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CRYOPRESERVATION OF SPERM OF RED ABALONE (*HALIOTIS RUFESCENS*)

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ABSTRACT Abalone culture, a developing industry in Baja California, Mexico, would benefit from genetic improvement and controlled breeding. The use of cryopreserved sperm would allow germplasm availability, and this study was designed to develop sperm cryopreservation protocols for red abalone *Haliotis rufescens*. The acute toxic effects of the cryoprotectants dimethyl sulfoxide (DMSO), propylene glycol (PG), and glycerol (GLY) were assessed after suspending sperm in different concentrations, whereby cryoprotectant treatments of 10% DMSO and 10% GLY equilibrated for 10 min yielded the highest range of motile sperm in preliminary freezing trials and were used for cryopreservation studies. To determine effective cooling rates, three freezing chambers were tested. Replicate samples of sperm from 4 males were placed in 0.5-mL French straws and frozen using a commercial freezing chamber (CFC) used for bull sperm, a programmable rate chamber (PRC), and a manually controlled styrofoam chamber (MCC). For the CFC, the cooling rate was 16°C/min, from 4°C to −140°C. For the PRC and MCC, it was 1°C/min, from −20°C to −30°C. The samples were held at −30°C for 5 min before being plunged into liquid nitrogen (−196°C) for storage, and each sample was thawed in a water bath at 45°C for 8 s. The quality of thawed sperm was determined by estimating percent motility, evaluating membrane integrity using a dual-staining technique and flow cytometry, and estimating fertilization rate. Statistical analyses were performed using 2-way ANOVA where chamber and treatment were the independent variables. Sperm quality parameters were independent. For motilities, a significant interaction was noted between the cryoprotective treatment and the chamber type, whereby motilities for DMSO and GLY were higher ($P = 0.0055$) using MCC. Membrane integrities were significantly lower after using the PRC than the CFC or the MCC ($P = 0.0167$). The highest post-thaw motility (48 ± 7%) was found using sperm suspended in 10% glycerol and frozen in the MCC. The highest percent of intact membranes (56 ± 11%) was for sperm suspended in 10% glycerol and frozen in the CFC. The highest fertilization rate (29 ± 10%) was with samples frozen with 10% glycerol in the CFC. The use of cryopreserved sperm from red abalone provides an alternative breeding option for culture and the protocols delineated are the first developed for this species.

KEY WORDS: red abalone, *Haliotis rufescens*, cryopreservation, sperm, flow cytometry, fertilization

INTRODUCTION

Abalone inhabit the coasts of most continents, especially in the tropical western Pacific, near Australia, Japan, South Africa and along the northeastern coast of the Pacific margin (Lindberg 1992). Fisheries began in Asia more than 1,500 y ago, and in the 1970s, abalone became economically important as a gourmet food to the Pacific Coasts of Mexico and the United States (Guzmán del Próo and Ortiz-Quintanilla 1972). Five species, popular for human consumption, occur along coasts of Baja California, Mexico. These species include red abalone (*Haliotis rufescens*), pink abalone (*Haliotis corrugata*), green abalone (*Haliotis fulgens*), black abalone (*Haliotis cracherodii*) and white abalone (*Haliotis sorenseni*) (Leighton 2000, Guzmán del Próo 1992). Nonregulated fishing activities and anthropogenic impacts have heightened conservation awareness for abalone, whereby habitat restoration and stocking of native species need to be considered. The creation of germplasm banks would aid in such restoration efforts for abalone species.

Interest in the cryopreservation of gametes and other cell types from abalone has increased in the past decade. Cryopreservation of gametes can improve the production of commercially reared aquatic animals and can assist in the conservation of genetic resources for cultured and endangered species. The main goal of cryopreservation is to establish specific protocols that allow the long-term storage of functional cells (Koebe et al. 1993). At present, there are fewer than 10 publications concerning cryopreservation of gametes or larvae of abalone, and most of these studies have focused on the most important commercial species of Asia (small abalone *H. diversicolor*) (Table 1); however, cryopreservation studies of the most important species of the coast of California in the United States and Baja California, Mexico have not been reported.

The goal of this study was to develop cryopreservation protocols for sperm of red abalone from Baja California, Mexico. The objectives were to: (1) evaluate the acute toxic effect on sperm motility of concentrations of 3 cryoprotectants: dimethyl sulfoxide (DMSO), propylene glycol (PG) and glycerol (GLY); (2) determine effective cooling rates by testing three freezing chambers: a commercial freezing chamber (CFC) used for bull sperm, a manually controlled styrofoam chamber (MCC) and a programmable cooling rate chamber (PRC) and (3) evaluate the quality of thawed sperm by estimating percentage of motility, membrane integrity and fertilization rate.

MATERIALS AND METHODS

Spawning

Ripe red abalone were obtained from a private company, Abulones Cultivados S. de R. L. de C. V., in Ejido Eréndira, Baja California, Mexico (31°20′, 116°29′) and were transported cold inside styrofoam boxes to the Department of Aquaculture of Centro de Investigación Científica y Educación Superior de Ensenada

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TABLE 1.

Relevant cryopreservation studies of abalone species.

Species	Cell Types Studied	Reference
Haliotis gigantean	Sperm	Matsunaga et al. 1983
Haliotis discus	Sperm	Matsunaga et al. 1983
Haliotis diversicolor	Sperm	Tsai & Chao. 1994
Haliotis diversicolor	Eggs	Lin et al. 1992
Haliotis diversicolor	Eggs	Lin et al. 1995
Haliotis diversicolor diversicolor	Eggs and embryos	Lin & Chao 2000
Haliotis diversicolor supertexa	Sperm	Gwo et al. 2002
Haliotis iris	Sperm	Roberts et al. 2000
Haliotis tuberculata	Haemocytes	Poncet and Lebel 2003

(CICESE). The organisms were kept in a recirculating system containing 250 L of natural seawater with salinity of ~35‰ at 15°C. Males, identified by a yellowish color of the gonad (Fallu 1991), were placed together in a plastic container containing 3 L of filtered seawater (FSW). Spawning was induced by adding 2 M TRIS (Sigma Chemical Co., St. Louis, Missouri) for 15 min before adding 0.66 mL of 30% hydrogen peroxide (Clarkson Laboratory and Supplies, Chula Vista, CA) per liter of FSW. Gamete release began ~2 to 3 h after the addition of hydrogen peroxide. When spawning began, males were placed in individual containers with 50 mL of FSW, and sperm were collected in 50-mL centrifuge tubes (Corning Inc., Corning, New York). Sperm were centrifuged at ×200*g* for 5 min, the debris was decanted, and the sperm were centrifuged at ×1000*g* for 10 min (Kopf et al. 1983). Centrifugation is a common procedure to concentrate abalone sperm (Gwo et al. 2002) without affecting sperm quality. The sperm pellet was resuspended in 5 mL FSW and maintained at 4°C until use. The sperm cell concentration was determined by diluting sperm in FSW (1:9) and counting the cells by microscopy in a Neubauer chamber. Sperm from males with >90% motility were used for experimentation.

Acute Toxic Effect of Cryoprotectants on Sperm Motility

Four males were spawned separately, and prior to the addition of cryoprotectants, sperm motility was evaluated for each male with 10-µL aliquots of sperm and observation at ×400 magnification (Olympus, BH-2, Olympus America Inc, New York). Twenty µL of FSW were added to dilute the sample and motility was again evaluated. Motility was also evaluated after fresh sperm samples were suspended in 4 different concentrations (5%, 10%, 15% and 20%: v/v) of DMSO, PG or GLY for 6 equilibration times (5, 10, 15, 20, 25 and 30 min). Each of the solutions was prepared with reagent grade chemicals (Sigma Chemical Co., St. Louis, Missouri). Following the data analyses of the acute toxic effects and after a preliminary freezing trial showing 10% DMSO and 10% glycerol allowing for higher post-thaw motilities than 5% DMSO, these two cryoprotectant treatments were selected for cryopreservation and sperm quality assessments.

Cryopreservation

The same day of collection, samples of concentrated sperm (>90% motility) from four red abalone were transported individually in 15-mL centrifuge tubes (Corning Inc., Corning, New York) via overnight priority delivery to the Louisiana State University Agricultural Center, Aquaculture Research Station, Baton Rouge, Louisiana. All experiments were performed ~24 h after spawning. Upon sample delivery, sperm from each individual were immediately resuspended in 20 mL of FSW, and motility was evaluated as described above. The sperm received at the Aquaculture Research Station maintained the same motility as at CICESE (>90% motility). Sperm were equilibrated in 10% DMSO or 10% glycerol for 10 min, and samples were placed in 0.5-mL French straws and frozen in the three freezing chambers described later.

Manually Controlled Styrofoam Chamber

Samples were placed in a styrofoam cooler containing liquid nitrogen. Freezing was performed in the vapor phase at −20°C; samples were cooled at −1°C/min to −30°C and held at this temperature for 5 min. Temperature was recorded with a thermocouple (Cole-Palmer Instrument Company, Vernon Hills, Illinois) placed at the same distance from the liquid nitrogen as the straws.

Commercial Freezing Chamber

Samples were transferred in a styrofoam cooler at ~5°C to Genex, Inc., at the Dairy Improvement Center of the Louisiana State University, Baton Rouge, Louisiana. The samples were frozen in a tank with the temperature starting at −140°C, and then raised to −60°C during the first 3 min of the program by the heat load of the straws. Liquid nitrogen vapor was added to lower the chamber temperature at −16°C/min to −140°C and held for 10 min (Chandler 2000).

Programmable Rate Chamber

A programmable freezing chamber (KRYO 10 series II, Planer Products, Sunbury-on-Thames, United Kingdom) was used. The cooling rate was −1°C/min to −30°C and held for 10 min. Samples were plunged into liquid nitrogen (−196°C) and stored for further examination.

Assessment of Sperm Quality

Motility

Immediately before use, samples were thawed in a 45°C water bath for 8 s. Motility was expressed as the percentage of sperm actively swimming in a forward direction. Samples of thawed sperm were observed by microscopy as described above.

Membrane Integrity

Samples of thawed sperm were stained using the Live-Dead Sperm Viability Kit (Molecular Probes, Eugene, Oregon) with the fluorescent dyes SYBR 14 and propidium iodide (PI). The SYBR 14 stock solution contained 1 mM of dye in 100 µL of DMSO. For the experiment, a 1:50 dilution in FSW was prepared from the stock solution resulting in a final concentration of 20 nM. The PI stock solution was used at a 2.4 mM final concentration. For staining, 5 µL of diluted SYBR 14 were added to a 1-mL sample and incubated in the dark for 10 min at 24°C, and then 5 µL of PI were added and incubated for another 10 min in the dark at 24°C. Stained samples were analyzed with a flow cytometer (FACSCalibur, Becton Dickinson Immunocytometry Systems (BDIS), San Jose, California), equipped with an air-cooled, 480-nm argon laser. The FACS Comp Software (BDIS, San Jose, California) was used to calibrate the instrument settings. Sperm cells (10,000) were

analyzed per sample using forward-scatter and side-scatter profiles. Density plots using green fluorescence (nondisrupted membranes) and red fluorescence (disrupted membranes) were used to assess membrane integrity.

Fertilization Rate

Frozen sperm was transported in shipping dewars by priority delivery to the Department of Aquaculture of CICESE and fertilization of fresh eggs with fresh and thawed sperm were performed in Mexico. One thousand eggs, from each of three females, were fertilized with thawed sperm from each of four males, previously frozen in the three chambers using each of the cryoprotectant treatments. The range of sperm numbers in the 0.5-mL French straws was 1×10^6 to 1×10^7 cells per mL. As a control for egg quality, eggs from all three females were fertilized with 1×10^6 cells per mL of fresh sperm from a fifth male in FSW.

On the addition of sperm, the eggs remained undisturbed for 45 min. Excess of sperm was eliminated by rinsing and the fertilized eggs were placed in 50 mL of FSW. Additional washes were performed every 3 h. After 24 h, a sample of 100 eggs from each treatment was evaluated under a stereoscopic microscope at ×50 magnification. The fertilization rate was expressed as the number of eggs that reached the trochophore stage divided by the total number of eggs evaluated.

Statistical Analysis

A covariance analysis (ANCOVA) using time as a covariable was performed using STATISTICA 5.0 (Statsoft, Inc. Tulsa, Oklahoma) to analyze the data from the acute toxic effects experiment, after an arcsine-square-root transformation of motility percentages. Sperm quality parameters were independent as shown by noncorrelated residual error terms generated by 2-way ANOVA for motility, membrane integrity and fertilization rate. To analyze the sperm quality data, to account for nonhomogeneity, these data were linearly ranked and transformed for use in a 2-way ANOVA using cryoprotective treatment and chamber as the independent variables and analyzed with SAS (SAS Institute, Cary, North Carolina, 1989). A value of $P < 0.05$ was chosen as the level for significance.

RESULTS

Acute Toxic Effect of Cryoprotectants on Sperm Motility

Sperm motility decreased according to cryoprotectant concentration and equilibration time (Fig. 1). ANCOVA results showed the highest motility (88% ± 1%) was found in sperm suspended in 5% DMSO for 5 min, and the lowest motility (4% ± 6%) was found in 20% DMSO after suspension for 30 min. For PG, the highest motility was 51% ± 19% for sperm suspended in a concentration of 5% for 5 min, whereas the lowest motility (1% ± 1%) was found in samples incubated for 25 min at a concentration of 15%. For GLY, samples suspended in a concentration of 5% for 5 min had the highest motility (80% ± 24%), whereas the lowest motility (2% ± 4%) was observed for samples suspended in 20% for 25 min. No significant differences were found in the motility of sperm from different males suspended in 5% GLY for 30 min ($P > 0.05$). No significant differences were found between DMSO and GLY at concentrations of 5% and 10% for 5 or 10 min ($P > 0.05$). Based on these results and after running preliminary cryopreservation tests, 10% DMSO, 10% GLY and an equilibration time of 10 min were selected to perform the rest of the cryopreservation experiments.

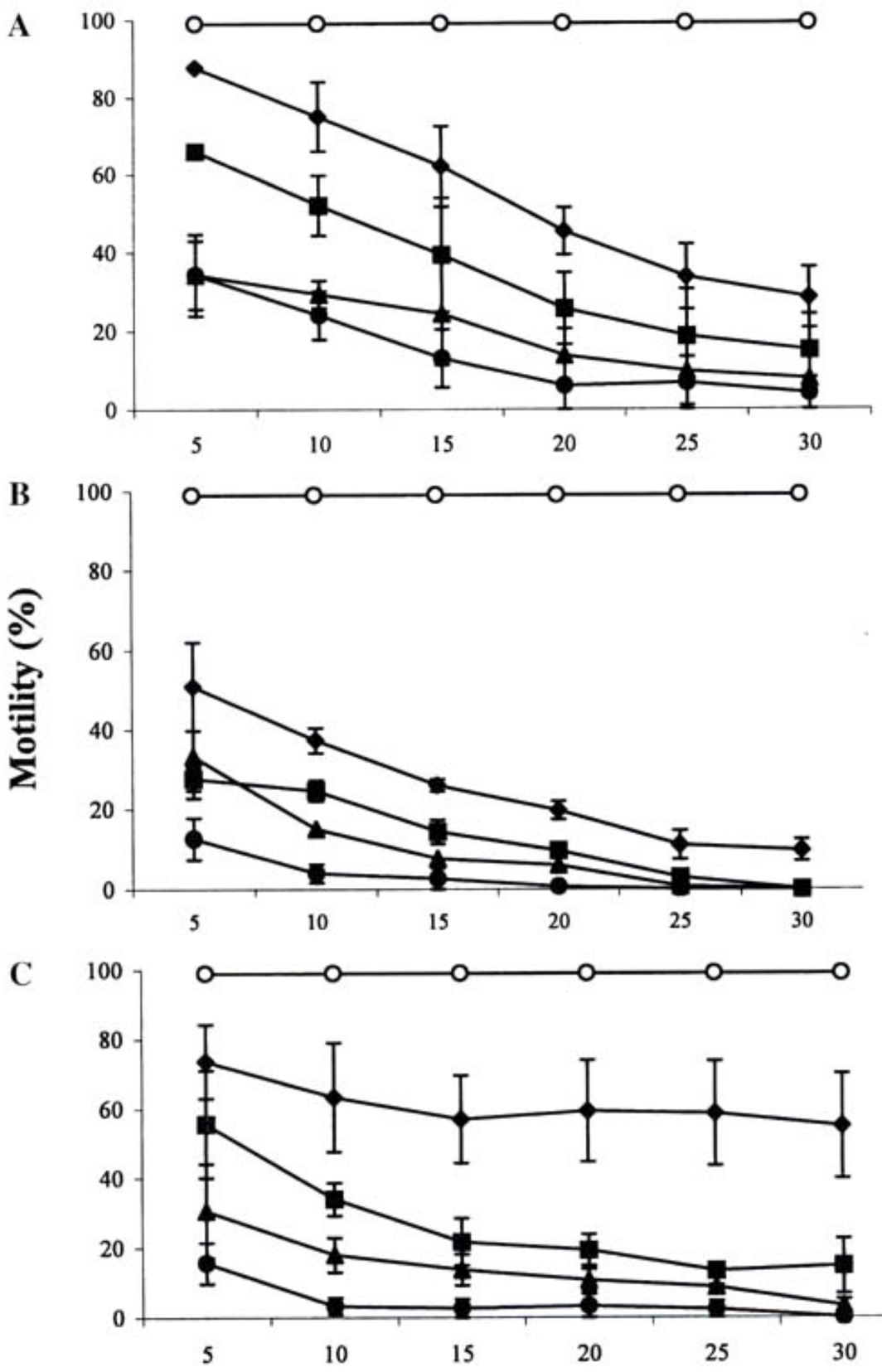

Figure 1. Acute toxic effect on sperm motility of three cryoprotectants (A, dimethyl sulfoxide; B, propylene glycol; C, glycerol) at different concentrations and equilibration times for sperm of red abalone Haliotis rufescens (fresh sperm control, open circles; 5%, diamonds; 10%, squares; 15%, triangles; 20%, filled circles) ($n = 4$).

Assessment of Sperm Quality

Motility

There was an interaction between the cryoprotective treatment and the chamber type, where for DMSO and GLY, motility results were significantly higher ($P = 0.0055$) using MCC than either CFC or PRC. Motilities were not significantly different ($P > 0.05$) for either DMSO or GLY treatments using CFC or PRC, whereas for MCC, motilities using DMSO were significantly lower than GLY. The highest post-thaw motility (48 ± 7%) was observed for sperm suspended in 10% GLY and frozen in the MCC, and the lowest (<10%) was for sperm suspended in 10% GLY and frozen in the PRC and the CFC (Table 2).

Membrane Integrity

Membrane integrity was significantly lower after using the PRC than the CFC or the MCC ($P = 0.0167$). Flow cytometric

TABLE 2.

Quality assessments of thawed red abalone sperm after 10 min equilibration with 10% glycerol (GLY) or 10% dimethyl sulfoxide (DMSO) and freezing in a manually controlled chamber (MCC), a programmable freezing chamber (PRC), or a commercial freezing chamber (CFC).

	Motility (%)		Membrane Integrity (%)*		Fertilization (%)	
	GLY	DMSO	GLY	DMSO	GLY	DMSO
MCC	48 ± 7	22 ± 8	28 ± 8	40 ± 7	20 ± 9	23 ± 11
PRC	<10	<10	21 ± 7	28 ± 4	18 ± 10	16 ± 4
CFC	<10	<10	44 ± 11	48 ± 2	29 ± 10	18 ± 13

* Dual-stain flow cytometry.

analysis showed highest membrane integrity for those samples frozen in the CFC with 10% DMSO (48 ± 2%) and 10% GLY (44 ± 11%). Samples frozen in the PRC showed highest values when suspended in 10% DMSO (28 ± 4%) and when suspended in 10% GLY (21 ± 7%). The higher values for samples frozen in the MCC were 40 ± 7% when suspended in 10% DMSO and 28 ± 8% when suspended in 10% GLY (Table 2).

Fertilization Rate

No differences were found in fertilization rates ($P > 0.05$) between the chamber types, cryoprotectants, or their interactions. The highest fertilization rate (29 ± 10%) was found for samples frozen in the CFC and suspended in 10% glycerol, whereas the lowest fertilization rate (16 ± 4%) was for samples frozen in the PRC and suspended in 10% DMSO (Table 2).

DISCUSSION

Many factors contribute to successful sperm cryopreservation including the type and concentration of cryoprotectant, equilibration time and freezing and thawing rates. Cells can suffer damage before, during or after cryopreservation, and therefore detecting when damage occurs assists in optimizing procedures. Estimating the acute toxic effects of cryoprotectants on live cells is necessary prior to freezing. As found with our study, sperm of *H. diversicolor supertexa* were motile in cryoprotectant solution containing 10% DMSO (3,000 mOsmol/kg) before freezing (Gwo et al. 2002). Motilities decreased; however, when the equilibration time was extended to 30 min (Gwo et al. 2002) indicating that cellular proteins may be denatured at high cryoprotectant concentrations during prolonged equilibrations (Shafer 1981). Also, the predominance of phosphatidylcholine in marine animal sperm (Drokin 1993) could lessen the impact of osmolarity differences among cryoprotectants. In our experiments, cells appeared to be disrupted as they agglutinated, and this was likely due to an acrosome reaction. Sperm with reacted acrosomes or with damaged mitochondria can yield low fertilization rates. For example, a motile sperm may contact the egg but not penetrate it because of a deformity of the acrosome.

High percentages of malformation of the acrosomal region were observed in sperm of the Pacific oyster, *Crassostrea gigas*, with exposure to concentrations of DMSO higher than 12% and during equilibration that exceeded 15 min (Kurokura et al. 1990). In *H. diversicolor*; however, high hatching rates (>90%) were obtained when sperm were suspended in 8% DMSO, 8% DMSO combined with 5% glucose, and 8% DMSO combined with 5% sucrose after being stored at 15°C or 5°C for 2 h. High hatching rates (>80%) were also observed using sperm suspended in 8% DMSO combined with 5% glucose, or 8% DMSO combined with 5% sucrose after 8 h of storage at 15°C, 10°C or 5°C (Tsai & Chao 1994). This indicates that the toxicity of cryoprotectants can be reduced when sperm is stored at temperatures lower than 5°C.

Cryoprotectant selection is species-specific for aquatic invertebrates (Tsai & Chao 1994, Paniagua-Chávez & Tiersch 2001, Gwo 2000). For example, PG was a suitable cryoprotectant for sperm of the eastern oyster *Crassostrea virginica* (Paniagua-Chávez & Tiersch 2001). In this study however, PG was observed to have a toxic effect on sperm of red abalone. Preliminary studies have shown that glycerol was a suitable cryoprotectant for sperm of medaka abalone, *H. gigantea*, (Matsunaga et al. 1983), but not for *H. diversicolor* (Gwo et al. 2002). In our study with red abalone, 10% DMSO or 10% glycerol were successfully used for cryopreservation.

To date, a wide variety of methods have been designed to freeze cells. These range from sophisticated devices that electronically control cooling rates to simple dry ice blocks that are used to produce frozen sperm pellets. Each of these methods has its own advantages, but if not used properly, they may produce excessive variability from sample to sample (Leibo 2000). In this study, the efficiency of three freezing devices ranging from the economic MCC, which can be easily used in the field, to commercial equipment used in the dairy industry (PRC) were tested. With the MCC, temperature conditions are difficult to replicate (Leibo 2000). Dairy industry cryopreservation procedures and equipment for cattle have been adapted for use with aquatic species including sperm of eastern oyster (Paniagua-Chávez 1999) and blue catfish, *Ictalurus furcatus* (Lang et al. 2003). This technology allows repeatable freezing conditions and the storage and inventory of large volumes of sperm. The PRC allows great control and precision of freezing rates; however, differences in the cooling temperatures occur at different locations within the chamber (Koebe et al. 1993), and sample through-put is limited.

To estimate the efficiency of these three chambers, we used three methods to evaluate sperm quality. According to several authors, visual estimation of sperm motility has not always proven to be a reliable indicator of fertilizing ability in fish (e.g. Stein & Bayrle 1978, Stoss & Holtz 1983, Piironen 1987, Gwo 2000) or invertebrates (e.g. Paniagua-Chávez & Tiersch 2001, Matsunaga et al. 1983). Still, motility is the most common method used to evaluate sperm viability. The use of technologies such as flow cytometry to assess membrane integrity of fresh and thawed fish sperm has been used recently in fish (Ogier de Baulny et al. 1997, Segovia et al. 2000, Cabrita et al. 2001) and in some invertebrates (Paniagua-Chávez 1999, Adams et al. 2003, Lezcano et al. 2004). Despite the information that we can obtain using flow cytometry,

fertilization and hatching rates remain the most significant methods to evaluate fertilizing ability of thawed sperm.

Low correlations of sperm motility and fertility may result from low accuracy and limited precision of the visual method, low sample sizes sometimes used when assessing fertility, or other factors such as nondetectable sperm damage, variable egg quality and problems in hatchery management (Gwo 2000). In our study, the motility of thawed sperm from the CFC and PFC chambers was low (10%) although the percentages of intact membranes were relatively high for the CFC (~45%). The dual-staining method to detect membrane damage was used to evaluate damage to the heads of sperm; however, midpiece and tail damage cannot be detected with this method. Studies using rhodamine 123 and propidium iodide to evaluate mitochondrial function and membrane integrity of black lip abalone, *Haliotis iris*, showed that sperm with damaged mitochondria had an intact cell membrane (Adams et al. 2003). Mitochondria provide energy for flagellar action and damage to the sperm midpiece could be responsible for deficiencies in sperm motility. Thus, compromised mitochondrial activity could also contribute to low fertilization rates.

However, in artificial breeding practices, physical contact of nonmotile sperm is accomplished by mixing of gamete suspensions. Nonmotile sperm can fertilize following an acrosomal reaction in proximity of an egg. Direct injection of sperm heads into mature oocytes is a common practice carried out at human infertility clinics (Kuretake et al. 1996). Therefore, spermatozoa without flagella or with damaged plasma membranes can potentially provide fertilization.

The sperm concentration used in this study (1×10^6 cells per mL) is comparable to that used for *H. diversicolor* (Gwo et al. 2002), and with this concentration we obtained a maximum fertilization rate of 48%. High fertilization rates (~91%) have been obtained for *H. diversicolor* using thawed sperm suspended in 8% DMSO (Tsai & Chao 1994); however, the authors did not report the sperm density used in their fertilization trials. Also, a fertilization rate of 93% has been reported for *H. gigantea* using thawed sperm suspended in 12% glycerol (Matsunaga et al. 1983). As with the *H. diversicolor* study, sperm density was not reported in this study.

Agglutination of sperm was observed in our experiments. This agglutination is likely due to spontaneous acrosome reactions which could have occurred at some point during freezing or thawing. Agglutinated sperm may or may not be viable because of membrane integrity differences. Direct microscopic observations using fluorescent dyes such as SYBR 14 and propidium iodide to assess membrane integrity indicated that sperm without an acrosome could be considered to have intact plasma membranes (unpublished observations); however, such sperm might not be available for fertilization. Thus, the effective density of sperm used after thawing to fertilize could be less than the calculated density of sperm before freezing, thereby affecting the fertilization rates determined in these experiments.

The evaluation of the equipment used to freeze sperm allowed us to determine their overall feasibility and convenience. Manually controlled styrofoam chambers (MCC) can be considered more convenient freezing devices than CFC and PRC because they are inexpensive and can be easily used in hatcheries. Unfortunately, reliability among different freezing batches can be poor because of the inability to control the chamber temperature. Commercial chambers (CFC) designed to freeze bull sperm are a good alternative for freezing abalone sperm, if there is one readily available. Programmable freezing chambers are expensive, but because temperature can be controlled, freezing can be more accurate and reliable with them.

Gene resource conservation of aquatic organisms of Baja California, Mexico has become a priority for the Mexican government. This study is a foundation in the creation of germplasm banks that will aid in restoration efforts for abalone species. Also, commercial species can benefit from this work because frozen sperm can be used to enhance breeding programs at hatcheries.

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