greater volume of semen and more convenience in using. Ultimately, it increases the chance of sperm to inseminate with egg. However, the optimal ratio of sperm and egg in Bagrid catfish was still unclear thus further research in finding the optimum ratio is recommended in the future.

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