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Laboratory Studies of Cryopreservation of Sperm and Trochophore Larvae of the Eastern Oyster

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The eastern oyster, *Crassostrea virginica*, is the most important cultured oyster species of the Atlantic and Gulf coasts of the United States. Cryopreservation of gametes and larvae of aquatic organisms has increased in importance in recent years. However, studies on the cryopreservation of sperm and larvae of mollusks have focused on the Pacific oyster, *Crassostrea gigas*. The present study was conducted to improve cryopreservation of sperm and trochophore larvae and to assess fertilizing ability and male-to-male variation of thawed sperm of the eastern oyster. Sperm were diluted in 12 cryoprotectant solutions composed of Hanks' balanced salt solution without calcium and 0, 5, 10, 15, 20, and 25% (v/v) propylene glycol with or without 0.25 M sucrose. Trochophore larvae were suspended in artificial seawater and 10 or 15% propylene glycol (v/v). Sperm or trochophore larvae were placed in 5-mL macrotubes and allowed to equilibrate for 15 min. The macrotubes were cooled in a controlled-rate freezer at a rate of 2.5°C per min until reaching a final temperature of −30°C and were plunged into liquid nitrogen. After storage for 2 weeks, the samples were thawed in a water bath at 70°C for 15 s. Overall, for cryopreservation of sperm and larvae, best results were obtained using 10 or 15% propylene glycol. Thawed sperm presented significant male-to-male variation in fertilizing ability. Survival of thawed larvae decreased as the concentration of larvae per macrotube increased. The procedures developed in this study for sperm and larvae are suitable for production of seedstock in commercial oyster hatcheries.

Key Words: eastern oyster; sperm; trochophore larvae; cryopreservation.

INTRODUCTION

Freezing of sperm dates back more than 50 years to the discovery by Polge *et al.* (34) that the addition of glycerol allowed survival of human and fowl sperm after thawing. Today, cryopreservation techniques in domestic animals are well known and are applied commercially, such as in the dairy industry (11). In contrast to the research and advances made in cryopreservation of sperm and embryos of domestic animals and humans, few methodologies are available for the application of cryopreservation to aquatic organisms other than fish, and most of these have addressed sperm. To date, it is estimated that spermatozoa of some 200 species of freshwater and marine fishes have been cryopreserved, with the majority of publications and protocols addressing four groups of aquacultural importance: salmonids, tilapias, carps, and catfishes (35, 40). Little research has been reported on the cryopreservation of embryos and larvae in aquatic organisms, and research in aquatic invertebrates has concentrated on spermatozoa of the Pacific oyster, *Crassostrea gigas* (22, 13). Cryopreservation of advanced developmental stages of aquatic organisms is difficult because of their structural complexity. The first successful cryopreservation of a fully developed aquatic organism, the ragworm, *Nereis virens*, was reported recently (28). This helped to reinvigorate study of cryopreservation in embryos and larvae of aquatic organisms and encouraged application in aquaculture (27).

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Interest in cryopreservation of gametes and larvae of aquatic organisms is increasing due to potential applications in conservation efforts and aquaculture. Despite this, only ~30 species of invertebrates have been studied (12). To date, fewer than 50 papers have been published about cryopreservation of sperm of invertebrates (12) and fewer than 15 papers have been published about cryopreservation of embryos and larvae.

In oysters, cryopreservation techniques were used initially to study self-fertilization (16) after animals underwent sex reversal. The need for cryopreservation of gametes and larvae of oysters has expanded due to potential applications in the oyster industry such as improved management and production of seedstock, increased availability and distribution of selected lines, and development and maintenance of genetically modified stocks. At present, there are fewer than 25 publications concerning cryo-preservation of gametes or larvae of oysters (Tables 1 and 2). Most of these studies have focused on the Pacific oyster due to its commercial importance worldwide. Several protocols have been designed for cryopreservation of sperm and larvae of this species; however, results range from successful to ineffective, and research is focusing on the optimization of these techniques.

Cryopreservation studies of the eastern oyster began in the 1970s. Only two studies addressing sperm cryopreservation have been reported (14, 47) (Table 2). Twenty years have passed without further study of cryopreservation in this species, despite an average annual economic value during this time of 74 million dollars in the United States (with most of this coming from the Gulf coast). Production of eastern oysters has been of historic and economical importance in the United States, and for centuries the Chesapeake Bay was considered one of the most productive oyster areas. Unfortunately, it has experienced steady declines in production over the past century. Annual production was ~480 million kg in 1890, but declined to ~160 million kg by 1905 (5). Between 1930 and 1980, the annual production was relatively stable at 65 to 95 million kg. Nevertheless, in the early 1980s diseases such as Dermo caused by the protozoan *Perkinsus marinus* and MSX caused by *Haplosporidium nelsoni* decimated remaining natural stocks and made traditional culture methods unproductive

TABLE 1
Relevant Cryopreservation Studies of Gametes and Larvae of the Pacific Oyster *Crassostrea gigas*

Material frozen	Use	Reference
Sperm	Self-fertilization studies	(16)
Sperm	Self-fertilization studies	(42)
Sperm	Self-fertilization studies	(6)
Embryos	Breeding and conservation of strains	(37)
Oocytes	Breeding management	(10)
Sperm	Breeding management	(15)
Embryos	Conservation of strains	(36)
Sperm	Breeding management	(46)
Larvae	Monitoring of water quality	(23)
Embryos	Breeding management	(18)
Embryos	Breeding management	(8)
Embryos	Development of cryopreservation techniques	(19)
Embryos and oocytes	Development of cryopreservation techniques	(17)
Sperm	Breeding and monitoring of water quality	(22)
Embryos	Breeding management	(13)
Embryos and early larvae	Development of cryopreservation techniques	(9)
Oocytes, embryos, and larvae	Development of cryopreservation techniques	(25)
Sperm	Breeding management	(41)
Embryos	Development of cryopreservation techniques	(20)

TABLE 2
Relevant Cryopreservation Studies of Gametes and Larvae of Other Oyster and Clam Species

Species	Material frozen	Reference
Crassostrea virginica	Sperm	(14)
Crassostrea virginica	Sperm	(47)
Ruditapes philippinarum	Embryos	(37)
Pecten maximus	Embryos	(37)
Crassostrea tulipa	Sperm	(46)
Crassostrea iredalei	Sperm	(46)
Saccostrea cucullata	Sperm	(46)
Tapes philippinarum	Larvae	(23)
Meretrix lusoria	Embryos and early larvae	(9)
Crassostrea virginica	Larvae	(31)
Crassostrea virginica	Sperm and larvae	(30)

(32, 5). In 1996, only 1 million kg of oysters were landed. This catastrophic decline in oyster populations in Chesapeake Bay has left the Maryland and Virginia oyster industries in collapse. Development of procedures for cryopreservation of gametes and larvae of this species could assist recovery efforts for the eastern oyster industry.

Cryopreservation protocols for use in eastern oyster hatcheries could assist production by making top-quality larvae available year-round, preserving desirable lines, and enabling genetic and breeding studies in areas such as development of disease-resistant lines. Although there are protocols available to cryopreserve sperm of the Pacific oyster (growth beyond larval stages has not been achieved for cryopreserved embryos and larvae in this species), cryopreservation methods developed for one species are not always applicable to other species. Reproducible techniques for long-term storage of gametes and larvae of the eastern oyster are needed.

The goal of the present study in the eastern oyster was to improve cryopreservation techniques for sperm and larvae in the laboratory for subsequent use in the hatchery. The objectives were to (1) improve techniques for cryopreservation of sperm, (2) evaluate the effect of variability of thawed sperm from individual males on fertilizing ability, and (3) improve techniques for cryopreservation of trochophore larvae.

MATERIALS AND METHODS

Oyster Collection

Oysters were collected from a shellfish hatchery on Grand Isle, Louisiana (29°15′12″N, 90°03′26″W), maintained by the Louisiana Sea Grant College Program, and were transported to the Louisiana State University, Aquaculture Research Station in Baton Rouge. After 5 days of acclimation to laboratory recirculating systems (7), the oysters were opened and inspected visually for the presence of gonadal development and prominent genital canals (1). Gonad samples were collected with a capillary tube and were smeared on a glass microscope slide for examination at 200× magnification. Sex was identified based on the presence of eggs or sperm (Fig. 1).

Gamete Preparation

Gamete samples were removed from each oyster by the dry stripping method (1). The gonad was gently disrupted and material was collected with a Pasteur pipette. The gametes were placed in separate 50-mL beakers until suspension. Eggs were suspended in artificial seawater (Fritz Super Salt; Fritz, Inc., Dallas, TX) at 601 mOsmol/kg (ASW 601). After suspension, eggs were washed through a 70-μm screen (Aquacenter, Leland, MS), collected on a 15-μm screen, and resuspended in ASW 601. Sperm samples were suspended in calcium-free Hanks' balanced salt solution (C-F HBSS) and were filtered through 70- and 15-μm screens. Motility was estimated as described below. Ten-microliter samples were removed from the gamete suspension to measure osmolality with a vapor pressure osmometer (Model 5500; Wescor Inc., Logan, UT).

Motility Estimation

A 10-μL sample was removed from sperm suspensions to estimate motility. The sample was mixed with 20 μL of C-F HBSS (676 mOsmol/kg) on a glass microscope slide. The percentage of sperm exhibiting vigorous forward movement was estimated at 200× magnification using darkfield microscopy (Optiphot 2;

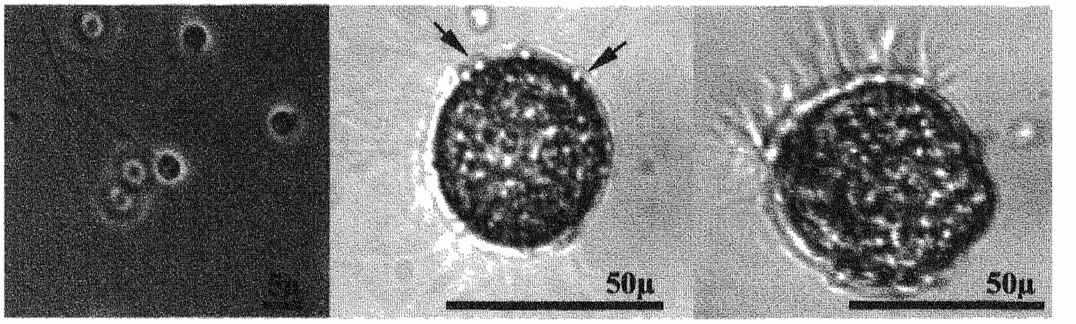

FIG. 1. Gametes and larva of the eastern oyster, *Crassostrea virginica*. Left, phase-contrast micrograph of sperm; middle, fertilized egg, arrows indicate sperm surrounding the egg; right, late trochophore larvae.

Nikon Inc., Garden City, NY). Sperm vibrating in place were not considered motile. Only males with actively swimming sperm (>90%) were selected for experimentation.

Cryopreservation of Sperm

Sperm from three males (96 ± 5% motility) were used for this experiment (30). Osmotic pressure was measured with a vapor pressure osmometer. Cryoprotectant solutions were prepared with fresh-made filtered C-F HBSS containing 0, 5, 10, 15, 20, or 25% (v/v) propylene glycol (PG), corresponding to 0.68, 1.36, 2.04, 2.72, or 3.40 M, for a final volume of 30 mL. Another series of cryoprotectants were prepared by adding 0.25 M sucrose to the solutions. Sperm suspensions (15 mL) were placed in plastic weighing boats (VWR Scientific Inc., St. Louis, MO) and cryoprotectant was added to yield a final volume of 30 mL. Sperm aliquots (5 mL) were placed in 5-mL macrotubes (Minitube of America, Inc., Madison, WI) and equilibrated for 20 min at 21°C. Osmotic pressure and motility were measured after the addition of cryoprotectants (before cryopreservation) to determine the toxic effect of cryoprotectants and after cryopreservation.

After equilibration, sperm were cooled in a controlled-rate freezer (Kryo 10 Series II; Planer Products, Sunbury-on-Thames, UK). The initial temperature was 15°C, and the samples were cooled at a rate of 2.5°C per min until reaching a final temperature of −30°C, which was held for 5 min. The macrotubes were plunged into liquid nitrogen and held for 2 weeks.

The tubes were thawed in a water bath at 25°C for 30 s or at 70°C for 15 s. After thawing, sperm samples (5 mL) were placed in 50-mL plastic beakers (VWR Scientific Inc.) and an equal volume of C-F HBSS was added. Motility and osmotic pressure were measured after dilution in C-F HBSS. Thawing rates and temperatures were determined after preliminary experiments.

Eggs of three ripe females were used to determine the fertilizing ability of thawed sperm. The concentration of eggs was determined by the counting of 1-mL aliquots in a Sedgewick–Rafter chamber (Hausser Scientific Parthership, Horsham, PA), which is a fixed-volume slide used to examine water samples. Twelve hours after fertilization, the number of trochophore larvae was determined. Gamete and larvae counts were performed in duplicate. A suspension containing ~304,000 eggs/mL was incubated in ASW for 1 h in a 500-mL plastic beaker. The osmotic pressure of the egg suspension was 512 mOsmol/kg. Approximately 14,000 eggs (35 eggs/mL) were placed in a 500-mL plastic beaker (VWR Scientific Inc.) and fertilized with 500 µL of thawed sperm (~5×10^8 sperm/mL). Eggs from the same stock solution were fertilized with fresh (nonfrozen) sperm (>90% motility) suspended in C-F HBSS (679 mOsmol/kg). After fertilization, fil-

ter-sterilized (0.45 μm) ASW (699 mOsmol/kg; pH 7.5) was added to yield a final volume of 400 mL. The samples and control treatments were incubated at room temperature (25°C) for 12 h. After incubation, the number of larvae/mL was counted in a Sedgewick–Rafter chamber. All gamete and larvae counts were performed in duplicate.

Variability of Thawed Sperm from Individual Males

Gametes from five females and five males (>90% motility) were placed in individual plastic beakers. Sperm samples were suspended in C-F HBSS (641 mOsmol/kg). The total volume per male was 6 mL and mean sperm concentration was $4.4 \pm 1.4 \times 10^8$/mL. Eggs from individual females were placed in separate 500-mL plastic beakers at a concentration of 35 eggs/mL. Eggs from each female were fertilized with sperm of each of the five males in a 5×5 experimental matrix.

Sperm suspended in C-F HBSS (641 mOsmol/kg) from the individual males were placed in plastic weighing boats and propylene glycol was added to yield a final concentration of 15% and a volume of 5 mL. Sperm aliquots were placed in 5-mL macrotubes and equilibrated for 20 min at 21°C. After equilibration, the macrotubes were frozen in a controlled-rate freezer under the conditions described above. Macrotubes were plunged in liquid nitrogen and after a month of storage the macrotubes were thawed at 70°C and the sperm were diluted (1:1) in C-F HBSS.

Eggs from five ripe females (different from the females used in the fertilization experiment above) were used to assess the fertilizing ability of thawed sperm. Eggs from each female were fertilized individually with thawed sperm of each of the five males, yielding a 5×5 matrix. A different set of males was used as a control. Fresh sperm samples of five males were pooled in the same proportions as those used for individual males and were used to fertilize the eggs from the five females. Fertilized eggs were incubated for 12 h at room temperature (25°C). After incubation, the number of larvae/mL was counted in a Sedgewick–Rafter chamber. All gamete and larvae counts were performed in duplicate.

Cryopreservation of Trochophore Larvae

Six million eggs (35 eggs/mL) were suspended in 2 L of ASW and fertilized with fresh (nonfrozen) sperm in an approximate ratio of 1 egg to 5 to 10 sperm. Concentrations of eggs were determined by counting of 1-mL aliquots in a Sedgewick–Rafter chamber. Fertilized eggs were incubated until ~50% were in first cell division at 25°C (~1 h). After incubation, fertilized eggs (Fig. 1) were placed in 15-L plastic buckets at 21°C. Twelve hours after fertilization, the number of trochophore larvae/mL was counted in a Sedgewick–Rafter chamber. Gamete and larvae counts were performed in duplicate. Temperatures varied during the experiment based on the location of specific activities. Fertilizations were performed in the wet laboratory (25°C) and larvae were incubated on the laboratory bench (21°C) until cryopreservation at 12 h after fertilization (trochophore stage).

Two cryoprotectant solutions were prepared with filtered ASW containing 10 or 15% PG (12). Trochophore larvae were concentrated on a 30-μm screen, yielding a total volume of 24 mL (3 million larvae). After concentration, the larvae were mixed with cryoprotectant. Five different larval concentrations (10, 100, 1000, 10,000, and 100,000 larvae/mL) were tested for each cryoprotectant. Osmotic pressure of the suspensions was measured before (642 mOsmol/kg) and after addition of cryoprotectant (1461 mOsmol/kg for larvae suspended in 10% PG, and 1627 mOsmol/kg for larvae in 15% PG). Five-milliliter aliquots were placed in 5-mL macrotubes and equilibrated for 20 min at 21°C. After equilibration, the macrotubes were frozen under conditions described for sperm.

Macrotubes were thawed in a water bath at 70°C for 15 s. After thawing, the larvae were placed in 50-mL plastic beakers containing 5 mL of filtered ASW. The number of motile trochophore larvae/mL was estimated by counting in a Sedgewick–Rafter chamber.

Data Analysis

Statistical analyses were performed using SAS software for Windows (SAS Institute, Cary, NC). Motility of sperm was analyzed using a three-way analysis of variance for the effect of cryoprotectant concentration, presence or absence of sucrose, and thawing temperature. Fertilizing ability of sperm was analyzed using a three-way analysis of variance for the effect of cryoprotectant concentration, presence or absence of sucrose, and thawing temperature. One-way factorial analysis of variance was used to test male-to-male variability. For cryopreserved larvae, two-way analysis of variance was used to test the effect of concentration of larvae per macrotube and cryoprotectant concentration. Data were arcsine square-root transformed before analysis. Specific differences among treatment groups were identified by the least square difference test. A value of $P < 0.05$ was chosen as the level for significance.

RESULTS

Cryopreservation of Sperm

Sperm motility of samples suspended in different concentrations of cryoprotectant with or without addition of sucrose before cryopreservation was significantly different ($P = 0.001$) (Fig. 2). Sperm suspended without cryoprotectant in 0.25 M sucrose declined by as much as 40% in motility. Sperm suspended in the different cryoprotectant concentrations with the addition of 0.25 M sucrose declined in motility by as much as 30%. Sperm suspended in 0, 5, and 10% propylene glycol without addition of sucrose maintained high motility (>90%). Sperm suspended in 15% propylene glycol declined in motility by as much as 76% and, beyond that concentration, motility dropped to 4%. After 20 min of equilibration, sperm suspended in 0, 5, 10 and 15% propylene glycol with or without sucrose were resuspended in fresh C-F HBSS and regained their original motility (>90%), indicating that a lingering

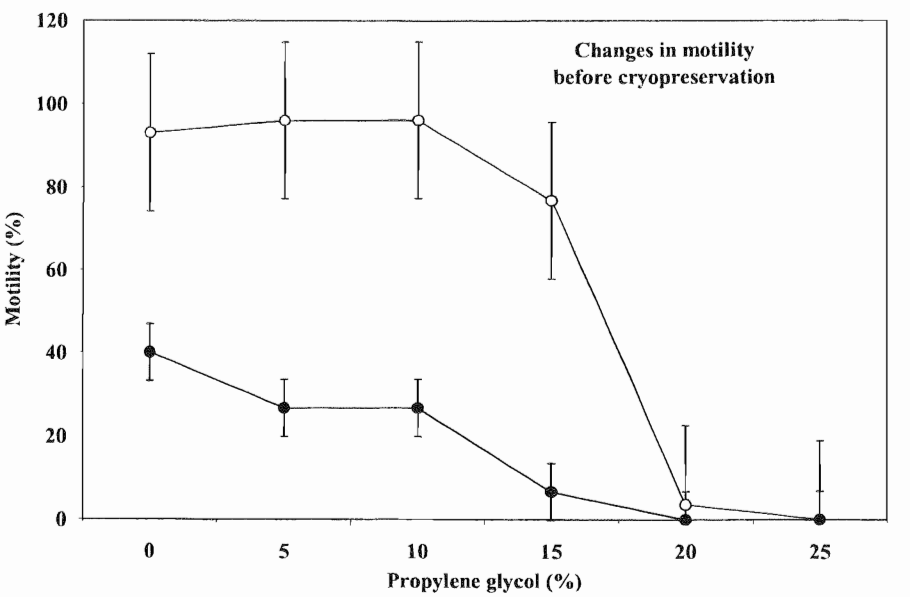

FIG. 2. Changes in the motility of sperm before cryopreservation (mean ± SE). Motility of sperm suspended in cryoprotectant solution without addition of sucrose (open circles) and motility of sperm suspended in cryoprotectant solution with addition of sucrose (closed circles).

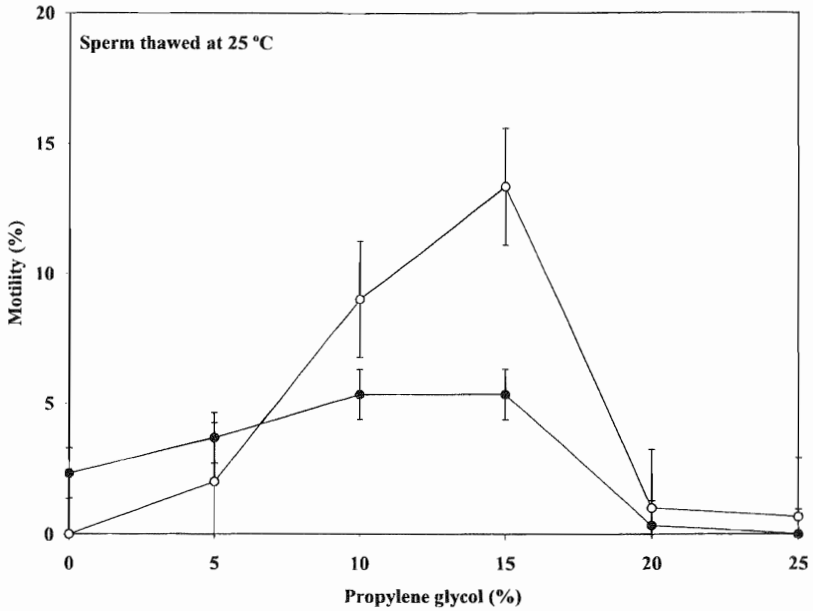

FIG. 3. Motility (mean ± SE) of sperm thawed at 25°C used to fertilize eggs. Open circles, motility of sperm without sucrose; closed circles, motility of sperm with sucrose.

toxic effect of these cryoprotectant solutions was not present.

For sperm thawed at 25°C, the highest motility (13%) was found for samples suspended in 15% propylene glycol without the addition of sucrose (Fig. 3). The highest fertilization (29%) was found for sperm suspended in 10% propylene glycol without the addition of sucrose (Fig. 4). For sperm thawed at 70°C, the highest motility (22%) was found for sperm suspended in 15% propylene glycol without the addition of sucrose (Fig. 5), and the highest fertilization (57%) was found for sperm suspended in 10% propylene glycol without the addition of sucrose (Fig. 6). Sperm motility and fertilizing ability were affected by the concentration of cryoprotectant ($P = 0.0001$). Addition of sucrose did not enhance sperm motility ($P = 0.9020$) or fertilizing ability ($P = 0.4314$). Thawing rate had a significant effect on sperm motility ($P = 0.0163$) and fertilizing ability ($P = 0.0145$).

Variability of Thawed Sperm from Individual Males

It was not possible to use the same batches of eggs to assess the fertilizing ability of thawed and fresh (nonfrozen) sperm from the same males because the sperm were stored frozen for a month. Thus, two groups of females were used. As control, eggs were fertilized with pooled fresh sperm from a new set of five males, yielding 90 ± 21% fertilization, which indicated that the eggs were of good quality. The fertilizing ability of fresh sperm from individual males was not significantly different ($P = 0.8609$) (Fig. 7), and differences in fertilization were attributed to females ($P = 0.0001$). However, the fertilizing ability of thawed sperm from the same males was significantly different ($P = 0.006$) (Fig. 7).

Cryopreservation of Trochophore Larvae

Larval survival was affected by the concentration of larvae per macrotube ($P = 0.001$).

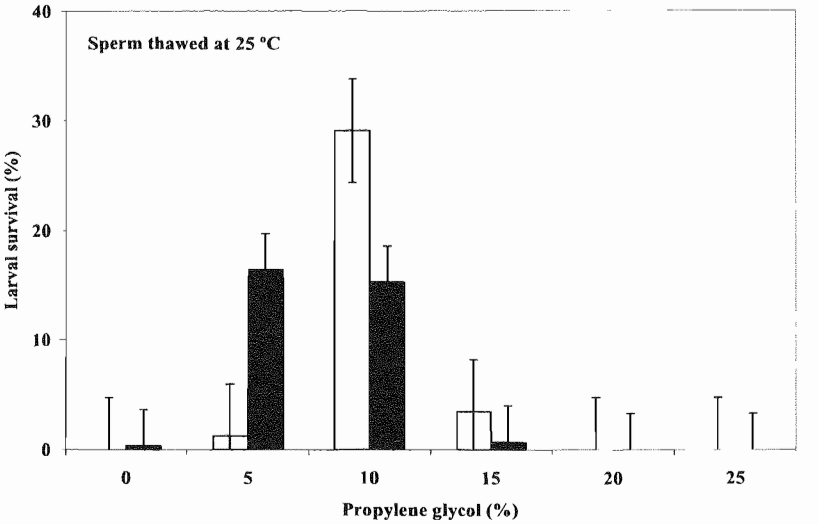

FIG. 4. Survival (mean ± SE) of trochophore larvae 12 h after fertilization by sperm thawed at 25°C. Open bars, fertilization by sperm without sucrose; shaded bars, fertilization by sperm with sucrose.

The highest numbers (4000) of surviving larvae were found for a concentration of ~50,000 larvae per macrotube, although the highest percentage of survival (~100%) was found for a concentration of 125 larvae/mL (Fig. 8).

DISCUSSION

Embryo cryopreservation has been applied to a wide variety of domestic and wild species since the first reports 30 years ago (43, 44). These studies have emphasized mammalian

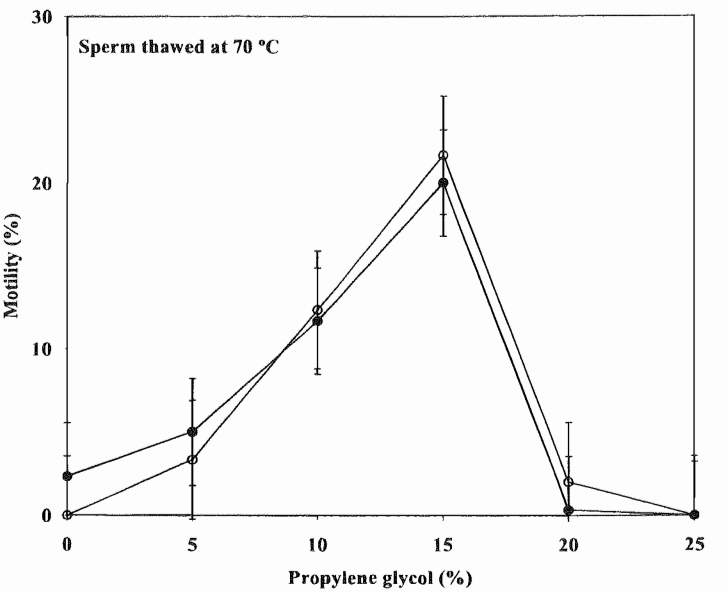

FIG. 5. Motility (mean ± SE) of sperm thawed at 70°C used to fertilize eggs. Open circles, motility of sperm without sucrose; closed circles, motility of sperm with sucrose.

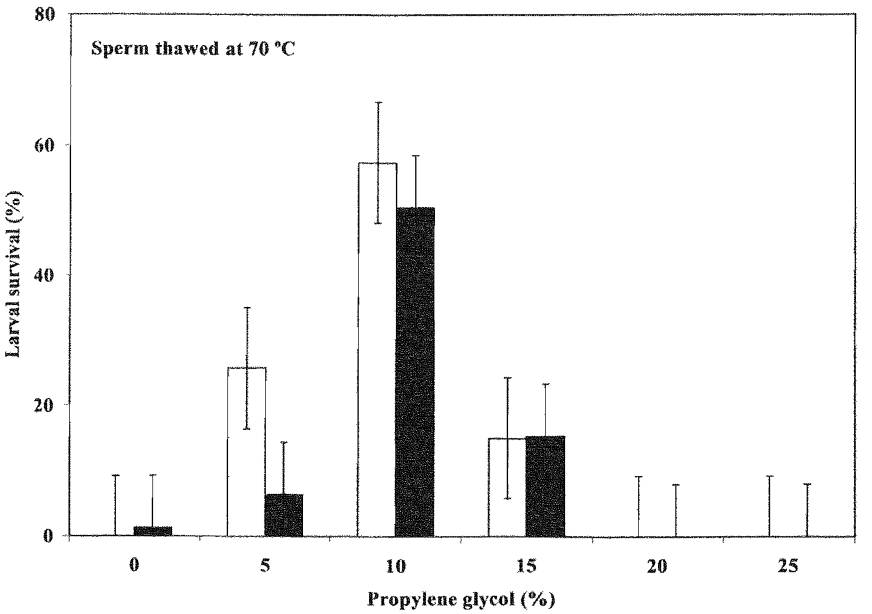

FIG. 6. Survival (mean ± SE) of trochophore larvae 12 h after fertilization by sperm thawed at 70°C. Open bars, fertilization by sperm without sucrose; shaded bars, fertilization by sperm with sucrose.

species such as humans, cattle, pigs, and mice. Studies in aquatic species are limited to less than 50 publications. The successful cryopreservation of juveniles of the polychaete *N. virens* (28) provided a needed model for aquatic species, which have physiological characteristics different from those of mammals. Clearly, additional research models are needed for

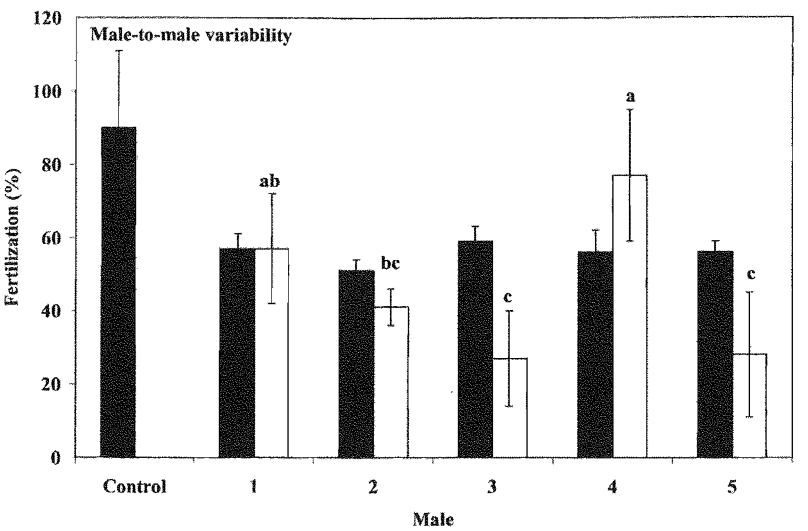

FIG. 7. Fertilizing ability (mean ± SE) of eastern oyster sperm from five males before (shaded bars) and after (open bars) cryopreservation. Bars sharing a letter were not significantly different.

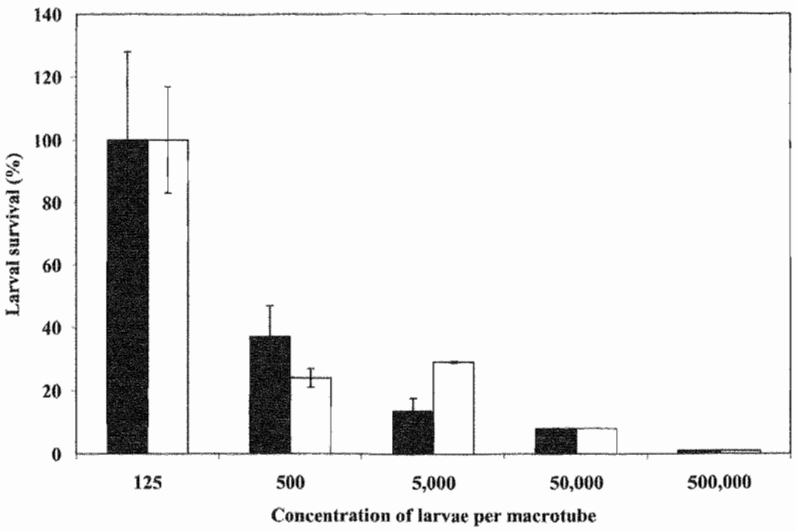

FIG. 8. Survival of trochophore larvae (mean ± SD) after thawing and suspension in artificial seawater at five larval concentrations in 5-mL macrotubes. Shaded bars, larvae cryopreserved in 10% propylene glycol; open bars, larvae cryopreserved in 15% propylene glycol.

aquatic species. For example, estuarine organisms are subjected to large and rapid osmotic changes (e.g., from 400 to 1000 mOsmol/kg) not experienced by freshwater or terrestrial species. Specifically, oysters are adapted to intertidal zones, which experience rapid changes in salinity and temperature, including exposure to freezing temperatures, and could provide useful models for other aquatic species including endangered mollusks.

The first study on cryopreservation of eastern oyster sperm reported that eggs presented with thawed sperm yielded 2% fertilization (35 min after insemination) (14). A later study reported growth of D-stage larvae 24 h after insemination from naturally spawned eggs fertilized with thawed sperm of the eastern oyster (47). However, the experimental conditions in the latter study were difficult to reproduce. The samples were intended to be cooled at 5°C/min, but the actual cooling rates were variable, and in some trials sperm samples were combined. Thus, the fertilizing ability of thawed sperm from individual males could not be tested. No other work addressing cryopreservation in the eastern oyster has been reported except for our studies of sperm and larvae in a commercial-scale hatchery (31, 29). This work included the first production of oyster spat and seed oysters (larvae that progressed past the free-swimming stages of the first week after insemination and metamorphosed into sessile juveniles).

The use of sugars in the cryopreservation of sperm of domestic livestock is a common practice. However, sugars such as trehalose or sucrose have not been used in the cryopreservation of oyster sperm. It believed that sugars can contribute to stabilization of membranes by forming hydrogen bonds between the sugar hydroxyl groups and the phospholipid polar heads, substituting for water molecules under dehydrating conditions during freeze-drying or low-rate freezing (2, 39). Also, it is believed that sugars can change the properties of the bulk solution in addition to acting on the lipid bilayer. Therefore, sugars could affect the pattern of crystallization and the mechanical properties of the solidified medium (26). Another potential benefit of sugars is that they may help to reduce the salt concentration of the unfrozen fraction or may help to prevent solute injury in eutectic freezing by trapping salts in viscous phase (45).

In the present study, we found that motility decreased from 90 to 5% within 20 min when sperm were suspended in C-F HBSS plus sucrose with and without cryoprotectant, although motility was restored when sperm were resuspended in C-F HBSS (676 mOsmol/kg). In some fishes, it is reported that a change in osmotic pressure of the diluent activates sperm motility (24, 4). However, for the eastern oyster, motility (90%) was maintained over a range of osmolality from 600 to 1500 mOsmol/kg. Furthermore, the presence of sucrose did not affect sperm survival after thawing. Previous studies have yielded various results when the effects of sugars were considered in isolation from other factors such as cryoprotectant extender medium and cooling rate. Thus, the combined effect of these factors should be considered to improve protocols when sugars are used (45).

The highest fertilization found in the present study for cryopreservation of eastern oyster sperm was with 10% propylene glycol, although the highest motility was found with 15% propylene glycol. This indicates that motility was not a direct indicator of fertilizing ability and that other techniques such as fluorescence microscopy or flow cytometry will be required to evaluate the quality of thawed sperm and to evaluate damage of specific compartments in the head of the sperm such as the acrosome, which is responsible for lysis of the egg membrane in the fertilization process. Inconsistency in the quality of sperm after thawing has been discussed in several studies (3). Motility is used routinely to predict the fertilizing ability of sperm, but it does not always correlate with fertilization rates before freezing or after thawing (38, 33). This discrepancy has been attributed partly to biological variation in factors such as genetic traits and health of broodstock (3). In the present study, there was no difference in fertilizing ability of fresh sperm from five males; however, the fertilizing ability of sperm from these males varied upon thawing. It is possible that the sperm of different males vary in response to freezing and thawing. More studies need to be performed to investigate variation in the fertilizing ability of thawed oyster sperm.

For larvae, techniques previously reported for the Pacific oyster (13) were adapted in this work. However, this previous report did not identify thawing rates, concentration of larvae, or type of freezing tube used. These factors are important for experiments at the hatchery level. For practical purposes, in the present study 5-mL macrotubes were used to store larger numbers of trochophore larvae (thousands or millions per macrotube) than were possible using 0.5-mL French straws. In other studies, greatest attention has been placed on physical and chemical factors that affect survival of thawed trochophore larvae (20). However, biological factors need to be considered also. One of the most important is the quality of larvae, which can vary from batch to batch. Broodstock oysters mature at different rates due to genetic differences, food availability, stress, or other environmental factors. Consequently, these factors can influence the meiotic events, yielding oocytes with differential fertilization potential or asynchrony in developmental stage. Also, the density of oocytes can play an important role in the quality of larvae produced. For the eastern oyster, an optimum of 30,000 eggs/L has been recommended to obtain successful fertilization (21). We found that larvae from batches with poor fertilization (e.g., $<40\%$) did not respond well to cryopreservation.

Another important factor that affected larval survival was the concentration of larvae per macrotube. If embryos are allowed to settle atop one another, toxic substances can accumulate and oxygen necessary for normal metabolic functions can become limiting. This creates problems for cryopreservation procedures, which require the concentration of larvae. When cryopreserved at low densities ($\sim$150 per macrotube) larvae showed almost complete survival and strong swimming movements upon thawing. Higher densities were associated with reduced survival and vigor. It is possible that larvae cryopreserved at high densities would benefit from improved culture conditions after thawing. Although we did not rear the thawed larvae in these laboratory studies beyond the trochophore stage, protocols based on these ex-

periments have been used elsewhere to rear larvae produced from thawed sperm and thawed larvae beyond metamorphosis (settlement) in a research hatchery (31, 29).

The present study details cryopreservation of sperm and larvae of the eastern oyster in the laboratory and provides useful protocols for the application of cryopreservation for oyster production at a commercial scale. In summary, best fertilization results for sperm were obtained using C-F HBSS at ~640 mOsmol/kg in 5-mL macrotubes with 10% propylene glycol as the cryoprotectant. The samples were cooled at 2.5°C per min until reaching a final temperature of −30°C, which was held for 5 min before plunging into liquid nitrogen. They were thawed at 70°C for 15 s. Best survival for trochophore larvae was obtained using the same procedures used for sperm, but the optimal density was ~50,000 larvae per macrotube.

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