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High-throughput sperm cryopreservation of eastern oyster *Crassostrea virginica*

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ABSTRACT

Sperm cryopreservation is a valuable tool in germplasm preservation and breeding. Despite many studies, reliable and routine cryopreservation of oyster sperm remains a challenge. The goal of this study was to develop a reliable protocol for sperm cryopreservation of the eastern oyster *Crassostrea virginica* with high-throughput processing by using two types of 0.5-ml straws (French and CBS™ high security straws). The objectives were to: 1) evaluate the effect of 10% methanol, dimethyl sulfoxide (DMSO), and propylene glycol as cryoprotectants at cooling rates of 5, 20 and 40 °C/min from 5 to −80 °C and thawing at 30, 40 and 50 °C; 2) evaluate the effect of cooling rates of 10, 15, 20, 25 and 30 °C/min with 10% DMSO as cryoprotectant and thawing at 40 °C; 3) evaluate the effect of equilibration time (10–60 min) before freezing; 4) evaluate the effect of sperm concentrations from 1×10^8 to 1×10^9 for freezing, and 5) verify the established protocol by freezing sperm from 16 individual males. Among the three cryoprotectants, DMSO yielded the highest post-thaw motility at a cooling rate of 20 °C/min when thawed at 30 or 40 °C. Further evaluation of cooling rates of 10, 15, 20, 25 and 30 °C/min showed that 20 or 25 °C/min yielded the highest post-thaw motility ($34\pm5\%$) and fertility ($77\pm12\%$) for French straws and CBS straws ($28\pm3\%$ and $69\pm14\%$). Equilibration times of 10 to 60 min did not cause significant differences in post-thaw motility when freezing with 10% DMSO at a cooling rate of 25 °C/min. Also, sperm concentrations ranging from 1×10^8 to 1×10^9 at freezing did not cause significant differences in post-thaw motility. Sperm concentration after thawing was not different compared to that before freezing, and no agglutination was observed in the post-thaw samples. Finally, after thawing, sperm cryopreserved from 16 males with this protocol showed $58\pm24\%$ fertility (from 18 to 86%) for French straws, and $54\pm21\%$ fertility for CBS straws (from 18 to 95%). Overall, this study provided a reliable protocol for sperm cryopreservation in the eastern oyster with potential for high-throughput processing which can produce thousands of straws per day with homogenous and reliable quality.

1. Introduction

The eastern oyster *Crassostrea virginica* is naturally distributed along the Atlantic coast of America from the Canadian Gulf of St. Lawrence to the Gulf of Mexico, the Caribbean, and the coasts of Brazil and Argentina (Kennedy et al., 1996). It is an important commercial species and an important member of brackish habitats with critical roles in coastal ecosystems. Due to overfishing, disease, and pollution, wild oyster harvest has dramatically decreased, such as in Chesapeake Bay, and harvest of the eastern oyster is now estimated to be just 2% of its peak historical abundance (www.chesapeakbay.net). The genetic resources of natural populations in some areas are threatened. During its long history, the oyster industry (especially in the Gulf region) has been reliant on the harvest of wild spat with few breeding programs or biotechnologies involved. Catastrophic events, such as the Deepwater Horizon oil spill in 2010, can cause heavy losses and disruption of natural life processes. To cope with the ongoing issues of coastal erosion, pollution, over harvesting, shell budgeting, cultch replenishment, and oyster diseases, research efforts have focused on improving and sustaining this fishery through aquaculture.

The success of aquaculture production depends on the availability of domesticated or improved stocks with desirable traits. Research on oyster genetics and breeding has received considerable attention in recent decades. For the eastern oyster, a basic genetic linkage map has been established (Yu and Guo, 2003) and disease-resistant quantitative trait loci were identified (Yu and Guo, 2006). With these molecular techniques, further analyses showed that strains subjected to long-term selection for disease resistance shared frequency shifts at several loci (Guo et al., 2008; Yu and Guo, 2004), indicating that the markers were linked to disease-resistance genes and could be used for selective breeding programs. Artificial selection has produced eastern oyster strains with dual resistance to diseases caused by the

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parasites *Haplosporidium nelsoni* and *Perkinsus marinus* (Guo et al., 2008; Haskin and Ford, 1979; Ragone-Calvo et al., 2003). This strain showed higher growth and survival rates and low susceptibility to infection. In addition, tetraploid oysters have been developed that can produce 100% triploids for commercial production (Guo et al., 2009). Triploid oysters are sterile and exhibit faster growth and better summer meat quality than diploids. Triploids are produced by mating of diploid females with tetraploid males, and the ability to preserve and store tetraploid sperm would be extremely helpful to triploid production.

Cryopreservation is a technique to preserve genetic material in perpetuity. It can be applied to oysters in establishing pedigreed families or lines for breeding programs and preservation of genetic resources in the following ways. 1) *Creation of inbred lines by self-fertilization*. Eastern oysters are protandric, beginning life as males and changing sex as they age (Galtsoff, 1964). This characteristic allows for fertilization by cryopreserved sperm of eggs from the same oyster after sex change. 2) *Preservation of tetraploid sperm*. This offers several benefits. It can extend the commercialization of triploid–tetraploid technology by sale of frozen tetraploid sperm, provides cost savings and security for maintaining tetraploids, and can protect intellectual property by sale of sperm instead of reproductive broodstock. In addition, this can be used to expand genetic diversity of tetraploid populations. 3) *Preservation of specific lines or strains and valuable genetic resources*. Ongoing selective-breeding programs have yielded specific strains and lines of oysters that require secure long-term preservation.

Sperm cryopreservation in mollusks was first reported in *Crassostrea gigas* in 1971 (Lannan, 1971). Since then studies have been reported in other oysters, mussels, clams, and abalones (Adams et al., 2011). A single species, *C. gigas*, has been the focus of more than 25 publications (Dong et al., 2011). The cells and tissues used for cryopreservation have included sperm, eggs, embryos, and larvae at different stages. In general, wide variations exist in the protocols reported in these publications, even with the same species. For eastern oysters, cryopreservation has been studied for sperm (Hughes, 1973; Paniagua-Chavez and Tiersch, 2001; Zell et al., 1979) (Table 1) and trochophore larvae (Paniagua-Chavez et al., 1998b). Overall, the cryoprotectants used were DMSO (dimethyl sulfoxide, 5–10%) or propylene glycol (PG, 10 or 15%). The extenders used were artificial sea water or calcium-free Hanks' balanced salt solution (Ca-free HBSS). The nominal cooling rates studied were 1–5.5 °C/min, and the packaging materials were 2-ml ampoules or plastic straws (0.25-ml or 5-ml). The results varied from 2% to 91% fertilization (which is itself a poorly defined term) after thawing. Trochophore larvae were reported to develop after thawing beyond metamorphosis to the spat stage (Paniagua-Chavez et al., 1998b). These previous studies were based on laboratory-scale (protocol development) experiments, and although useful at that level, there has been no systematic evaluation of different cryoprotectants and cooling rates, which are believed to be the two critical factors in cryopreservation techniques. Furthermore, the existing protocols were not developed for high-throughput cryopreservation with consistency in quality and quantity, which has become a trend for aquatic germplasm (Varga and Tiersch, 2012). For oyster production where the fertilization of billions of eggs is required, high-throughput cryopreservation of sperm is essential.

The goal of this study was to develop a reliable protocol for sperm cryopreservation of the eastern oyster with high-throughput processing by using two types of 0.5-ml straws: French straws and CBS™ high security straws (CBS straw) (IMV Technologies USA, Maple Grove, MN). The objectives were to: 1) evaluate the effect of 10% methanol, DMSO, and propylene glycol as cryoprotectants at cooling rates of 5, 20 and 40 °C/min from 5 to −80 °C and thawing at 30, 40 and 50 °C; 2) evaluate the effect of cooling rates of 10, 15, 20, 25 and 30 °C/min with 10% DMSO as cryoprotectant and thawing at 40 °C; 3) evaluate the effect of equilibration time (10–60 min) before freezing; 4) evaluate the effect of sperm concentrations from 1×10^8 to 1×10^9 for freezing, and 5) validate the established protocol by freezing sperm from 16 individual males.

2. Materials and methods

2.1. Oysters

The male oysters used in this study came from the Louisiana Sea Grant Bivalve Hatchery at Grand Isle, Louisiana, and were transported (3-h driving time) dry in a cooler on ice to the Aquaculture Research Station in Baton Rouge, Louisiana for sperm collection and cryopreservation. Female oysters came from the Haskin Shellfish Research Laboratory (HSRL) at Rutgers University (New Jersey), and were used for fertility testing of cryopreserved samples at HSRL. The collection season was April and May in Louisiana, and June in New Jersey.

2.2. Sperm collection and concentration determination

Sperm were collected by dissection in this study. Before dissection, the oysters were cleaned by brushing off mud and removal of attached barnacles and other animals, and were measured for height (from hinge to the opposite edge), length, and body weight. After opening the oysters, sex was determined by viewing the presence of eggs or sperm from a gonadal smear by use of a microscope at 200-× magnification. The gonads from males were stripped individually into a sterilized petri dish with a scalpel blade, and weighed. Sperm were released by crushing of the testis in Ca-free HBSS at an osmolality of 650 mOsmol/kg (Ca-free HBSS650) with a volume of 10 times the testis weight, and the sperm suspensions were filtered through 100-μm and a 20-μm Nitex screens to remove debris. Sperm concentration was determined by measuring the absorbance of the suspension with a microspectrophotometer (NanoDrop®, Thermo Fisher Scientific, Wilmington, DE), and calculating with an equation deduced from a standard curve developed between absorbance and sperm concentration ($R^2=0.996$): $Y=(2\times X+0.05)\times10^8$ where Y was the sperm concentration and X was the absorbance measured at 600 nm

Table 1
Summary of sperm cryopreservation protocols developed for the eastern oyster *Crassostrea virginica* since 1973.

Material cryopreserved	Extender	CPA[a]	Cooling rate	Packaging container	Thawing	Viability	References
Sperm	Filtered seawater	DMSO 5, 10%	1 °C/min from 0 to −8 °C; 5.5 °C /min to −25 °C	2-ml glass ampoules	21 °C in air	1–5% motility; 2% fertilization	Hughes, 1973
Sperm	2.6-×HBSS[b] with 80 mM glycine, 55 mM $NaHCO_3$	DMSO 8%	5–7.5 °C /min from 0 to −20 °C, 13.5 °C/min to −80 °C	0.25-ml French straws	55–60 °C waterbath	7–91% fertilization; larvae harvested at day 11	Zell et al., 1979
Sperm and trochophore larvae	Calcium-free HBSS	PG 10, 15%	2.5 °C/min from 15 °C to −30 °C; hold for 5 min	5-ml macrotube	70 °C for 15 s in water bath	59% fertilization; larvae developed to spat	Paniagua-Chavez and Tiersch, 2001

[a] CPA = cryoprotectant agents; DMSO = dimethyl sulfoxide; PG = propylene glycol.
[b] HBSS = Hanks' balanced salt solution.

(Cuevas-Uribe and Tiersch, 2011; Tan et al., 2010). Ca-free HBSS650 was prepared by adjusting the water volume from for the standard recipe of HBSS 1 L to around 450 ml without the addition of $CaCl_2$ (0.137 M NaCl, 5.4 mM KCl, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.55 mM glucose, pH = 7.8) (Hanks, 1975; Paniagua-Chavez et al., 1998a). The osmolality was measured with a vapor pressure osmometer (Model 5520, Wescor Inc., Logan, UT), and pH was measured with an AB15 pH meter (Fisher Scientific, Pittsburgh, PA). The sperm suspensions were held on ice before and during use in the experiments.

2.3. Motility estimation

Eastern oyster sperm begin to swim when suspended in sea water or buffers at suitable osmolalities (Paniagua-Chavez et al., 1998a), and in contrast to sperm of most fishes, can swim continuously for as long as 5 h or longer (Kennedy et al., 1996). In this study, motility of sperm was estimated within 1 h after suspending in Ca-free HBSS650 by use of computer-assisted sperm analysis (CASA) (Hamilton Thorne, Inc., Beverly, MA; CEROS model) with 20-μm 2-cell slides (Hamilton Thorne, Inc.). The parameter settings for CASA were: minimum contrast, 30; minimum cell size, 3; number of frames for recording, 30; average path (VAP) cut off, 20 μ/s; straight line (VSL) cut off, 10 μ/s; static intensity gate, 0.15–2.82; static size gate, 0.72–3.29; elongation gate, 56–99, and slow cell, static. For each sample, at least three CASA measurements of different fields were performed, and the average was used as the motility for that sample. These parameter settings were verified with the "playback" function of the software. However, one phenomenon observed during the process of CASA analysis was the non-homogeneous distribution of sperm cells on the slide sample area, which could cause variation in the motility readings at different observation fields. To minimize this difference, CASA readings were taken at the center of the sample loading area on slides, and multiple readings (at least three) from each sample were used for estimating motility.

2.4. Sperm cryopreservation process

In this study, the final concentration of sperm for freezing was set at 1×10^8 cells/ml (Dong et al., 2007) except for Experiment IV. The procedure for cryopreservation was to adjust the sperm concentration to double the targeted concentrations (e.g., 2×10^8 cells/ml), prepare a double-strength solution of cryoprotectant in Ca-free HBSS650, mix the sperm suspension and double-strength cryoprotectant at a ratio of 1:1 and record the equilibration time (from mixing of sperm and cryoprotectant until the samples were at 5 °C at the start of the freezing program), and load 0.5 ml sample into 0.5-ml French straws by hand or into CBS straws by use of an automated system (MAPI, CryoBioSystem Co. Paris, France). For each treatment, 4–8 straws were produced for the different analyses. A proprietary computer program (SIDE, CBS) controlled the system to automatically fill, seal, and label the straws, which were arrayed by hand on racks and placed in a commercial-scale programmable freezer (Micro Digitcool, IMV, France) to freeze the samples after a 20-min equilibration time (except for Experiment V) with a pre-programmed cooling rate from 5 °C to −80 °C (based on probe temperature within a straw), and transfer of frozen samples into liquid nitrogen for storage.

2.5. Artificial fertilization

Artificial fertilization was performed at Rutgers University with frozen samples shipped overnight from Louisiana in a commercially available shipping dewar (LD 10, Taylor-Wharton, Thoedore, AL). Eggs were collected by dissection of 1-yr-old females. Gonads were minced individually with a scalpel into 1-μm filtered seawater at a salinity of 20 ppt, and allowed to hydrate for 1–2 h. The egg suspensions were filtered through a 200-μm screen to remove gonadal debris, and were collected and washed on a 20-μm screen using filtered sea water. To obtain enough eggs for fertilization, the output from several females (3–5) was combined, and the concentration was determined by counting the number of eggs in a 1-ml sample with a Sedgewick–Rafter counting cell (S50, Pyser-sgi Limited, Kent, UK). The eggs were aliquoted into 50-ml samples in 1-L beakers, and were ready for fertilization within 1 h.

After storage for more than one week in liquid nitrogen, the samples were thawed at 40 °C in a water bath for 8 s for French straws or 20 s for CBS straws, and the sperm suspensions were released into 1.5-ml centrifuge tubes on ice for motility estimation and fertilization.

For fertilization, thawed sperm (a pool of samples from 4 straws) were mixed with 50 ml of egg suspension (concentration of ~10,000 eggs/ml) in a 1-L beaker at room temperature (23 °C) (the sperm:egg ratio was about 400–500 sperm per egg). After 30 min, additional fresh seawater was added to increase the final volume to 1 L. Embryos at the 4-cell stage or beyond were counted as fertilized eggs, and the percentage based on total eggs was used to calculate fertility. To evaluate egg quality, two aliquots from the same suspension used for testing of cryopreserved sperm were fertilized with fresh sperm obtained from 2 to 3 male oysters.

2.6. Experiment I. Effect of cryoprotectant, cooling rate, thawing rate, and their interactions

Three cryoprotectants (methanol, DMSO, and PG) with a final concentration of 10% (v/v) were chosen based on their common use in molluscan gamete cryopreservation. Cooling rates of 5, 20 and 40 °C/min from 5 to −80 °C were tested for samples in 0.5-ml French straws and CBS straws. Samples were thawed at 30 °C (10 s for French straws, and 25 s for CBS straws), 40 °C (8 s for French straws, and 20 s for CBS straws), and 50 °C (5 s for French straws, and 15 s for CBS straws). Motility after thawing was used to evaluate the effects of treatments. Four replicates were produced using sperm from 1 to 2 males in each replicate.

2.7. Experiment II. Re-evaluation of cooling rates on post-thaw motility and fertility

Based on the results from Experiment I, cooling rates of 10, 15, 20, 25 and 30 °C/min were tested with 10% DMSO as cryoprotectant, and 0.5-ml French straws and CBS straws were used as packaging containers. Samples were thawed at 40 °C for 8 s (French straw) or 20 s (for CBS straw). Motility and fertility after thawing were used to evaluate the effects of cooling rate. Three replicates were produced for each treatment by using sperm from one male in each replicate.

2.8. Experiment III. Effect of equilibration time on post-thaw motility

Based on the results from Experiments I and II, equilibration times of 10, 20, 30, 40, 50, and 60 min were tested for freezing with 10% DMSO as cryoprotectant at a cooling rate of 25 °C/min in 0.5-ml French straws, and samples were thawed at 40 °C for 8 s. Motility after thawing was used to evaluate the effects of treatments. Seven replicates were produced using sperm from one male in each replicate.

2.9. Experiment IV. Effect of sperm concentration for cryopreservation

In previous research on Pacific oysters, the suitable concentration for freezing sperm to avoid agglutination was 1×10^8 cells/ml (Dong et al., 2007). To evaluate this in eastern oysters, concentrations of 1×10^8, 3×10^8, 5×10^8, 7×10^8, and 1×10^9 cells/ml were tested for freezing with 10% DMSO at a cooling rate of 25 °C/min in 0.5-ml French straws, and thawing at 40 °C for 8 s. Motility after thawing

was used to evaluate the effects of treatments. Five replicates were produced using sperm from one male in each replicate.

2.10. Experiment V. Verification of fertility of post-thaw sperm from individual oysters

To verify the protocols developed from the experiments above, sperm samples from 16 individual males were cryopreserved as follows: sperm concentration was set at 1×10^8 cells/ml; cryoprotectant was 10% DMSO; equilibration time was 20 min; cooling rate was 25 °C/min; packaging used was 0.5-ml French straws and CBS straws, and thawing temperature was 40 °C. Motility and fertility after thawing were used to evaluate post-thaw quality for this experiment.

2.11. Data analysis

Data in this study were analyzed using SYSTAT 13 (Systat Software, Inc., Chicago, IL). Treatment effects were evaluated by use of t-test and ANOVA (three-way analysis in Experiment I, and a one-way analysis in the other Experiments). Pairwise comparisons were made by use of Tukey test. Percentage data were arcsine-square-root transformed before analysis. The significance level was set at $P<0.050$.

3. Results

3.1. Effect of cryoprotectant, cooling rate, thawing temperature, and their interactions

Cryoprotectant, cooling rate, and thawing temperature were all significant factors that influenced post-thaw motility of cryopreserved sperm with CBS straws ($P\leq0.006$) and French straws ($P\leq0.006$) (Fig. 1). Among these three factors, cooling rate and cryoprotectants showed significant interactions for both straw types ($P=0.000$), but thawing temperature did not show an interaction with cooling rate and cryoprotectant ($P\geq0.081$ for CBS straws or $P\geq0.091$ for French straws). Pairwise comparison showed that cooling rates of 5, 20 and 40 °C/min caused significant differences in post-thaw motility for both straw types ($P\leq0.001$), and the highest post-thaw motility was observed at a cooling rate of 20 °C/min for the three cryoprotectants and the two types of straws (Fig. 1). DMSO as cryoprotectant yielded significantly higher post-thaw motility for CBS straws and French straws than did methanol ($P=0.000$) and PG ($P=0.000$). A thawing temperature of 40 °C yielded significantly higher post-thaw motility for CBS straws and French straws than did 50 °C ($P\leq0.005$), but showed no difference with 30 °C ($P\geq0.159$). The combination of 20 °C/min cooling rate and DMSO yielded significant higher post-thaw motility for CBS straws than did all other combinations ($P\leq0.001$). The highest post-thaw motility was 19 ± 5% (mean ± SD) for CBS straws and 18 ± 6% for French straws when thawing at 40 °C compared to the motility of fresh sperm 44 ± 13%.

3.2. Effect of cooling rates on post-thaw motility and fertility with DMSO as cryoprotectant

With 10% DMSO as cryoprotectant, cooling rate (from 10 to 30 °C/min with 5 °C/min intervals) significantly affected post-thaw motility ($P=0.000$), but not fertility ($P=0.050$) possibly due to the high standard deviation among the replicates (Fig. 2). Straw type did not cause significant differences in post-thaw motility ($P=0.068$) or fertility ($P=0.491$), nor were there interactions with straw type or cooling rate for post-thaw motility ($P=0.808$) and fertility ($P=0.428$).

The motility of fresh sperm was 63 ± 6%. The highest post-thaw motility was at the cooling rate of 25 °C/min for French straws (34 ± 5%) and CBS straws (28 ± 3%), and was not significantly different

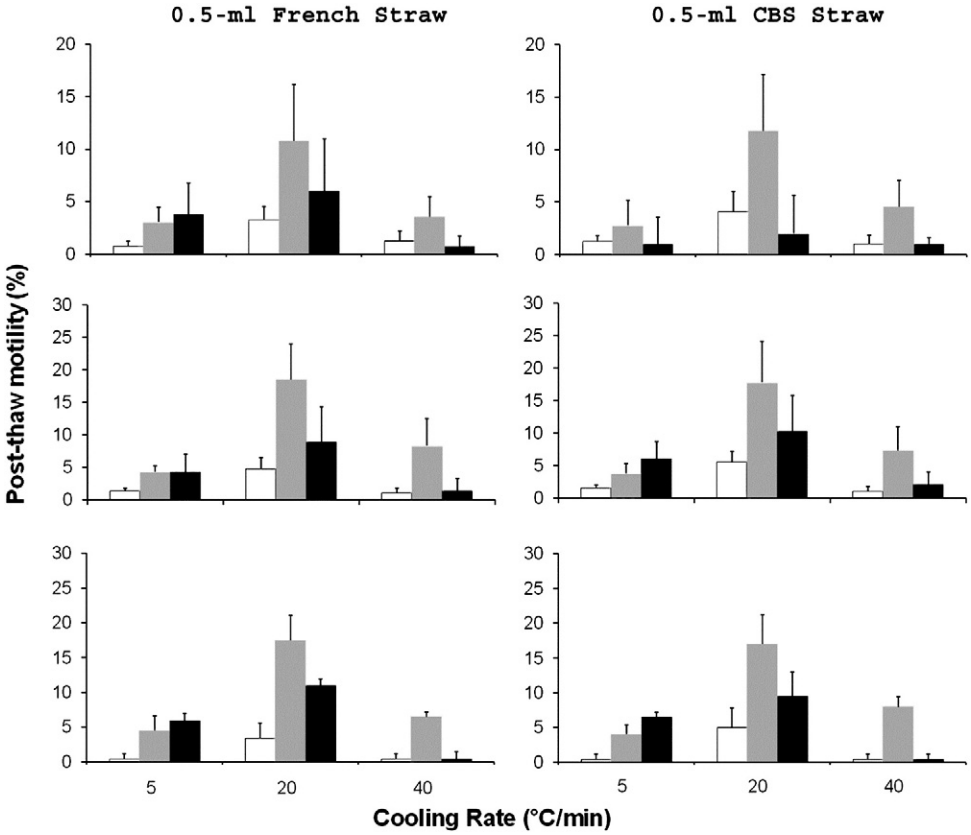

Fig. 1. Post-thaw motility (mean ± SD) of eastern oyster *Crassostrea virginica* sperm after cryopreservation with the cryoprotectants methanol (white bars), dimethyl sulfoxide (grey bars), and propylene glycol (black bars) at cooling rates of 5, 20 or 40 °C/min from 5 to −80 °C and thawing at 30 °C (top row), 40 °C (middle row) or 50 °C (bottom row) in 0.5-ml French straws (left column) or CBS straws (right column).

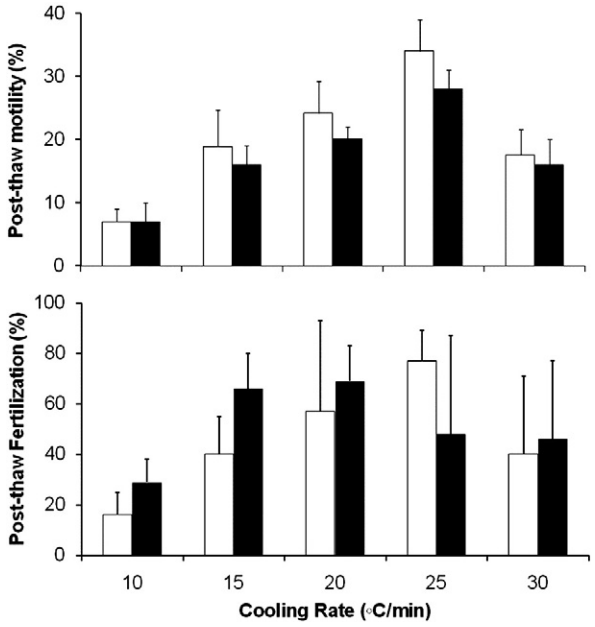

Fig. 2. Motility and fertility (mean ± SD) of post-thaw sperm from eastern oyster *Crassostrea virginica* after cryopreservation with 10% dimethyl sulfoxide at cooling rates of 10, 15, 20, 25, or 30 °C/min from 5 to −80 °C and thawing at 40 °C for 8 s in French straws (white bars) or 20 s in CBS straws (black bars).

from that at 20 °C/min (24 ± 5% for French straws, and 20 ± 3% for CBS straws). Similarly, the highest fertility of post-thaw sperm was at 25 °C/min for French straws (77 ± 12%), and at 20 °C/min for CBS straws (69 ± 14%), but no significant differences were detected in fertility among the five cooling rates for French straws ($P = 0.092$) and CBS straws ($P = 0.376$). Also, there was no correlation between post-thaw motility and fertility for French straws ($P = 0.556$) or CBS straws ($P = 0.397$). Fertilization from the same batches of eggs with fresh sperm was 90 ± 2%, indicating good egg quality.

3.3. Effect of equilibration time on post-thaw motility

Equilibration of sperm cells with 10% DMSO for 10, 20, 30, 40, 50 and 60 min did not yield significant differences in post-thaw motility when cooled at 25 °C/min ($P \geq 0.436$) (Fig. 3). This result indicated that there would as long as 60 min available for handing of samples such as for packaging, sealing, and labeling after mixing with cryoprotectant and before the start of freezing. But, compared to fresh sperm motility (45 ± 8%), post-thaw motility from all equilibration groups was significantly decreased ($P = 0.000$).

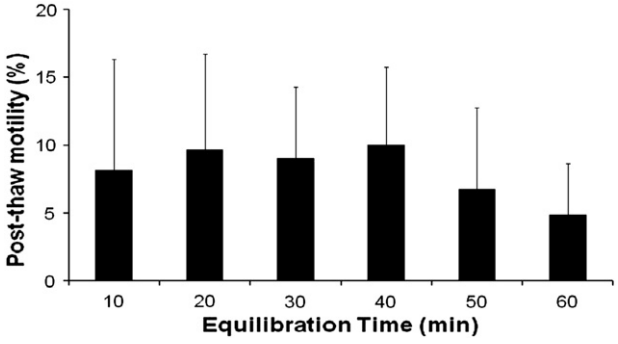

Fig. 3. Post-thaw motility (mean ± SD) of eastern oyster *Crassostrea virginica* sperm after equilibration with 10% of dimethyl sulfoxide for 10, 20, 30, 40, 50 and 60 min, and freezing at cooling rates of 25 °C/min from 5 to −80 °C and thawing at 40 °C for 8 s in French straws.

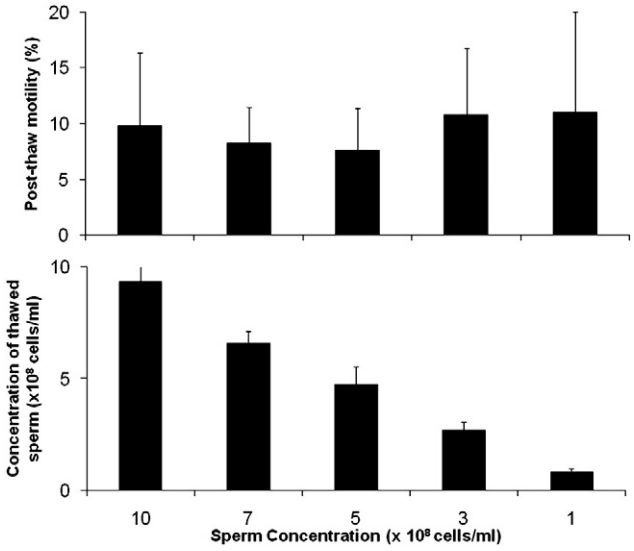

Fig. 4. Motility (upper row) and concentration (lower row) of thawed sperm from eastern oyster *Crassostrea virginica* after cryopreservation at 1, 3, 5, 7, or 10×10^8 cells/ml with 10% dimethyl sulfoxide for 20-min equilibration at a cooling rate of 25 °C/min from 5 to −80 °C and thawing at 40 °C for 8 s in French straws.

3.4. Effect of sperm concentration for cryopreservation

The motility of fresh sperm was 36 ± 16%. After freezing at concentrations of 1, 3, 5, 7, and 10×10^8 cells/ml, the post-thaw motility ranged from 8 ± 3% to 11 ± 9% (Fig. 4 upper), and there were no differences ($P = 0.889$). Sperm agglutination was not observed in the thawed samples. In addition, the sperm concentration after thawing did not change significantly compared to that before freezing ($P \geq 0.052$) (Fig. 4 lower).

3.5. Protocol verification by freezing sperm from 16 individual oysters

After freezing with 10% DMSO at a cooling rate of 25 °C/min, post-thaw sperm showed motility ranging from 2 to 15% for French straws and 5–15% for CBS straws (Table 2). The fertilization of post-thaw

Table 2
Weight and sizes of male oysters, and percent fresh motility, post-thaw motility, and post-thaw fertility (percentage of embryos at 4-cell stage or beyond in total eggs) of sperm from 16 individual males of the eastern oyster *Crassostrea virginica*. Sperm samples were cryopreserved in two types of plastic 0.5-ml straws with 10% DMSO at a cooling rate of 25 °C/min from 5 °C to −80 °C, and sperm concentration was 1×10^8 cells/ml. Eggs from the same batches had 81 ± 6% fertilization with fresh sperm.

Oyster	Total weight (g)[a]	Shell height (mm)	Shell length (mm)	Fresh sperm motility (%)[b]	Post-thaw motility (%)		Post-thaw fertility (%)	
					French straw	CBS straw	French straw	CBS straw
1	85.14	67.87	53.21	–	10	9	79	57
2	40.89	58.10	45.90	–	10	10	79	50
3	102.90	69.18	58.73	–	6	5	52	95
4	67.11	70.62	41.59	–	4	7	71	63
5	69.59	66.26	45.57	–	6	10	86	46
6	82.61	77.96	46.27	–	3	10	83	74
7	96.72	77.08	46.94	–	2	5	19	49
8	87.08	63.43	62.29	41	15	8	26	53
9	112.60	74.77	61.83	41	13	7	48	30
10	162.74	67.82	57.95	26	12	5	74	52
11	143.78	64.52	55.95	23	9	8	30	74
12	100.00	71.86	60.90	30	10	5	45	20
13	106.22	72.74	60.36	32	10	5	70	53
14	100.00	74.90	60.21	30	11	15	64	53
15	90.16	69.51	57.25	33	11	10	76	80
16	175.13	60.02	52.94	19	10	15	18	18

[a] Shell plus meat.
[b] Data for first seven males were lost due to computer malfunction.

sperm was 58 ± 24% for French straws (ranging from 18 to 86%) and 54 ± 21% for CBS straws (18 to 95%), which were not significantly different ($P = 0.649$). The fertilization was 81 ± 6% with fresh sperm (same volume at 10^8 cells/ml) demonstrating good egg quality. There were no significant correlations between post-thaw motility and fertility for French straws ($P = 0.877$) or CBS straws ($P = 0.543$), and no correlation between fresh sperm motility and post-thaw motility or fertility ($P \geq 0.321$). No correlations were detected ($P \geq 0.263$) among body weight, body height, and body length, and these body factors also did not have correlations with fresh motility, post-thaw motility, or fertility ($P \geq 0.055$).

4. Discussion

Through systematic evaluation of cryoprotectants, cooling rates, thawing rates, equilibration times, and sample concentrations, this study developed an optimized and standardized protocol for sperm cryopreservation in eastern oysters. Because of the use of 0.5-ml straws, which are standard for the bull semen industry (O'Connor, 2011), this protocol can be adopted to high-throughput processing by using automated semen processing systems for loading, sealing, and labeling of straws.

4.1. The need for standardized protocols for sperm cryopreservation in shellfish

Cryopreservation is a technique comprising a series of steps including sample collection, sample dilution in extender, choice and addition of cryoprotectants, sample packaging, cooling, storage, and thawing for artificial fertilization (Tiersch, 2011). These steps interact with each other, and can have cumulative effects on sperm survival because errors at any step can cause damage leading to failure of the overall cryopreservation process (Leibo, 2011). Therefore, it is necessary to set quantified standards for each step of the process to ensure the repeatability and reliability of any protocol developed. Sperm cryopreservation in shellfish has been studied in oysters, clams, mussels, and abalones with 64 publications from 1971 to 2011, and with at least 28 publications addressing a single species, the Pacific oyster. The protocols concluded from these publications have varied greatly, even within the same species such as Pacific oyster (reviewed by Dong et al., 2011). This was mostly because steps in protocols were not quantified or standardized, such as the use of different packaging containers, sperm concentrations, cooling and thawing rates, and loading volumes. Although these factors can seem minor to those outside of cryobiology, they reduce the repeatability among laboratories and can cause unnecessary controversy in comparing results. To use sperm cryopreservation for broad application, it would be necessary to establish standardized (e.g., within laboratories) and harmonized (e.g., among laboratories) protocols to guarantee outcomes and enable comparisons (Cuevas-Uribe and Tiersch, 2011).

4.2. Factors for development of sperm cryopreservation protocol

As indicated above, to develop a reliable cryopreservation protocol for any species, basic factors should be evaluated, such as cryoprotectant type and concentration, cooling rate, and thawing rate, and this should be followed by establishment of a pathway for standardization with quality control and streamlined processing, leading to application of the finalized pathway for specific purposes (Tiersch, 2011). For eastern oysters, the three currently existing protocols (Table 1) used three different packaging containers, and lacked systematic evaluation of cryoprotectants and cooling rates. Also, the three protocols focused on laboratory-scale research with only one or two trials. The current study sought to develop a protocol that would be compatible with existing high-throughput equipment, and accordingly based on the sample availability from oysters, two types of 0.5-ml straw were chosen as packaging containers.

For selection of cryoprotectant, this study evaluated methanol, DMSO and PG, the three most widely used cryoprotectants for gamete cryopreservation in molluscs (used in more than 50% of the 64 publications from 1971 to 2011). Based on post-thaw motility, DMSO was the most suitable, which agreed with two of three previous publications in eastern oysters (Hughes, 1973; Zell et al., 1979). However, this was not always the case in the present study, for example, at lower cooling rates such as 5 °C/min, PG provided better protection than did DMSO. This agreed with the results in the other publication on eastern oysters (Paniagua-Chavez and Tiersch, 2001), in which PG was used for freezing samples packaged in 5-ml straws at a slow cooling rate of 2.5 °C/min. This illustrates that due to interactions among cryoprotectant, cooling rate, thawing temperature, and the type of container, there is no absolute "best" protocol, and selection of factors remains a relative concept aimed at achieving an overall balance (Tiersch, 2011).

Cooling rate is a critical factor in cryopreservation, and usually interacts with cryoprotectants (Muldrew et al., 2004). Generally, when cooling slowly, cells lose water to maintain osmotic equilibrium with the outside medium, and encounter dehydration and prolonged exposure to concentrated solutions; whereas when cooling fast, cells do not have sufficient time for water to leave, and intracellular ice crystals can be formed (Mazur, 1984; 2004). The cooling rate that allows cell survival is the one which is rapid enough to minimize the time of exposure to concentrated solutions, yet slow enough to minimize intracellular ice crystal formation (Mazur, 1984; 2004). Therefore, the cooling rate and cell survival usually display an inverted "U"-shaped relationship, and are related to the choice of cryoprotectant (Mazur, 2004). This is true in most species tested so far, and in this study the evaluation of cooling rate also showed these results.

4.3. Effect of sperm concentration on cryopreservation

In this study, the sperm concentration used (except in Experiment V) was 1×10^8 cells/ml. This was based on the findings from previous study of sperm cryopreservation in Pacific oysters (Dong et al., 2007). In that study, agglutination of sperm after thawing was observed and found to be affected by interaction of sperm concentration, type of cryoprotectant, cryoprotectant concentration, and cooling and thawing methods. Under the conditions used in that study, a sperm concentration of greater than 1×10^8 cells/ml led to the formation of higher levels of agglutination. To develop a high-throughput protocol for sperm cryopreservation and to use the samples efficiently, higher sperm concentrations are preferred because less time is required for processing and there are less costs in terms of labor, packaging materials, and storage space. As such, an increase of concentration from 1×10^8 to 1×10^9 cells/ml could reduce cost by about 90% on a per-sperm basis (for example by enabling use of 50 straws to hold the same number of sperm that would be held in 500 straws at the lower concentration). More specifically, based on our experience on sperm collection in eastern oysters, sperm suspensions at 1×10^8 cells/ml can typically be obtained by suspending the testis in buffer with a volume of 60 times the testis weight. This means 1 g of testis can yield about 60 ml of suspended sample, which will require about 120 straws for packaging and considerable space for storage. For an oyster of about 5–7 cm in height and 4–6 cm in length, it is typical to collect 1 g of testis at the peak of spawning season. If the packaging concentration could be increased to 1×10^9 cells/ml, only 12 straws would be needed for the sperm of this oyster with storage space and transportation costs reduced accordingly.

In this study, the testing of serial concentrations ranging from 1×10^8 and 1×10^9 cells/ml did not show differences in post-thaw motility and agglutination was not observed. This indicated that it would be practical to adjust the sperm concentration to between

1×10^8 and 1×10^9 for freezing at the specified conditions. In an early study of eastern oysters (Hughes, 1973), agglutination was observed in post-thaw sperm samples with 5% or 10% DMSO as cryoprotectants, but sperm concentration was not controlled or reported in that study, and as such it is impossible to directly compare those results with the current study. However, it is important to note that such failures in cryopreservation are usually ascribed to problems with cryoprotectants or cooling rates, and the effect of sperm concentration on post-thaw outcomes is typically not considered.

4.4. The high-throughput processing for sperm cryopreservation

In this study, French straws and CBS straws were used to package samples for freezing. This was in consideration of high-throughput processing in which these straws can be used with automated filling, labeling, and sealing equipment. French straws are made from polyvinyl chloride and polyethylene terephthalate glycol, and CBS straws are made from an ionomeric resin with an external polyvinyl chloride identification jacket for labeling. Therefore, the heat transfer of these two types of straws is different (our unpublished data, Hu et al., 2009) and can influence the choice of cooling and thawing rates. However, in the current study, there was no difference in suitable rates for these two types of straws based on post-thaw parameters.

In this study we used the automated MAPI system from IMV technologies (CryoBio System, www.cryobiosystem-imv.com) for filling, sealing, and labeling of CBS straws. This system can process 900 straws per h, and is specially designed for CBS straws. However, there are other commercially available high-throughput processing systems, for example, from Minitube International (CombiSystem, www.minitube.com) that can process 4200 French straws per h for filling and sealing (MPP Uno type) or 15,500 straws per h (MPP Quattro type, using simultaneous processing of 4 straws), and can be combined with a printer that can label 20,000 straws per h.

After processing with automated equipment, the packaged samples can be reliably frozen with programmable freezers, and freezer capacity can influence high-throughput output. With cooling rates ranging from 0.01 to 60 °C/min, freezer capacities can range to as many as 3000 straws (TurboFreezer™ from Minitube International or Digitcool from IMV Technologies) per freezing cycle. Thus systems such as these can routinely produce thousands of straws per day, providing a practical solution for management of oyster germplasm, repository development, and genetic improvement at an industrial scale.

4.5. Indicators for evaluating post-thaw sperm fertility

High fertility after thawing is the goal for any sperm cryopreservation program. Identification of indicators related to fertility of thawed sperm would assist quality control. In this study, oyster body weight, height, and length were not correlated with fertility. This is understandable because molluscan shells can vary widely with environmental conditions, season, age, and other factors, and are not necessarily related to gonadal condition (Galtsoff, 1964). However, in this study fresh sperm motility and post-thaw motility also did not show correlations with fertility. This is different from some studies with fish in which fresh sperm motility or post-thaw motility can be correlated with fertility, such as in blue catfish *Ictalurus furcatus* (Hu et al., 2011), zebrafish *Danio rerio* (Yang et al., 2007) and medaka *Oryzias latipes* (Yang et al., 2010). The lack of correlation in the present study could have resulted from an insufficient number of sampled individuals, the overall low values measured for motility by CASA (which minimized the differences among individuals), or the usual difficulties in standardizing fertilization in strip-spawning of oysters (Kennedy et al., 1996). Theoretically, CASA is considered to provide an objective, accurate assessment of sperm motility because it relies on actual counts and measurements rather than subjective observation and estimations (Douglas-Hamilton et al., 2011). It was developed for use in human and livestock applications, and has recently been applied in aquatic species (reviewed in Yang and Tiersch, 2011). Proper settings are still being developed for aquatic species including oysters. Similar to sperm from humans and livestock, oyster sperm can swim continuously for hours upon suspension in seawater or buffer (considerably longer than for sperm of most fishes), providing convenience for motility analysis by use of CASA.

4.6. Conclusions

This study systematically evaluated the effects of cryoprotectants, cooling rates, thawing temperatures, equilibration times, and sperm concentrations on cryopreservation with 0.5-ml French and CBS straws as packaging containers. The concluded protocol was: collection of oyster sperm by dissection and stripping of the gonad into Ca-free Hanks' balanced salt solution at an osmolality of 650 mOsmol/kg (Ca-free HBSS650), mixing with an equal volume of 20% DMSO in HBSS650 to yield a final concentration of 10^8–10^9 sperm/ml, packaging in 0.5-ml French or CBS straws, cooling at 25 °C/min for French straws or 15–25 °C/min for CBS straws from 5 to −80 °C/min, transfer into liquid nitrogen for storage, thawing at 40 °C for 8 s (for French straws) or 20 s for CBS straws, and fertilization with freshly collected eggs immediately (within 1 min) after thawing. This protocol was tested for germplasm preservation with high-throughput processing by use of automated systems for filling, sealing, labeling, and freezing, and can routinely yield a throughput of thousands of straws per day depending on the configuration of automated equipment and the capacity of freezer used.

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