Efficient Cryopreservation of Humpback Grouper, *Cromileptes altivelis* (Valenciennes, 1828) Spermatozoa

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ABSTRACT

Chilled storage of humpback grouper, *Cromileptes altivelis* (Valenciennes, 1828) sperm cells with five extenders was investigated to find the most suitable type of extender for cryopreservation. Five extenders, namely Marine Fish Ringer (MFR), Extender 251 (E 251), Extender 189 (E 189), 0.1 M Sodium Citrate (CT) and 0.9% NaCl (NaCl) were tested. They proved to be appropriate extenders to use since no sperm were motile and all were still alive at 216 h (60 h after chilled storage). There was also no significant difference (p<0.05) in sperm motility when sperm was diluted with extender at 1:1, 1:4 and 1:9. Toxicity to sperm cells was studied with 5 different cryoprotectants i.e. Dimethyl acetamide (DMA), Dimethyl sulfoxide (DMSO), Methanol, Glycerol and Trehalose at 5, 10 and 15% concentrations. The result shows a significant difference (p<0.05) in sperm motility at 5 and 10% of DMA and DMSO; they were the least toxic cryoprotectants. The hatching rates of Humpback grouper eggs from fresh and cryopreserved sperm in 5% DMA and DMSO were high and had no significant difference (p<0.05). [There was no significantly difference (p>0.05) between fresh sperms and sperms cryopreserved in 5% DMSO, but had a significant difference (p<0.05) between fresh sperms and those cryopreserved in 5% DMA.]

Keywords: spermatozoa, Humpback grouper, Cromileptes altivelis, cryopreservation, extender, cryoprotectant

INTRODUCTION

Humpback grouper is a marine fish of high commercial value with tasty meat. It is mainly consumed in Hong Kong and China. In 1998, China and Hong Kong imported 2,000 and 6,500 t of live grouper, respectively (Rimmer *et al*., 2004). There has always been a high demand for humpback grouper; however, since the supply is not enough, it commands a high price in the market. As majority of the grouper are still captured

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from the wild, the high demand and high price have resulted in overfishing, thereby reducing its population. It is now seldom found in the wild. Even though the Department of Fisheries has succeeded in breeding the humpback grouper in captivity many years ago, the quantity of seed produced is still low due to problems with broodstock, especially with the male. Several problems exist in humpback grouper seed production and larval nursing. One critical factor is the availability of healthy brood fish because it usually takes at least 3 to 5 years of culture before they can be functional broodstock. In general, the species naturally reverses its sex at a certain age and size. This often results in unsynchronized maturation between male and female broodstock in captivity, i.e. the females have mature eggs but males have no sperm, or vice versa.

Majority of domesticated groupers such as *Epinephelus* spp. change sex from female to male (protogynous hermaphrodite); after a period of time, a particular female fish turns to male (Yashiro, 1998). Humpback groupers caught by Tridjoko (2002) which were less than 50 cm were females and they started changing to male when they reached 50 to 55 cm in length, and they eventually become fully male when longer than 55 cm. Yashiro *et al.* (1993) found that *E. malabaricus* started to develop its ovaries at the age of 15 months with 89% functional females, 3.5% in the transition stage and 7.0% functional males. This indicated that at the beginning of its reproductive stage, the number of females was greater than the males.

There are two methods of preservation of fish sperm, i.e. short term preservation for 2 – 4 days at 0 – 4ºC, and long term preservation in liquid nitrogen at –196ºC. As long as the liquid nitrogen is always topped up, the sperm is preserved in good condition (Mongkonpunya, 1993). Therefore, this technique is very important and if the humpback grouper sperm is successfully preserved, seed production will also be successful because the problem of lack of males to provide the sperm when needed will be overcome.

MATERIALS AND METHODS

Experimental design and statistical analysis

The research was divided into three phases, namely, 1) study on type and effect of extenders on cryopreservation; 2) study on type and concentration of cryoprotectant; and, 3) study on the hatching rate of humpback grouper eggs with fresh and chilled sperm. A Completely Randomized Design (CRD) was used to design the experiment. The percentage of motility and viability of sperm cells were recorded. The motility percentage was analyzed by non-parametric test since the data were from the means and neither from the normal population nor heterogeneous population variance. Both viability and motility percentages were Arcine transformed before analysis because they had great differences in variance (Bartlett, 1973). Single factor and multi-factor analyses were also done, as well as testing differences between treatments using Duncan's Multiple Range Test (DMRT).

Experiment 1. Study on types and dilution ratio of extender and its effect on cryopreservation of humpback grouper

Mature humpback grouper (*C. altivelis*) males from the Coastal Fisheries Research and Development Center (CFRDC) in Rayong were selected on December 25, 2007 and tagged with microchips to prepare them for the experiment. Out of 25 fish, 6 were mature males with milt. The fish were tranquilized with 5 ppm of Quinaldine, then the superficial mucus and water on the abdomen were removed. The sperm was collected by pressing the genital area and stroking downwards towards the urogenital pore, letting the milt drop into a sterile beaker, making sure that there was no contamination from seawater or other agents from the fish such as urine and faeces. The collected milt was then centrifuged at 20,000 rpm at 4ºC for 10 minutes. The clear portion on the top (seminal fluid) was collected to measure osmolality using an osmometer. The remainder of the milt was used for quality assessment.

The five types of extenders were Marine Fish Ringer (MFR), Extender 251 (E 251), Extender 189 (E 189), 0.1 M Sodium Citrate (CT), and 0.9% Saline (NaCl). Their osmolalities were also measured.

Study on sperm's motility

One drop each of distilled water (20 µl) and milt (1 µl) were mixed together on a glass slide, then the motility was checked under a microscope (40X). The motility was graded into 10 levels (Mangampun, 1990).

Study on viability percentage by Eosin-Nigrosin stain

A drop each of Eosin-Nigrosin (5 µl) and milt (1 µl) were put on a glass slide and then mixed. Another glass slide or cover glass was pressed once on the mixture. The slide was then put over a flame once or twice to dry the mixture. A drop of topcoat was put onto the glass slide, covered and viewed under a microscope (100X).

The dead cells were counted randomly in 5 different locations and 20 cells per location. Cells which remained white and unstained were considered alive and those which were stained, either purple or pink were considered dead.

Sperm concentration

This was determined by counting the number of sperms in one quantity unit. First, fresh milt was diluted in saline or seawater at ratios of 1:1000, 1:1500 and 1:2000. The mixtures were then shaken then let to stand for 5 minutes to settle the sperms. After this they were shaken well again, then some amount was transferred by micropipette to a hematocrit, where they were left 5 minutes before counting the sperm cells under the microscope at 100X or counted with a Makler counting chamber (Sefi-Medical Instruments) by pipetting 5 µl of milt and dropped onto the center of the chamber and covered with a slide. When a spectrum could be seen, the cover and the chamber were marked. This showed that the sperm distribution was in 10 microns (there must be no air). Both motile and non-motile sperms were counted using a microscope (20X). The number of cells counted in 10 slits on the chamber represented concentration of sperms in a million per milliliter.

The efficiency of the different types of extenders was tested based on motility and viability percentage of sperms. The tests were done at milt to extender ratios of 1:1, 1:4 and 1:9 and after 60 hrs chilled storage (0º – 4ºC)

Experiment 2. A study on types and concentration of cryoprotectants and their effect on cryopreservation of humpback grouper sperm

This process was possible only after Experiment 1 was conducted and the appropriate extender and sperm ratio was obtained

Preparation of cryoprotectant

Each of the five types of cryoprotectants i.e. DMA, DMSO, MeOH, glycerol and trehalose were prepared at concentrations of 5, 10 and 15%. The cryoprotectants were diluted with an appropriate extender from Experiment 1, and divided into 2 equal portions. The first portion was diluted with the appropriate extender without cryoprotectant (portion A). The other portion was diluted with the appropriate extender in cryoprotectant at double concentration of the desired final concentration (portion B). In diluting the sperm, the first portion was added to the sperm, then shaken well and the mixture of cryoprotectant was added. It was shaken again before storing in an ice box. When mixing A and B, the concentration of cryoprotectant becomes half and the final concentration will be obtained. For example, if the final concentration of 5% is desired, prepare B at 10% concentration. Before deep freezing, the temperature of preserved sperms was reduced by using a temperature controller. After dilution with cryoprotectant, 480 µl of sperm was put into a tube, which was then closed and sealed by flamed forceps tips. The temperature was reduced at the rate of 10ºC per minute until the final temperature of –80ºC was reached. The tubes were kept in liquid nitrogen at –196ºC. After 48 hrs of preservation, the sperm was thawed by soaking the tubes in a water bath at 37ºC for 10 seconds. A sampling of the sperm was done to check motility of cells following the procedure by Mangampun (1990).

Experiment 3. A study on the effect of fresh and chilled sperm on hatching rate of humpback grouper

This process was possible only after obtaining results from Experiment 2 in determining the appropriate cryoprotectant for humpback grouper.

In preparing the fresh sperm, male humpback groupers were induced 1:9 for spermiation by mixed hormone (Puberogen) prior to stripping. The stripped milt was then mixed with the appropriate extender at a ratio of 1:9 (milt:extender).

Each cryoprotectant i.e. DMA, DMSO, MeOH, glycerol and trehalose was prepared to obtain their final concentration of 5, 10 and 15% to find out the best type and concentration which could give the highest percentages of motility and viability.

Female humpback groupers were induced by injecting mixed hormone (Puberogen) in order to obtain ripe eggs. The broodfish were stripped and 300 µl of eggs were chosen and counted. The sampled eggs were transferred to a Petri dish. Then fresh sperm and chilled sperm were respectively added to the eggs for fertilization. The fertilized eggs were put into the hatching tanks. The hatched eggs were again sampled and counted under low magnification microscope.

Regarding the test on the effect of fresh and chilled sperms on hatching, 0.9% NaCl and 5% DMA and DMSO were used as extender and cryoprotectants, respectively.

A two-step temperature reduction at 10°C per min to the final temperature of -80°C was followed, with equilibration time of 20 min after 52 hrs in liquid nitrogen, then thawing at 32°C for 20 sec after 24 hrs hatching.

RESULTS

Experiment 1. Study on types and dilution ratio of extender and its effect on cryopreservation of humpback grouper

The effect of extenders on cryopreservation of humpback grouper sperms

Viability, motility percentages and sperm concentration were analyzed. Six samples of humpback grouper sperms were prepared to check sperm concentration. The average concentration was 7.3 – 8.4 x 10^9 cells/ml. The average motility was 75–100% and average viability was 77-98% (stained with Eosin-Nigrosin). The osmolality of the five extenders and the humpback grouper seminal fluid ranged from 228 to 438 mOsmol/kg (Table 1).

Table 1. Osmolality of extenders and seminal fluid of humpback grouper from Experiment 1

Types	Osmolality (average) (mOsmol/kg)
Marine Fish Ringer (MFR)	438 ± 1.00
Extender 251 (E 251)	329 ± 1.41
Extender 189 (E 189)	295 ± 1.00
0.1 M Sodium Citrate (CT)	228 ± 1.15
0.9% NaCl	274 ± 0.58
Seminal fluid of humpback grouper	320 ± 0.50

The effect of extenders on preservation by chilling at 0–4°C was also studied. Motility and viability were assessed and it was observed that sperms mixed with the extenders stopped moving at 216 hrs. After 60 hrs of chilling the average percentages of motility and viability from each extender were not significantly different ($p>0.05$) (Tables 2 and 3).

Study on the optimum dilution (sperm to extender ratio) for the preservation of humpback grouper sperm

The effect of three dilution ratios of sperm to extender i.e. 1:1, 1:4 and 1:9 on preservation by chilling at 0–4°C was studied. Motility and viability were assessed and it was observed that sperms stopped moving after 216 hrs when mixed with each of the five extenders at three dilution ratios. After chilling for 60 hours, the average percentages of motility and viability from the three dilution ratios of sperm to extender were not significantly different ($p>0.05$) (Tables 4 and 5).

Table 2. The motility percentage of humpback grouper in five extenders from 0 to 60 hrs

Hour	Extender				
	MFR	E 251	E 189	0.1 M CT	NaCl 0.9%
0	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]
0.5	88.89 ± 3.33[a]	87.78 ± 4.41[a]	88.89 ± 3.33[a]	87.78 ± 4.41[a]	88.89 ± 3.33[a]
1.5	87.78 ± 4.41[a]	86.67 ± 5.00[a]	88.89 ± 3.33[a]	87.78 ± 4.41[a]	88.89 ± 3.33[a]
3	86.67 ± 5.00[a]	86.67 ± 5.00[a]	88.89 ± 3.33[a]	87.78 ± 4.41[a]	88.89 ± 3.33[a]
6	86.67 ± 5.00[a]	85.56 ± 5.27[a]	88.89 ± 3.33[a]	87.78 ± 4.41[a]	88.89 ± 3.33[a]
12	86.67 ± 5.00[a]	84.44 ± 5.27[a]	85.56 ± 7.26[a]	86.67 ± 7.07[a]	88.89 ± 3.33[a]
24	83.33 ± 5.00[a]	75.56 ± 5.27[a]	82.22 ± 6.67[a]	84.44 ± 10.14[a]	87.78 ± 4.41[a]
36	81.11 ± 7.82[a]	68.89 ± 6.01[a]	8.89 ± 7.82[a]	82.22 ± 9.72[a]	85.56 ± 5.27[a]
48	73.33 ± 8.66[a]	62.22 ± 6.67[a]	70.00 ± 14.14[a]	77.78 ± 9.72[a]	81.11 ± 9.28[a]
60	71.11 ± 9.28[a]	46.67 ± 13.23[a]	57.78 ± 13.94[a]	70.00 ± 12.25[a]	72.22 ± 9.72[a]

Remarks: The same superscripts in the same row show that differences are not significant at 95% confidence

Table 3. The viability percentage of humpback grouper in 5 extenders from 0-60 hrs

Hour	Extender				
	MFR	E 251	E 189	0.1 M CT	NaCl 0.9%
0	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]
0.5	97.22 ± 1.79[a]	97.44 ± 1.59[a]	95.22 ± 2.28[a]	95.56 ± 1.67[a]	97.67 ± 1.41[a]
1.5	88.11 ± 2.67[a]	84.56 ± 5.34[a]	84.44 ± 4.36[a]	89.00 ± 4.00[a]	90.22 ± 4.24[a]
3	87.44 ± 6.09[a]	86.11 ± 4.59[a]	89.44 ± 5.57[a]	88.67 ± 6.65[a]	94.44 ± 2.24[a]
6	83.11 ± 4.99[a]	85.67 ± 5.07[a]	84.78 ± 4.29[a]	83.89 ± 5.42[a]	86.56 ± 2.79[a]
12	76.78 ± 5.61[a]	79.00 ± 5.63[a]	76.22 ± 6.78[a]	74.22 ± 8.50[a]	79.44 ± 7.02[a]
24	76.33 ± 6.20[a]	80.56 ± 4.10[a]	77.56 ± 5.83[a]	73.78 ± 11.68[a]	77.67 ± 5.96[a]
36	73.11 ± 3.44[a]	72.22 ± 6.85[a]	74.67 ± 3.08[a]	70.22 ± 6.10[a]	71.00 ± 6.00[a]
48	70.78 ± 4.35[a]	71.67 ± 2.87[a]	71.22 ± 2.68[a]	69.67 ± 3.71[a]	75.22 ± 3.07[a]
60	67.44 ± 3.09[a]	69.22 ± 4.89[a]	68.11 ± 3.33[a]	66.11 ± 4.04[a]	69.22 ± 2.95[a]

Remarks: The same superscripts in the same row show that differences are not significant at 95% confidence.

Table 4. The motility percentage of humpback grouper in 3 dilution ratios of 5 extenders after 60 hrs of chilling

Extenders	Dilution ratios		
	1:1	1:4	1:9
MFR	63.33 ± 5.77 [a]	76.67 ± 5.77 [a]	73.33 ± 11.55 [a]
E 251	63.33 ± 5.77 [a]	40.00 ± 0.00 [a]	36.67 ± 5.77 [a]
E 189	88.11 ± 2.67 [a]	84.56 ± 5.34 [a]	84.44 ± 4.36 [a]
0.1 M CT	80.00 ± 10.00 [a]	73.33 ± 5.77 [a]	56.67 ± 5.77 [a]
NaCl 0.9%	66.67 ± 15.28 [a]	80.00 ± 0.00 [a]	70.00 ± 0.00 [a]

Remark: The same superscripts in the same row show that differences are not significant at 95% confidence.

Table 5. The viability percentage of humpback grouper in 3 dilution ratios of 5 extenders after 60 hrs of chilling

Extenders	Dilution ratios		
	1:1	1:4	1:9
MFR	65.33 ± 4.04 [a]	68.67 ± 1.15 [a]	68.33 ± 3.21 [a]
E 251	66.67 ± 2.31 [a]	72.33 ± 0.50 [a]	68.67 ± 8.08 [a]
E 189	68.67 ± 1.53 [a]	68.67 ± 2.89 [a]	67.00 ± 5.576 [a]
0.1 M CT	68.67 ± 4.93 [a]	67.33 ± 1.15 [a]	62.33 ± 2.52 [a]
NaCl 0.9%	66.33 ± 2.89 [a]	69.67 ± 1.53 [a]	71.67 ± 1.53 [a]

Remark: The same superscripts in the same row show that differences are not significant at 95% confidence.

Experiment 2. A study on types and concentration of cryoprotectants and their effect on cryopreservation of humpback grouper sperm

Toxicity tests were conducted on the five cryoprotectants i.e. DMA, DMSO, methanol, glycerol and trehalose at three concentrations i.e. 5, 10 and 15 by diluting sperm in 0.9% NaCl solution (from Experiment 1). The motility and viability of sperm were also assessed after thawing.

This study found that increased concentration of up to15% resulted in low motility rates compared to concentrations at 5 and 10%, when tested with DMA, DMSO, methanol and glycerol ($p<0.05$) (Table 6). Three concentrations of 5, 10 and 15% DMA, methanol and trehalose did not affect viability rates ($p>0.05$) (Table 7). Average viabilities percentage at DMSO concentrations of 5 and 10% were not significantly different ($p>0.05$) but they were significantly different at 15% DMSO concentration (Table 7).

Table 6. The average motility of humpback grouper sperm after cryopreservation using different cryoprotectants

Cryoprotectants	Concentration (%)		
	5	10	15
DMA	80.00 ± 0.00 [a]	80.00 ± 0.00 [a]	0.00 ± 0.00 [b]
DMSO	80.00 ± 0.00 [a]	80.00 ± 0.00 [a]	56.30 ± 1.80 [b]
MeOH	67.50 ± 1.60 [a]	62.50 ± 1.60 [a]	12.50 ± 1.60 [b]
Glycerol	10.00 ± 0.00 [a]	10.00 ± 0.00 [a]	0.00 ± 0.00 [b]
Trehalose	60.00 ± 0.00 [a]	47.50 ± 3.10 [a]	42.50 ± 1.60 [a]

Remark: Different superscripts in the same row show significant differences at 95% confidence

Table 7. The average viability of humpback grouper sperm after cryopreservation using different cryoprotectants

Cryoprotectants	Concentration (%)		
	5	10	15
DMA	75.00 ± 1.81 [a]	73.25 ± 2.05 [a]	75.63 ± 2.45 [a]
DMSO	76.88 ± 0.99 [a]	74.38 ± 0.84 [a]	57.88 ± 1.75 [b]
MeOH	69.00 ± 2.60 [a]	66.83 ± 1.29 [a]	61.38 ± 1.32 [a]
Glycerol	34.75 ± 2.14 [a]	26.88 ± 1.04 [b]	25.88 ± 0.83 [b]
Trehalose	41.25 ± 3.97 [a]	42.88 ± 1.67 [a]	33.13 ± 1.88 [a]

Remark: Different superscripts in the same row show significant differences at 95% confidence.

The study on motility and viability of humpback grouper sperm

Considering a single factor i.e. type of cryoprotectant, DMSO was found to maximize the motility and viability percentages of humpback grouper sperm after cryopreservation as they were higher than those from DMA, MeOH, trehalose and glycerol.

In terms of cryoprotectant concentration, 5% DMSO provided the best result, and since there was no difference with the 10% concentration, it could be assumed that the lower concentration is better.

In conclusion, the use of DMA, DMSO, MeOH, glycerol and trehalose at three concentrations (5, 10 and 15%) resulted in significant differences ($p<0.05$) in viability of humpback grouper sperm. The results show that DMSO at 5% concentration was the most effective cryoprotectant for humpback grouper cryopreservation, followed by DMA at 15 and 5%, DMSO at 10%, DMA at 10%, and MeOH at 5% (Tables 6 – 7).

Experiment 3. A study on the effect of fresh and chilled sperm on the hatching rate of humpback grouper

After injecting puberogen to female humpback grouper, they were stripped within 77 hours. From the random sample of 300 µl of eggs, the average number of eggs was 450 eggs or 150 eggs/100 µl). The eggs were tested by mixing them with fresh and chilled sperms mixed with 5% DMA and DMSO, which contained 7.6 x 10^9 sperm cells. Twenty-four hours after hatching, eggs mixed with fresh sperm had an average hatching rate of 14.87 ± 0.02% while eggs mixed with chilled sperm in DMA at 5% concentration as cryoprotectant had an average hatching rate of 6.60 ± 0.02%. On the other hand, eggs mixed with chilled sperm in DMSO at 5% had an average hatching rate of 16.53 ± 0.02% (Table 8).

Table 8. Average hatching rates of humpback grouper eggs fertilized with fresh and chilled sperm

Type / cryoprotectants	Average (% hatched)
Fresh sperm	14.87 ± 0.02 ^{a}
Chilled sperm with DMA	6.60 ± 0.02 ^{b}
Chilled sperm with DMSO	16.53 ± 0.02 ^{a}

Remark: Different superscripts show significant differences at 95% confidence

DISCUSSION

Types and effect of extenders on cryopreservation of humpback grouper sperm

Motility and viability percentages of sperms chilled with the five types of extenders did not show any significant differences (p>0.05) during the first 60 hours. The osmolality of the following extenders, namely 0.9% NaCl, E 189, MFR, E 251and 0.1M CT were 274, 295, 438, 329 and 228 mOsm kg^{-1}, respectively, which are nearly similar to the osmolality of the body fluid in humpback grouper sperms (320 mOsm kg^{-1}). Studies by Chomputawat (2007) which tested the efficiency of 10 extenders, namely MFR, E 251, 0.1M CT, Calcium Free Hank's Balance Salt Solution (CF-HBSS), Hank's Balance Salt Solution (HBSS), 0.85% NaCl, Cortland, Saline Base (SB), Ringer (R) and Ca-F Saline (CFS), revealed that their osmolalities were 941, 878, 651, 317, 618, 342, 408, 250, 415 and 525 mOsm kg^{-1}, respectively, and that of the red snapper, *Lutjanus argentimaculatus* body fluid at 421 mOsm kg^{-1}. It also found that three extenders i.e. SB, R and CFS were effective in the cryopreservation of red snapper sperm. The osmolalities of the three extenders were obviously closer to that of the fluid in red snapper sperm, especially R whose osmolality was one of the lowest and closest. This caused the sperms to move the least or not at all. Therefore, the osmolality of the chosen extender is important to keep sperm cells alive and viable after the cryopreservation process. This is also true with other species such as yellowfin sea bream, *Acanthopagrus latus* (Gwo, 1994) and black grouper, *Epinephelus malabaricus* (Gwo, 1993). Osmolality is necessarily supportive to sperm motility, thus it can be used as an index of sperm motility. Considerably, osmolality

outside the marine fish sperm cell is higher than inside which causes the sperm to move. These findings were in accordance with Morisawa and Suzuki (1980), and Oda and Morisawa (1993) who stated that osmotic pressure is the key factor controlling the movement of the fish sperm from the very beginning. On the other hand, isotonic phenomenon possibly prevents the movement of the sperm before freezing, thereby reducing energy loss of the sperm cell after thawing.

Results of the study on extender to sperm ratio (1:1, 1:4 and 1:9) with five extenders showed that there was no significant difference (p>0.05) on the motility or viability of sperm among them. Therefore, in practice, 1:9 ratio is considered the most practical since the quantity of sperm required from humpback grouper male is small. Although dilution does not affect the sperm, given that the diluting agent contains appropriate ions and osmotic pressure, it is not advisable to make a dilution because this will use up space and containers for storage. Moreover, there is more possibility to cause frosting when temperature is reduced with more diluting agent (Chomputawat, 2007). If dilution is really necessary, then the ratio should be 1:1 to 1:9 (Mongkonpunya, 1993).

It was observed that the type and dilution of the five extenders did not affect the motilities and viabilities of the humpback grouper sperm differently. However, sperms coagulated when diluted with E189 and E 251. This could be caused by the extenders which contained soy lecithin, causing a lower motility efficiency than the other extenders. This resembles the studies with giant grouper, *E. lanceolatus*, wherein the sperm, which were diluted in E 189 and E 251, showed a lower performance in motilities and viabilities than those diluted in MFR and CT (Yashiro *et al*., 1999). For the humpback grouper sperm, motility and viability percentages depend on age, fertility, environment and size of the brood fish at the time of sperm collection. Sperm motility and viability after diluting in extender decreased gradually over time, which was also in accordance with previous studies conducted by Guest *et al*. (1976) and Yashiro *et al*. (1999). Cryopreservation with the five extenders i.e. 0.9% NaCl, E 189, MFR, E 251, and 0.1 M CT showed that after 60 hours, sperm motilities and viabilities were different, with the sperm chilled in 0.9% NaCl showing the highest efficiency.

Type and concentrations of cryoprotectant for the efficient cryopreservation of humpback grouper sperm

Motility percentages of humpback grouper sperm after being chilled in cryoprotectants (DMA, DMSO, MeOH, glycerol and trehalose) at three concentrations (5, 10 and 15%) were determined. The motility percentage after thawing the chilled sperm was assessed and it was found that the use of DMA and DMSO at 5 and 10% concentration resulted in the highest motilities (Table 6). A good cryoprotectant must be water soluble, low in toxicity, and able to regulate intra and extra-cellular osmotic pressures. Different types and concentrations of cryoprotectants showed different motility percentages. The cryoprotectant to be chosen should be the least toxic and should give the longest and best motility. DMA and DMSO have lower toxicity than MeOH, glycerol and trehalose. It was also observed that sperm mixed with glycerol and trehalose coagulated after thawing, resulting in low motility.

There were significant differences ($p<0.05$) in sperm motilities of humpback grouper using different types and concentrations of cryoprotectants. The best result was found with DMA and DMSO at 5 and 10% concentrations. This agreed with a report which said that the cryoprotectant or cell protectant DMSO and a high concentration of glycerol (20% v/v) could stimulate DNA generation (Ashwood-Smith, 1980). However, cryoprotectants at high concentrations and high temperature become toxic to cells because they denature protein, therefore it was perilous to non-frozen cells (Chao, 1996)

Viability of sperm after preservation was assessed by Eosin and Nigrosin staining using two-step temperature reduction at 10^{o}C per minute to reach the final temperature of -80^{o}C, with equilibration time of 10 to 30 minutes after 48 hours in liquid nitrogen. It was found that the effect of different cryoprotectants on survival rate of sperm after cryopreservation was significantly different ($p<0.05$) when considering only the type of cryoprotectant used. DMA gave the highest viability of humpback grouper sperm after cryopreservation. The best concentration was at 5%, although this was not significantly different from 10%. It could be concluded that based on the significant differences in viabilities of humpback grouper sperm by the different types and concentrations of cryoprotectants, the best choice is DMSO at 5% concentration. This is followed by DMA at 15%, DMA 5%, DMSO 10%, DMA 10% and MeOH 5% (Table 7). The results agree with a report by Suquet *et al*. (2000) which stated that the best extenders for marine brood fish were saline and sugar solutions, with DMSO acting as the best cryoprotectant for marine fish. Nonetheless, DMSO showed higher toxicity in fish as concentration increased, as observed in studies done with Atlantic croaker, *Micropogonias undulates* (Gwo *et al*, 1991), barramundi, *Lates calcarifer* (Leung, 1987), black grouper, *E. malabaricus* (Gwo, 1993), and grouper, *E. tauvina* (Withler and Lim, 1982).

Effect of fresh and chilled sperms on the hatching rate of humpback grouper

The condition of the sperm (whether fresh or chilled) could affect the hatching rate of humpback grouper eggs. The use of DMSO as cryoprotectant at 5% concentration resulted in the highest hatching rate although it was not significantly different ($p>0.05$) from the others except with DMA at 5% concentration.

ACKNOWLEDGEMENTS

The authors wish to thank the Rayong Coastal Fisheries Research and Development Center, Coastal Fisheries Research and Development Bureau, Department of Fisheries for providing the facilities support and to Kasetsart University Research and Development Institute for the financial support, which made this study possible.

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