Available online at www.sciencedirect.com
SCIENCE DIRECT®
Cryobiology 51 (2005) 176–197
CRYOBIOLOGY
www.elsevier.com/locate/ycryo

# Systematic factor optimization for cryopreservation of shipped sperm samples of diploid Pacific Oysters, *Crassostrea gigas* ☆

Qiaoxiang Dong [a], Changjiang Huang [a,b], Benoit Eudeline [c], Terrence R. Tiersch [a,*]

[a] *Aquaculture Research Station, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, USA*
[b] *School of Marine Science and Technology, Zhejiang Ocean University, Zhoushan 316004, PR China*
[c] *Taylor Resources Hatchery, Quilcene, WA 98376, USA*

Received 13 May 2005; accepted 24 June 2005
Available online 29 August 2005

**Abstract**

Despite some 26 published reports addressing oyster sperm cryopreservation, systematic factor optimization is lacking, and sperm cryopreservation has not yet found application in aquaculture on a commercial scale. In this study, the effects of cooling rate, single or combined cryoprotectants at various concentrations, equilibration time (exposure to cryoprotectant), straw size, and cooling method were evaluated for protocol optimization of shipped sperm samples from diploid oysters. Evaluation of cooling rates revealed an optimal rate of 5 °C/min to −30 °C followed by cooling at 45 °C/min to −80 °C before plunging into liquid nitrogen. Screening of single or combined cryoprotectants at various concentrations suggested that a low concentration (2%) of polyethylene glycol (FW 200) was effective in retaining post-thaw motility and fertilizing capability when combined with permeating cryoprotetcants such as dimethyl sulfoxide (DMSO), methanol (MeOH), and propylene glycol (P-glycol). However, polyethylene glycol alone was not as effective as MeOH, DMSO, and P-glycol when using the same methods. The highest post-thaw motility (70%) and percent fertilization (98%) were obtained for samples cryopreserved with 6% MeOH. However, this does not exclude other cryoprotectants such as DMSO or P-glycol identified as effective agents in other studies. There was no significant difference in post-thaw motility between straw sizes of 0.25- and 0.5-ml. Equilibration time (exposure to cryoprotectant) of 60 min could be beneficial when the cryoprotectant concentration is low and solution is added in a step-wise fashion at low temperature. Differences in post-thaw sperm quality (e.g., motility or percent fertilization) among individual males were evident in this research. As a consequence, a generalized classification describing males with different tolerances (broad, intermediate, and narrow) to cryopreservation was developed. This classification could be applied to strain or species differences in tolerances to the cryopreservation process. The present study demonstrated that oyster sperm could be collected and shipped chilled to another facility for cryopreservation, and that it could be shipped back to the hatchery for fertilization performed at a

☆ This work was supported in part by funding from the USDA-SBIR Program, 4Cs Breeding Technologies, Inc., and the Louisiana Sea Grant College Program.
* Corresponding author. Fax: +1 225 765 2877.
*E-mail address:* ttiersch@agctr.lsu.edu (T.R. Tiersch).


doi:10.1016/j.cryobiol.2005.06.007

production scale yielding live larvae with >90% fertilization. Given the existence of facilities for commercial-scale cryopreservation of dairy bull sperm, the methods developed in the present study for oysters provide a template for the potential commercialization of cryopreserved sperm in aquatic species.


*Keywords:* Pacific oyster; *Crassostrea gigas*; Cryopreservation; Standardization; Optimization; Sample shipment; Dairy; Oyster; Sperm; Tolerance

Sperm cryopreservation in aquatic species offers many benefits in genetic improvement programs such as hybridization, selective breeding, gynogenesis and androgenesis, development of inbred lines, polyploidization, and domestication. Cryopreservation provides reliable supplies of sperm, without seasonal limitations and costly hatchery maintenance of adult males, and provides a safe repository for improved lines with desirable traits or founder stocks. The first studies of fish sperm cryopreservation were published 50 years ago [6], and since then more than 200 fish species have been studied [44,55]. In contrast to the extensive studies in cryopreservation of fish semen, similar work for invertebrates has been attempted for only about 30 species, and has been limited to echinoderms (sea urchins, sand dollars, and starfishes), mollusks (oysters and abalone), polychaetes, and crustaceans (shrimps and crabs) (for review see [22]). Among these, research has been primarily concentrated on spermatozoa from oysters, specifically focusing on the Pacific oyster, *Crassostrea gigas*.

Although these research efforts have yielded protocols that are being applied with varying levels of success in fish and invertebrates, they essentially are limited to laboratory scale, and sperm cryopreservation has not yet found application in aquaculture on a commercial scale [33]. The benefits of sperm cryopreservation can be fully realized only if this technology is commercialized. In the case of oysters, tetraploid broodstocks have been developed to facilitate the commercial production of triploid seedstock; thus, commercialization of cryopreserved sperm from tetraploid oysters would be especially beneficial for this industry [19]. Currently, human medicine and livestock agriculture are the only worldwide industries that have incorporated cryopreservation of semen into commercial artificial insemination practices [9,14,15]. If cryopreservation of fish or shellfish sperm is to be integrated into hatchery operations, the use of specialized cryopreservation centers such as dairy facilities should be considered as a time-saving and cost-effective option [57]. In fact, dairy protocols have been adapted to freeze sperm from blue catfish, *Ictalurus furcatus* [33], red snapper *Lutijanus campechanus* [48], and diploid and tetraploid Pacific oysters [19]. To achieve commercial-scale application of cryopreserved sperm in aquatic species, research studies in commercial settings are necessary. An under-studied, but especially important component involved in utilization of central cryopreservation facility is the shipment of broodstock or milt. Unlike other studies in oyster sperm cryopreservation, the present study employed the use of shipped sperm and sperm collected from shipped oysters.

Despite the fact that the Pacific oyster has been the focus of 26 sperm cryopreservation studies, with dimethyl sulfoxide (DMSO) as the primary cryoprotectant in the majority of these, the results have varied widely and there has been inconsistent reporting of cryoprotectant concentrations, equilibration time, cooling rates, and thawing conditions [17]. A lack of procedural standardization has been considered to be the most important factor responsible for the inconsistencies in results among various studies, not only in sperm cryopreservation of oysters per se, but of aquatic species in general [22,44,55]. Optimization of protocols without standardization offers little value for the improvement of existing methods and results, especially for the future development of commercial application. Researchers have begun to pay attention to this problem and recent studies address or incorporate standardization in protocol optimization [19,20,36]. The present study continued the efforts to optimize protocols for sperm cryopreservation

for the Pacific oyster through use of a systematic approach. Due to the marked effects of male variation observed in this study, males with broad, intermediate, and narrow tolerances of cryopreservation procedures, in combination with the stress related with shipping, were evaluated and this principle was extended to species variation in general.

The goal of this study was to optimize protocols used for sperm cryopreservation of diploid Pacific oysters, and the approaches taken were to use protocols optimized through laboratory studies and tested in commercial settings. Specifically, post-thaw motility was used as the main criterion for range-finding experiments and procedure optimization, and percent fertilization and hatch were used to test the results of optimized procedures. The objectives of this study were to evaluate the effects on post-thaw sperm quality of: (1) cooling rate; (2) single cryoprotectants and concentrations; (3) combined cryoprotectants and concentrations; (4) interactions between cooling rate and selected cryoprotectants at specific concentrations; (5) equilibration time; (6) straw size (0.25-ml vs. 0.5-ml); and (7) cooling methods for laboratory research-scale and hatchery commercial-scale production.

## Materials and methods

### *Sperm collection and motility estimation*

Diploid Pacific oysters were obtained in June and July, 2004, from Taylor Resources Quilcene Shellfish Hatchery (TRQSH; www.taylorshellfish.com) in Quilcene, Washington (47° 49′133″N, 122° 49′ 523″W), and were shipped chilled at 5–10 °C by overnight delivery to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS). For Experiments 1 and 2, intact oysters were shipped and sperm were collected by dry stripping of the gonad upon arrival. For other experiments, sperm samples were collected with the same method and placed separately into single 15-mL centrifuge tubes for each male, and each week 8–10 samples (undiluted) were shipped in a styrofoam shipper from TRQSH to the ARS. Because this project also included study of sperm from tetraploid oysters, the ploidy level of individual oysters was verified by flow cytometry [1,19]. Sperm samples were placed in a 4 °C refrigerator for temporary storage immediately upon arrival. Prior to experiments, undiluted sperm samples were suspended in calcium-free Hanks' balanced salt solution (C-F HBSS) at 1000 mOsm/kg [18] and suspensions were filtered through a 40-μm cell strainer (BD Biosciences Discovery Labware, Bedford, MA). The concentrations of sperm suspensions were adjusted to $2 \times 10^9$ cells/mL using readings at 581 nm from a spectrophotometer (Genesys 20, Thermo Spectronic, Rochester, New York) and derived standard curves [20]. A total of 27 males were used in this study, and each oyster or sperm sample was identified by a code (e.g., CG04M01) with the species designation (CG: *Crassostrea gigas*), year (04: 2004), sex (M: male) and the order in which it was processed (01: the first oyster of 2004). These codings were used for database entries, and was part of the information permanently printed on the French straws with a specialized laser printer (Domino Codebox 2, Domino Amjet, Gurnee, IL).

Motility was used as one of the indicators of sperm quality. The continuous and prolonged (hours to days) swimming behavior of oyster sperm is distinct from that of the vast majority of teleost fishes (the group most studied in aquatic species), in which sperm motility is elicited only upon contact with the external environment, and the duration of active motility is brief, usually lasting no longer than 1 or 2 min. Sperm motility was estimated visually at 200-× magnification using darkfield microscopy (Optiphot 2, Nikon, Garden City, New York) and was expressed as the percent of cells actively moving in a forward direction. Sperm vibrating in place were not considered to be motile. Initial motility refers to the sperm motility after dilution and shipping, but before the addition of cryoprotectants to the samples. Post-thaw motility was estimated immediately after thawing.

### *Cryoprotectant*

The cryoprotectants tested included permeating and non-permeating compounds. The permeating cryoprotectants were methanol, ethylene glycol, propylene glycol, dimethyl sulfoxide, *N*,*N*-dimethyl

acetamide, and glycerol. The non-permeating cryoprotectants were polymers of polyethylene glycol at formula weights of 200 or 600. Single or combined cryoprotectants were used (abbreviations are listed in Table 1). All solutions were prepared within 2 h of use with C-F HBSS at 1000 mOsm/kg as the diluent and were stored at 4 °C. All chemicals used for preparation of solutions were of reagent grade (Sigma Chemical Corporation, St. Louis, Missouri).

*Freezing and thawing procedures*

For freezing with a controlled-rate freezer (Kryo 10 Series II; Planer Products, Sunbury-on-Thames, UK), aliquots of sperm suspensions with cryoprotectant (detailed below) were drawn into 0.5- or 0.25-ml French straws (IMV International, Minneapolis) manually. Straws were held (equilibrated) for 10 min (except for the experiment addressing equilibration time) at room temperature (23–25 °C), for 2 min at 4 °C in the controlled-rate freezer before initiation of the various cooling rates. For the cooling rates of 0.5, 5, 16, and 30 °C/min, samples were cooled in two steps, initially to −30 °C at these rates, followed by cooling at 45 °C/min from −30 °C to −80 °C. For cooling rates of 45 and 50 °C/min, samples were cooled in a single step from 5 °C to −80 °C directly at the specified rate. All straws were held at −80 °C for 5 min before being plunged into liquid nitrogen in a storage dewar. After a minimum of 12 h, four straws were thawed for 7 s (for 0.5-ml straw) or 6 s (for 0.25-ml straw) in a 40 °C water bath (Model 1141, VWR Scientific, Niles, IL) to estimate the post-thaw motility.

Existing commercial freezing methods developed for dairy bulls were also evaluated in this study. Sperm samples were cryopreserved at the LSU T. E. Patrick Dairy Improvement Center through Genex Custom Collection in Baton Rouge (www.crinet.com/ccollect.htm). Sperm samples were mixed with the appropriate cryoprotectant before freezing and allowed 15 min for equilibration in a walk-in cooler held at 5 °C. The sperm solutions were placed into pre-labeled 0.5-mL French cryopreservation straws using an automated straw filler (model MRS 1, IMV Int., Minneapolis, MN). The straws were placed on horizontal racks with enough water-filled straws added to standardize the heat load within the freezing chamber (660 total straws). The samples were placed in the freezing chamber held at −140 °C. During the first 3 min of freezing, the chamber was allowed to warm from −140 to −60 °C as a result of the heat load of the samples. Liquid nitrogen was added to the chamber to cool at a rate of 16 °C/min returning the chamber to −140 °C [11]. Once frozen, the samples were removed and placed under liquid nitrogen for sorting and preparation for long-term storage. After 2

Table 1
Abbreviations for cryoprotectants

| First cryoprotectant (%) | Second cryoprotectant (%) | Abbreviation |
|---|---|---|
| Methanol | — | MeOH |
| Ethylene glycol | — | E-glycol |
| Propylene glycol | — | P-glycol |
| Dimethyl sulfoxide | — | DMSO |
| *N*,*N*-Dimethyl acetamide | — | DMA |
| Glycerol | — | Not abbreviated |
| Polyethylene glycol 200[a] | — | PEG200 |
| Polyethylene glycol 600[b] | — | PEG600 |
| Polyethylene glycol 200 | Methanol | PEG/MeOH |
| Polyethylene glycol 200 | Propylene glycol | PEG/P-glycol |
| Polyethylene glycol 200 | Dimethyl sulfoxide | PEG/DMSO |
| Polyethylene glycol 200[c] (2%) | Methanol (4%) | 2% PEG/4% MeOH |

[a] Polyethylene glycol at formula weight of 200.
[b] Polyethylene glycol at formula weight of 600.
[c] Example of abbreviation for combined cryoprotectants with specific concentrations.

days, 2–4 straws from each male were thawed in a 40 °C water bath for 7 s to estimate the post-thaw motility. General observations of sperm morphology, such as broken tails or sperm agglutination were also documented for thawed samples.

*Fertilization and larval evaluation*

Ten straws of each treatment were transported in a shipping Dewar (CP35, Taylor-Wharton, Theodore, AL) to the TRQSH for fertilization trials within 2 months of freezing. Diploid females were used for fertilization trials. Eggs from individual females were obtained by dissection, sieved, washed on a 25-μm mesh, and suspended in filtered seawater (34 ppt) at 25 °C. The number of eggs per ml was determined by Coulter counter (Z1 series, Beckman Coulter, Fullerton, CA). After counting, the eggs were held in seawater at 25 °C for at least 30 min to observe germinal vesicle breakdown at 100-× brightfield magnification. In general, unfertilized eggs (fresh) were pooled from three females and separated into beakers, and fertilization trials were conducted by mixing 5 ml of thawed sperm suspension (the pooled contents of ten 0.5-ml straws) with 500,000 eggs (fresh) held in 250 ml of seawater, yielding approximately 10,000 spermatozoa per egg. The gametes were incubated at 25 °C and percent fertilization was calculated by counting developing embryos at 2 h after insemination. Treatments held for further evaluation of percent hatching were transferred to 100-L tanks filled with fresh seawater. Twenty-four hours after fertilization, these tanks were drained through a 45-μm mesh and percent hatching was calculated by counting normal straight-hinge larvae with a dissecting microscope. For a negative control, eggs were monitored after treatment as described above without addition of sperm.

For controls of egg quality, fresh (non-frozen) sperm from diploid males were collected using the techniques described above and the sperm were washed through a 70-μm mesh and added to fresh eggs to obtain about 100 spermatozoa per egg. Sperm counts were performed with a spectrophotometer (DR/2000, Hach, Loveland, CO) at TRQSH based on the techniques developed by [20]. For controls of cryoprotectant toxicity, fresh sperm at the same concentration as those with thawed sperm samples were exposed to the same treatments (concentration, equilibration time, and batch of eggs), and percent fertilization was estimated. To avoid contamination of gametes among individuals, the animals were handled with care and all surfaces were washed with 0.01% bleach. The sexes were held separately in different containers to avoid unintended fertilization.

*Experimental design and data analysis*

A total of 11 experiments (Fig. 1) were carried out and the experimental design began with a preliminary evaluation of five cooling rates with four cryoprotectants (Experiment 1). Cooling rates of 5 and 30 °C/min were selected to test with eight cryoprotectants each at two concentrations (Experiment 2). A cooling rate of 5 °C/min was selected for subsequent extensive evaluations of single or combined cryoprotectants at various concentrations. Among the eight cryoprotectants tested in Experiment 2, six were selected to provide 16 single or combined treatment levels to see possible combinations between different cryoprotectants (Experiment 3, first selection). Subsequently, three combinations (PEG/MeOH, PEG/P-glycol, and PEG/DMSO) were selected for evaluation of optimal concentration combination (Experiment 4, second selection). Separate males were used for each combination because of the large number of treatments (for each combination: 16 treatments × 4 straws per treatment per male × 2 males = 128 straws). Therefore, in Experiment 5 (final selection) the same males were used to test 16 single or combined cryoprotectants selected from Experiment 4. Single or combined cryoprotectants that showed consistently high post-thaw motility from Experiments 2, 3, 4, and 5 were selected to evaluate straw size (Experiment 6), equilibration time (Experiment 8), and to re-evaluate cooling rate (Experiment 7). For all 8 experiments, samples were cooled with the controlled-rate freezer and the criterion used for selection was post-thaw motility. Subsequently, in addition to post-thaw motility, percent fertilization, and hatch were used to evaluate the selected cryoprotectants and cooling rate with the controlled-rate freezer

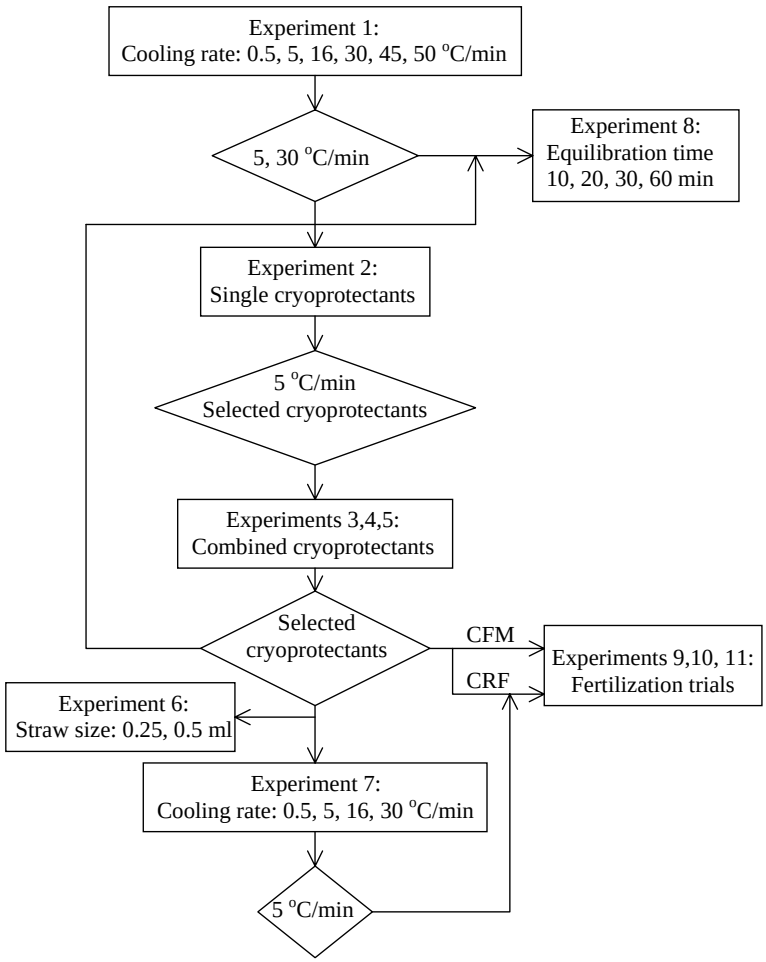


Fig. 1. Design of experiments for optimization of sperm cryopreservation from diploid Pacific oysters. Post-thaw motility was used as the main criterion for procedure optimization and percent fertilization and hatch were used to test the results of optimized procedures. CRF: cooled at 5 °C/min using a controlled-rate freezer; CFM: cooled using a commercial freezing method developed for dairy bulls. All fertilization trials were conducted in the hatchery at Quilcene, Washington. Rectangles indicate experiments, rhomboids indicate decisions made based on experiments.

(Experiment 9), the selected cryoprotectants with commercial freezing method (Experiment 10), and the difference between these two cooling methods with the same males (Experiment 11).

To minimize the effects of seasonality on the sperm quality of oysters used for different experiments, all 11 experiments were performed within the peak spawning month (June 4–July 7). Due to the large number of treatment factors and levels to be evaluated in initial range-finding experiments within this time constraint, most experiments were replicated only with two males (Table 2). However, the experimental design outlined above (Fig. 1) actually allowed factors such as cooling rate and cryoprotectant to be repeatedly evaluated in successive experiments (Table 2), and in some cases the same treatment (e.g., 2% PEG/4% MeOH) was tested on as many as 17 males.

Experiments involving two or three factors were all factorial designs (factors crossed with each other). Sperm suspensions from the same males were assigned to all treatments, and thus males were treated as blocks to reduce experimental error (Table 2). Data were analyzed using one-way,

Table 2
Experimental design and model statement for the 11 experiments

| Experiment | Factor (treatment level) | Male code[c] | ANOVA | Model statement[d] |
|---|---|---|---|---|
| 1 | Cooling rate (6); cryoprotectant (4) | 26, 27, 28, 29 | Two-way | $y = \mu + CR + cpa + cr \times cpa + male + \varepsilon$ |
| 2 | Cryoprotectant (8); concentration (2) cooled at 5 °C/min | 30, 31 | Two-way | $y = \mu + CPA + con + cpa \times con + male + \varepsilon$ |
| | Cryoprotectant (8); concentration (2) cooled at 30 °C/min | 59, 60 | Two-way | $y = \mu + CPA + con + cpa \times con + male + \varepsilon$ |
| 3 | Single or combined cryoprotectants (16) | 69, 70 | One-way | $y = \mu + CPA + male + \varepsilon$ |
| 4 | PEG/MeOH combination (16) | 80, 81 | One-way | $y = \mu + CPA + male + \varepsilon$ |
| | PEG/P-glycol combination (16) | 77, 78 | One-way | $y = \mu + CPA + male + \varepsilon$ |
| | PEG/DMSO combination (16) | 78, 79 | One-way | $y = \mu + CPA + male + \varepsilon$ |
| 5 | Selected cryoprotectants (16) | 87, 88 | One-way | $y = \mu + CPA + male + \varepsilon$ |
| 6 | Straw size (2); cryoprotectant (7); thawing (2) | 83, 84 | Three-way | $y = \mu + STRAW + cpa + thaw + straw \times cpa + straw \times thaw + cpa \times thaw + straw \times cpa \times thaw + male + \varepsilon$ |
| 7 | Cooling rate (4); cryoprotectant (5) | 97, 98 | Two-way | $y = \mu + CR + CPA + CR \times CPA + male + \varepsilon$ |
| 8 | Equilibration time (4); cryoprotectant (2); cooling rate (2) | 106, 111 | Three-way | $y = \mu + TIME + cpa + cr + time \times cpa + time \times cr + cpa \times cr + time \times cpa \times cr + male + \varepsilon$ |
| 9 | Selected cryoprotectant (7) with CRF[a] | 87, 88, 92 | One-way | $y = \mu + CPA + male + \varepsilon$ |
| 10 | Selected cryoprotectant (5) with CFM[b] | 95, 96 | One-way | $y = \mu + CPA + male + \varepsilon$ |
| 11 | Cooling method (2); cryoprotectant (3) | 105, 106 | Two-way | $y = \mu + CM + CPA + CM \times CPA + male + \varepsilon$ |

[a] Cooled at 5 °C/min using a controlled-rate freezer.
[b] Cooled using a commercial freezing method developed for dairy bulls.
[c] The full coding would include the designation "CG04M" preceding each number.
[d] *cr* is the cooling rate; *cpa* is the cryoprotectant; *con* is the cryoprotectant concentration; *cm* is the cooling method; $\mu$ is the the mean of the population; $\varepsilon$ is the error term. Terms in upper-case letters indicate fixed factors, lower case letters indicate random factors.

two-way (mixed model), or three-way (mixed model) analysis of variance (ANOVA) (SAS 9.0, SAS Institute, Cary, NC). When a significant difference ($\alpha = 0.05$) was observed among treatments, Tukey's honestly significant difference procedure was used for pair-wise comparisons. Results were presented as means ± SD, and probability values of $P < 0.05$ were considered to be significant. Data for sperm motility, percent fertilization and percent hatch were arcsine-square root transformed prior to analysis [53].

*Experiment 1: effect of cooling rate*

Sperm from four males (CG04M26, 27, 28, and 29) were used to evaluate six cooling rates: 0.5, 5, 16, 30, 45, and 50 °C/min. Ten percent of MeOH, E-glycol, P-glycol, and DMSO were used as individual cryoprotectants and sperm suspensions were frozen in 0.5-ml straws. Motilities were estimated after suspension and after thawing.

*Experiment 2: effect of single cryoprotectants*

Sperm from four males were used to test eight cryoprotectants each at 5 and 10%: MeOH, P-glycol, DMA, DMSO, E-glycol, glycerol, PEG200, and PEG600. Based on the results of the previous experiment, sperm suspensions were placed in 0.5-ml straws and were cooled at 5 °C/min (CG04M30 and 31) and 30 °C/min (CG04M59 and 60). Motilities were estimated after suspension and after thawing.

*Experiment 3: effect of combined cryoprotectants (first selection)*

Sperm from two males (CG04M69 and 70) were used to test 16 single or combined cryoprotectants at various concentrations (Table 3). Sperm suspensions were placed in 0.5-ml straws and cooled at 5 °C/min. Motilities were estimated after suspension and after thawing.

Table 3
Post-thaw motility (mean ± SD) of sperm samples cryopreserved in 16 single or combined cryoprotectants and cooled at 5 °C/min (males: CG04M69 and CG04M70)

| First cryoprotectant (%) | Second cryoprotectant (%) | Post-thaw motility (%) |
|---|---|---|
| PEG200 (4) | MeOH (6) | $10 \pm 12^{abc}$ |
| PEG200 (4) | P-glycol (6) | $23 \pm 13^{a}$ |
| PEG200 (4) | E-glycol (6) | $9 \pm 8^{abc}$ |
| PEG200 (4) | DMSO (6) | $16 \pm 14^{ab}$ |
| PEG600 (4) | MeOH (6) | $6 \pm 4^{abc}$ |
| PEG600 (4) | P-glycol (6) | $10 \pm 7^{abc}$ |
| PEG600 (4) | E-glycol (6) | $3 \pm 2^{bc}$ |
| PEG600 (4) | DMSO (6) | $3 \pm 4^{bc}$ |
| DMSO (4) | MeOH (4) | $2 \pm 3^{bc}$ |
| DMSO (4) | P-glycol (4) | $5 \pm 5^{bc}$ |
| DMSO (4) | E-glycol (4) | $13 \pm 12^{abc}$ |
| P-glycol (4) | E-glycol (4) | $5 \pm 7^{bc}$ |
| — | MeOH (8) | $1 \pm 2^{c}$ |
| — | P-glycol (8) | $4 \pm 7^{bc}$ |
| — | E-glycol (8) | $1 \pm 2^{c}$ |
| — | DMSO (8) | $9 \pm 11^{abc}$ |

Numbers in columns sharing the same superscript were not significantly different at $P = 0.05$.

Table 4
Post-thaw motility (mean ± SD) of sperm samples suspended in single or combined cryoprotectants and cooled at 5 °C/min.

| PEG 200 (%) | Second CPA (%) | Second cryoprotectant (CPA) | | |
|---|---|---|---|---|
| | | MeOH (CG04M80, 81)* | P-glycol (CG04M77, 78) | DMSO (CG04M78, 79) |
| 0 | 4 | $29 \pm 11^{a}$ | $20 \pm 11^{bc}$ | $5 \pm 4^{de}$ |
| | 6 | $39 \pm 6^{a}$ | $15 \pm 7^{cde}$ | $13 \pm 8^{b}$ |
| | 8 | $18 \pm 3^{b}$ | $27 \pm 7^{ab}$ | $13 \pm 8^{b}$ |
| 2 | 0 | $2 \pm 1^{f}$ | $4 \pm 2^{fg}$ | $1 \pm 0^{f}$ |
| | 4 | $39 \pm 2^{a}$ | $16 \pm 6^{bcde}$ | $12 \pm 8^{bc}$ |
| | 6 | $40 \pm 8^{a}$ | $32 \pm 10^{a}$ | $20 \pm 11^{a}$ |
| | 8 | $8 \pm 3^{cde}$ | $16 \pm 5^{bcde}$ | $13 \pm 8^{b}$ |
| 4 | 0 | $10 \pm 5^{bcd}$ | $8 \pm 2^{efg}$ | $4 \pm 3^{e}$ |
| | 4 | $37 \pm 9^{a}$ | $17 \pm 5^{bcd}$ | $13 \pm 8^{b}$ |
| | 6 | $10 \pm 0^{bcd}$ | $17 \pm 5^{bcd}$ | $7 \pm 5^{d}$ |
| | 8 | $3 \pm 2^{ef}$ | $11 \pm 2^{cdef}$ | $6 \pm 5^{de}$ |
| 6 | 0 | $13 \pm 4^{bc}$ | $19 \pm 7^{bc}$ | $8 \pm 5^{cd}$ |
| | 4 | $13 \pm 3^{bc}$ | $19 \pm 8^{bc}$ | $13 \pm 8^{b}$ |
| | 6 | $4 \pm 2^{ef}$ | $9 \pm 2^{defg}$ | $6 \pm 4^{de}$ |
| | 8 | $6 \pm 5^{def}$ | $3 \pm 2^{g}$ | $4 \pm 2^{e}$ |
| 8 | 0 | $16 \pm 6^{b}$ | $14 \pm 4^{cde}$ | $8 \pm 6^{d}$ |

Numbers in columns sharing the same superscript were not significantly different at $P = 0.05$.
* Male numbers.

*Experiment 4: effect of combined cryoprotectants (second selection)*

Based on the results of Experiment 3, PEG200 at 0, 2, 4, 6, and 8% was chosen as the non-permeating agent for use in combination with the permeating cryoprotectants MeOH, P-glycol, and DMSO each at 0, 4, 6, or 8% for a total of 48 combinations (Table 4). Based on the availability of sperm volume, sperm from five males were

Table 5
Post-thaw motility (mean ± SD) of sperm samples suspended in 16 single or combined cryoprotectants and cooled at 5 °C/min

| PEG200 (%) | Second cryoprotectant (%) | Post-thaw motility (%) |
|---|---|---|
| 0 | MeOH (4) | $15 \pm 10^{cde}$ |
| 0 | MeOH (6) | $29 \pm 14^{a}$ |
| 0 | P-glycol (4) | $2 \pm 2^{h}$ |
| 0 | P-glycol (8) | $11 \pm 7^{defg}$ |
| 0 | DMSO (6) | $4 \pm 2^{gh}$ |
| 0 | DMSO (8) | $10 \pm 4^{defg}$ |
| 2 | MeOH (4) | $27 \pm 11^{a}$ |
| 2 | MeOH (6) | $23 \pm 14^{abc}$ |
| 2 | P-glycol (4) | $8 \pm 4^{defg}$ |
| 2 | P-glycol (6) | $16 \pm 8^{bcd}$ |
| 2 | DMSO (4) | $7 \pm 3^{efgh}$ |
| 2 | DMSO (6) | $14 \pm 4^{cdef}$ |
| 4 | MeOH (4) | $26 \pm 15^{ab}$ |
| 4 | P-glycol (4) | $12 \pm 7^{def}$ |
| 4 | DMSO (4) | $16 \pm 7^{bcd}$ |
| 8 | — | $6 \pm 2^{fgh}$ |

Numbers in columns sharing the same superscript were not significantly different at $P = 0.05$.

allocated to these three groups: PEG/MeOH (CG04M80 and 81), PEG/P-glycol (CG04M77 and 78), and PEG/DMSO (CG04M78 and 79). Sperm suspensions were placed in 0.5-ml straws and cooled at 5 °C/min. Motilities were estimated after suspension and after thawing.

*Experiment 5: effect of combined cryoprotectants (final selection)*

Single or combined cryoprotectants that yielded the highest post-thaw motility at the lowest concentration combinations in Experiment 4 were chosen for use in this experiment (Table 5). Sperm from two males (CG04M87 and 88) were used and suspensions were placed in 0.25-ml straws and cooled at 5 °C/min. Motilities were estimated after suspension and after thawing.

*Experiment 6: effect of straw size (0.25-ml vs. 0.5-ml)*

Sperm from two males (CG04M83 and 84) were used to evaluate the two straw sizes. Based on previous experiments, the combined and single cryoprotectants used were 2% PEG/4% MeOH, 2% PEG/6% P-glycol, 2% PEG/6% DMSO, 6% MeOH, 8% P-glycol, 8% DMSO, and 8% PEG200, and the samples were cooled at 5 °C/min. To evaluate possible effects of thawing on the different straw sizes, samples were thawed at two temperatures in a water bath: 40 °C (6 s for 0.25-ml straws and 10 s for 0.5-ml straws) and 60 °C (5 s for 0.25-ml straws and 7 s for 0.5-ml straws). Motilities were estimated after suspension and after thawing.

*Experiment 7: effect of interactions between cooling rate and cryoprotectant*

Sperm from two males (CG04M97 and 98) were used to re-evaluate the cooling rates of 0.5, 5, 16, and 30 °C/min with selected single or combined cryoprotectants based on previous experiments, which included 2% PEG/6% DMSO, 2% PEG/4% MeOH, 2% PEG/6% P-glycol, 6% MeOH, and 8% PEG200. Sperm suspensions were placed in 0.25-ml straws and were thawed in a 40 °C water bath for 6 s. Motilities were estimated after suspension and after thawing.

*Experiment 8: effect of equilibration time*

Sperm from two males (CG04M106 and 111) were used to evaluate equilibration times of 10, 20, 30, and 60 min. Six percent of MeOH and 2% PEG/4% MeOH were used as cryoprotectants. Sperm suspensions were placed in 0.25-ml

straws and cooled at 5 and 30 °C/min, and thawed in a 40 °C water bath for 6 s. Motilities were estimated after suspension and after thawing.

*Experiment 9: evaluation of selected cryoprotectants on percent fertilization and hatch*

Sperm from three males (CG04M87, 88, and 92) were placed in 0.5-ml straws and used to test seven selected single or combined cryoprotectants at a cooling rate of 5 °C/min in the controlled-rate freezer. The selected cryoprotectants and their concentrations were 2% PEG/4% MeOH, 6% MeOH, 2% PEG/6% P-glycol, 8% P-glycol, 2% PEG/6% DMSO, 8% DMSO, and 8% PEG200. Motilities were estimated after suspension and after thawing, and percent fertilization and hatch were estimated as described above.

*Experiment 10: evaluation of selected cryoprotectants with commercial-scale freezing method*

Sperm from two males (CG04M95 and 96) were used to test commercial-scale freezing protocols developed for dairy bulls. Sperm samples were suspended in 2% PEG/4% MeOH, 6% MeOH, 2% PEG/6% P-glycol, 8% P-glycol, and 2% PEG/6% DMSO, and equilibrated for 15 min prior to freezing. Motilities were estimated after suspension and after thawing, and percent fertilization were estimated as described above.

*Experiment 11: evaluation of cooling methods*

Sperm from two males (CG04M105 and 106) were used to evaluate the two freezing methods detailed above: cooling at 5 °C/min using the controlled-rate freezer (CRF) and the commercial freezing method (CFM) developed for dairy bulls. The cryoprotectants used were 2% PEG/4% MeOH, 6% MeOH, and 5% E-glycol. Motilities were estimated after suspension and after thawing, and percent fertilization and hatch were estimated as described above.

## Results

*Initial sperm motility after shipment*

A total of 27 Pacific oysters were transported in 7 shipments from June 4 to July 7, 2004. For the first two shipments, intact oysters ($n = 8$) were shipped and sperm were collected upon arrival. For the other shipments ($n = 19$), undiluted sperm samples were shipped, and the samples were diluted in C-F HBSS immediately prior to freezing. The initial motility ranged from 5 to 95% with an average of 82 ± 22%. Except for three males, the sperm had initial motilities of >80% (Fig. 2). The lowest initial motility (5%) was found in male CG04M26, followed by CG04M98 (20%), and CG04M31 (50%). The initial motility of sperm samples collected from shipped oysters (70 ± 29%) had marginally ($P = 0.046$) lower initial motility than shipped undiluted sperm samples (87 ± 16%).

*Experiment 1: effect of cooling rate*

Post-thaw motility of sperm cooled at rates ranging from 0.5 to 50 °C/min were not significantly different from one another ($P = 0.098$),

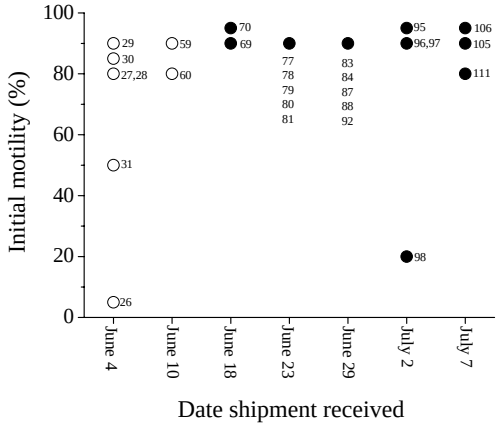


Fig. 2. The motility of sperm from 27 diploid oysters transported in 7 shipments from June 4 to July 7, 2004. Intact oysters (open circles) were transported in the first two shipments ($n = 8$), and undiluted sperm (filled circles) were transported in the other shipments ($n = 19$). The numbers identified each individual oyster at the order of their usage in experiments (the full coding would include the designation "CG04M" preceding each number).

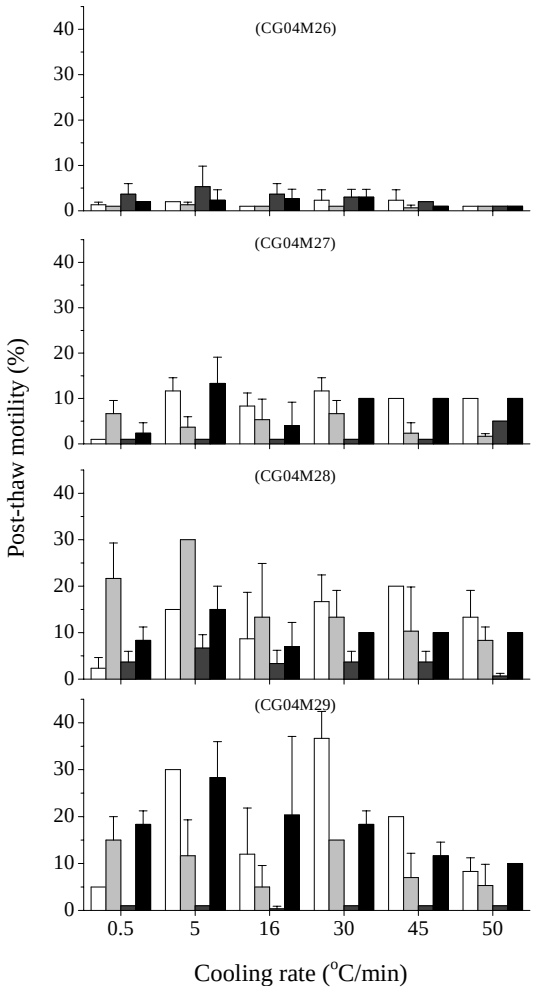


Fig. 3. Post-thaw motility (mean ± SD) of sperm samples suspended in 10% DMSO (white bars), E-glycol (light gray bars), MeOH (dark gray bars), P-glycol (black bars), and cooled at 0.5, 5, 16, 30, 45, and 50 °C/min. Four males were used (CG04M26, CG04M27, CG04M28, and CG04M29).

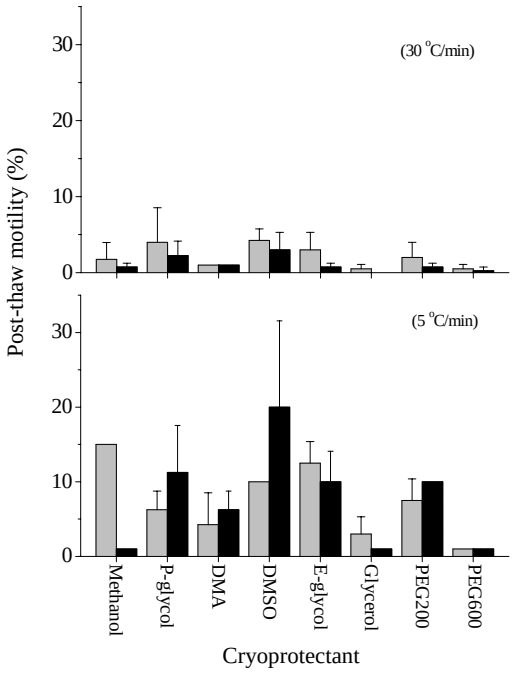


Fig. 4. Post-thaw motility (mean ± SD) of sperm samples suspended in eight cryoprotectants: MeOH, P-glycol, DMA, DMSO, E-glycol, glycerol, PEG200, and PEG600 each at 5 (light gray bars) and 10% (black bars), and cooled at 5 (CG04M30 and CG04M31) and 30 °C/min (CG04M59 and CG04M60).

although highest post-thaw motility was obtained with the rates of 5 and 30 °C/min (Fig. 3). There was a significant difference among the four cryoprotectants ($P < 0.001$) with 10% MeOH yielding the lowest post-thaw motility. A significant interaction was detected between the cryoprotectant and cooling rate ($P < 0.001$). The four males used were significantly different in post-thaw motility ($P < 0.001$) with the lowest post-thaw motility found for male CG04M26 (<10% for all treatments). After thawing, the highest motility (37 ± 6%) was found for samples suspended in 10% DMSO and cooled at 30 °C/min, followed by samples suspended in 10% DMSO (30 ± 0%), 10% E-glycol (30 ± 0%), 10% P-glycol (28 ± 8%), and cooled at 5 °C/min. Therefore, cooling rates of 5 and 30 °C/min were chosen for subsequent experiments.

*Experiment 2: effect of single cryoprotectants*

For samples cooled at 5 °C/min (Fig. 4), no significant differences were observed among the eight cryoprotectants ($P = 0.160$) or between the concentrations of 5 and 10% ($P = 0.837$). However, the interaction between cryoprotectant and concentration was significant ($P < 0.001$). Differences among males were significant ($P = 0.002$) with CG04M30 yielding the highest overall post-thaw motility. The highest post-thaw motility (20 ± 12%) was found in

samples suspended with 10% DMSO, followed by samples suspended in 5% methanol (15 ± 0%), 5% E-glycol (13 ± 3%), and 10% P-glycol (11 ± 6%).

For samples cooled at 30 °C/min (Fig. 4), significant differences were observed among the eight cryoprotectants ($P < 0.001$), and post-thaw motility of samples suspended in DMSO and P-glycol was found to be significantly higher than those suspended with glycerol and PEG600. There was also a significant difference between the concentrations of 5 and 10% ($P = 0.004$), but the interaction between cryoprotectant and concentration was not significant ($P = 0.930$). Both males yielded low post-thaw motility (<10%) and there was no significant difference between them ($P = 0.432$). Glycerol, DMA, and PEG600 yielded the lowest post-thaw motility among the eight cryoprotectants tested in both trials. However, better sperm morphology (most sperm with tails attached) was observed for samples cryopreserved with PEG200 and PEG600. Thus, glycerol and DMA were excluded from subsequent experiments.

*Experiment 3: effect of combined cryoprotectants (first selection)*

Significant differences ($P < 0.001$) were observed among the 16 single and combined cryoprotectants, with the combined cryoprotectants of PEG/P-glycol (23 ± 13%), PEG/DMSO (16 ± 14%), DMSO/E-glycol (13 ± 12%), and PEG/MeOH (10 ± 12%) yielding the highest post-thaw motility (Table 3). The two males used in this experiment did not show significant variation ($P = 0.066$). Combinations of non-permeating agent (PEG200) and the permeating agents MeOH, P-glycol, and DMSO were chosen for further optimization in the subsequent experiments.

*Experiment 4: effect of combined cryoprotectants (second selection)*

Significant differences ($P < 0.001$) were found for various concentration combinations within each combined cryoprotectant group (Table 4). Of the single cryoprotectants and cryoprotectant combinations of PEG200 and MeOH, the highest post-thaw motility was found in samples suspended in 2% PEG/6% MeOH (40 ± 8%) and 6% MeOH (39 ± 6%), followed by samples suspended in 2% PEG/4% MeOH (39 ± 2%), 4% PEG/4% MeOH (37 ± 9%), and 4% MeOH (29 ± 11%), which were all significantly higher than the other combinations ($P < 0.050$). The two males used did not show a significant difference in post-thaw motility ($P = 0.132$). Of the single cryoprotectants and cryoprotectant combinations of PEG200 and P-glycol, the highest post-thaw motility was found in samples suspended in 2% PEG/6% P-glycol (32 ± 10%), followed by 8% P-glycol (27 ± 7%) and 4% P-glycol (20 ± 11%). The two males used were not significantly different ($P = 0.325$). Of the single cryoprotectants and cryoprotectant combinations of PEG200 and DMSO, the highest post-thaw motility (20 ± 11%) was found in samples suspended in 2% PEG/6% DMSO, followed by 13 ± 8%, a value shared by 4% PEG/4% DMSO, 6% DMSO, 8% DMSO, and 6% PEG/4% DMSO. Differences between the two males were significant ($P < 0.001$). Five concentration combinations from each of the three combined cryoprotectants yielded high post-thaw motility at low total concentrations and were chosen to further compare their effectiveness with the same males in the subsequent experiment. PEG200 at 8% was also included as a single cryoprotectant comparison.

*Experiment 5: effect of combined cryoprotectants (final selection)*

Significant differences ($P < 0.001$) were observed among the re-grouped 16 single or combined cryoprotectants with the highest post-thaw motility observed in samples cryopreserved with 6% MeOH (29 ± 14%), followed by 2% PEG/4% MeOH (27 ± 11%), 4% PEG/4% MeOH (26 ± 15%), and 2% PEG/6% MeOH (23 ± 14%) (Table 5). The two males used in this experiment were significantly different in post-thaw motility ($P = 0.0001$). Based on the combined results of Experiments 3 through 5, 2% PEG/4% MeOH, 6% MeOH, 2% PEG/6% P-glycol, 8% P-glycol, 2% PEG/6% DMSO, 8% DMSO, and 8% PEG200 were selected for subsequent experiments.

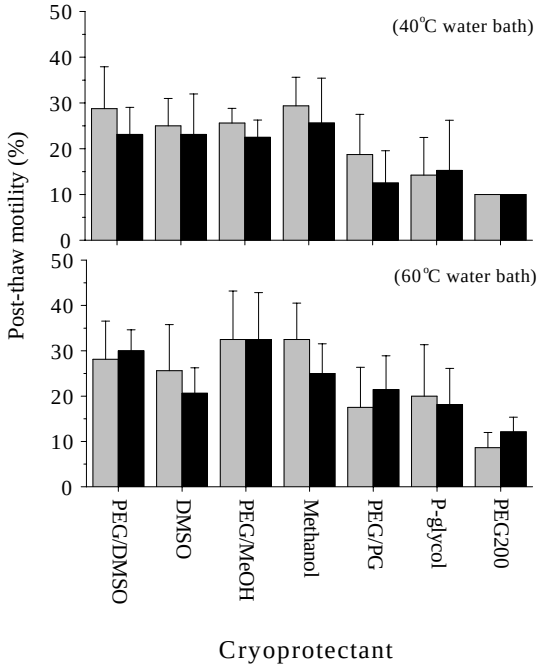


Fig. 5. Post-thaw motility (mean ± SD) of sperm samples suspended in 2% PEG/6% DMSO, 8% DMSO, 2% PEG/4% MeOH, 6% MeOH, 2% PEG/6% P-glycol, 8% P-glycol, and 8% PEG200, and equilibrated for 12 min in 0.25-ml (light gray bars) or 0.5-ml straws (black bars). Samples were cooled at 5 °C/min and thawed in a water bath at 40 °C (6 s for 0.25-ml straws, and 10 s for 0.5-ml straws), and 60 °C (5 s for 0.25-ml straws, and 7 s for 0.5-ml straws). Two males were used (CG04M83 and CG04M84).

*Experiment 6: effect of straw size (0.25-ml vs. 0.5-ml)*

To evaluate possible effects from straw size differences, 0.25-ml and 0.5-ml straws were compared in this experiment (Fig. 5). A significant difference in post-thaw motility was not observed for either straw size ($P = 0.465$) or thawing method (40 vs. 60 °C, $P = 0.208$).

All interactions among straw size, thawing method and cryoprotectant were found to be non-significant ($P > 0.125$). Significant differences were observed among the seven cryoprotectants ($P = 0.034$) and two males ($P < 0.001$) used in this experiment (Fig. 5). Similar to the previous experiment, samples suspended in 2% PEG/4% MeOH and 6% MeOH yielded the highest post-thaw motility, and those suspended in 8% PEG200 yielded the lowest post-thaw motility.

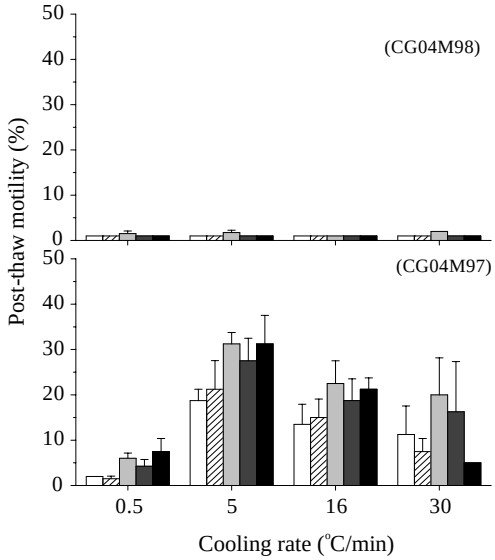


Fig. 6. Post-thaw motility (mean ± SD) of sperm samples suspended in 6% MeOH (white bars), 8% PEG200 (hatched bars), 2% PEG/6% DMSO (light gray bars), 2% PEG/4% MeOH (dark gray bars), 2% PEG/6% P-glycol (black bars), and equilibrated for 12 min in 0.25-ml straws. Samples were cooled at 0.5, 5, 16, and 30 °C/min, and thawed in a 40 °C water bath for 6 s. Two males were used (CG04M97 and CG04M98).

*Experiment 7: effect of interactions between cooling rate and cryoprotectant*

Samples cooled at 5 °C/min yielded the highest post-thaw motility, which was significantly different from that observed for cooling at 0.5, 16, and 30 °C/min ($P < 0.044$) (Fig. 6). Samples cooled at 16 and 30 °C/min were not significantly different from one another ($P = 0.082$), but these cooling rates yielded higher post-thaw motility than did samples cooled at 0.5 °C/min ($P < 0.001$). The interaction of cooling rate and cryoprotectant was not significant ($P = 0.645$). However, significant differences were observed for the cryoprotectant ($P = 0.002$) and the two males ($P < 0.001$) used in this experiment. The highest post-thaw motilities were found with samples from male CG04M97 suspended in 2% PEG/6% P-glycol (31 ± 6%), 2% PEG/6% DMSO (31 ± 3%), and 2% PEG/4% MeOH (28 ± 5%) and

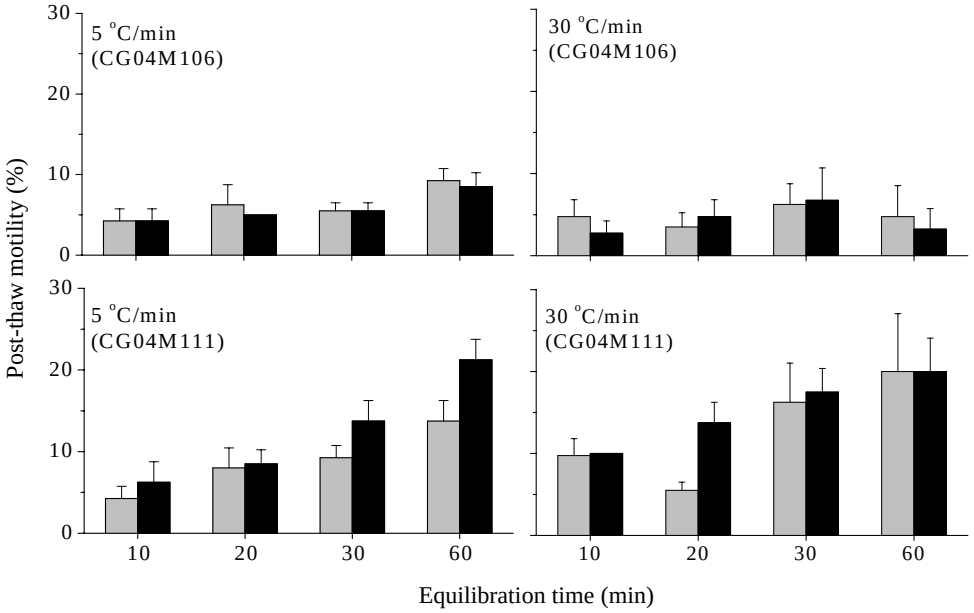


Fig. 7. Post-thaw motility (mean ± SD) of sperm samples suspended in 6% MeOH (light gray bars) and 2% PEG/4% MeOH (black bars), equilibrated for 10, 20, 30, and 60 min, and cooled at 5 and 30 °C/min. Two males were used (CG04M106 and CG04M111).

cooled at 5 °C/min. Consequently, a cooling rate of 5 °C/min was chosen for subsequent experiments.

*Experiment 8: effect of equilibration time*

Samples with equilibration times of 30 and 60 min yielded higher post-thaw motility than did those of 10 and 20 min, although they were not significantly different from one another ($P = 0.468$) (Fig. 7). All interactions among equilibration time, cooling rate, and cryoprotectant were found to be non-significant ($P > 0.051$). Cooling rates of 5 and 30 °C/min were not significantly different in this experiment ($P = 0.790$), but samples suspended in 2% PEG/4% MeOH yielded higher post-thaw motility than did those in 6% MeOH alone ($P < 0.001$). Post-thaw motility was significant for the two oysters used in this experiment ($P < 0.001$).

*Experiment 9: evaluation of selected cryoprotectants on percent fertilization and hatch*

For a cooling rate of 5 °C/min with the controlled-rate freezer (Table 6), the highest post-thaw motilities were obtained with samples suspended in 6% MeOH (35 ± 19%) and 2% PEG/4% MeOH (33 ± 15%), which were significantly higher than those of the other cryoprotectants although a significant difference in post-thaw motility ($P < 0.001$) was observed among the three males used. The highest percent fertilizations were also found in samples suspended in 6% MeOH (60 ± 35%) and 2% PEG/4% MeOH (50 ± 39%), which were not significantly different from the other cryoprotectants except for 6% PEG200 (9 ± 10%). Percent fertilization as high as 98% was observed for sperm samples cryopreserved with 6% MeOH for male CG04M87. Percent hatch was high (>50%) for treatments with fertilization above 80% (Table 6).

*Experiment 10: evaluation of selected cryoprotectants with commercial-scale freezing method*

The selected cryoprotectants and their combinations were also tested with the commercial freezing methods developed for dairy bulls (Table 7). Although the post-thaw motility was generally low (<5%), fertilization of greater than 50% was obtained with samples suspended in 2% PEG/4%

Table 6
Post-thaw motility (mean ± SD), percent fertilization, and hatch of sperm samples suspended in 2% PEG/4% MeOH, 6% MeOH, 2% PEG/6% P-glycol, 8% P-glycol, 2% PEG/6% DMSO, 8% DMSO, or 8% PEG200, equilibrated for 15 min, and cooled at 5 °C/min with a controlled-rate freezer

| Male | Criterion | PEG/MeOH | MeOH | PEG/P-glycol | P-glycol | PEG/DMSO | DMSO | PEG200 |
|---|---|---|---|---|---|---|---|---|
| Control* | Fertilization (%) | 99 | 98 | 98 | 97 | 92 | 90 | 100 |
| CG04M87 | Motility (%) | 45 ± 10 | 60 ± 8 | 33 ± 5 | 24 ± 5 | 28 ± 5 | 9 ± 2 | 13 ± 6 |
| | Fertilization (%) | 95 | 98 | 82 | 92 | 82 | 90 | 20 |
| | Hatch (%) | 52 | 59 | 61 | 67 | 55 | 58 | 13 |
| CG04M88 | Motility (%) | 39 ± 6 | 28 ± 5 | 16 ± 6 | 13 ± 5 | 15 ± 4 | 9 ± 2 | 9 ± 2 |
| | Fertilization (%) | 25 | 52 | 36 | 25 | 26 | 26 | 3 |
| CG04M92 | Motility (%) | 15 ± 4 | 19 ± 3 | 13 ± 3 | 10 ± 4 | 11 ± 3 | 7 ± 2 | 6 ± 1 |
| | Fertilization (%) | 30 | 29 | 9 | 16 | 8 | 8 | 3 |
| Average | Motility (%) | 33 ± 15[a] | 35 ± 19[a] | 20 ± 10[b] | 15 ± 8[bc] | 18 ± 8[b] | 8 ± 2[c] | 9 ± 4[c] |
| | Fertilization (%) | 50 ± 39[a] | 60 ± 35[a] | 42 ± 37[a] | 44 ± 42[a] | 39 ± 39[a] | 41 ± 43[a] | 9 ± 10[b] |

Numbers in rows sharing the same superscript were not significantly different at $P = 0.05$.
* Fresh sperm exposed to the same treatments as the thawed sperm (same cryoprotectants, concentration, equilibration time and batch of eggs).

Table 7
Post-thaw motility (mean ± SD) and percent fertilization of sperm samples suspended in 2% PEG/4% MeOH, 6% MeOH, 2% PEG/6% P-glycol, 8% P-glycol, and 2% PEG/6% DMSO, equilibrated for 15 min, and cooled using a commercial freezing method developed for dairy bulls

| Male | Criterion (%) | PEG/MeOH | MeOH | PEG/P-glycol | P-glycol | PEG/DMSO |
|---|---|---|---|---|---|---|
| CG04M95 | Motility | 1 ± 0 | 1 ± 0 | 1 ± 0 | 1 ± 0 | 3 ± 2 |
| | Fertilization | 4 | 1 | 2 | 2 | 4 |
| CG04M96 | Motility | 3 ± 2 | 4 ± 2 | 2 ± 0 | 1 ± 0 | 4 ± 2 |
| | Fertilization | 53 | 52 | 23 | 16 | 19 |
| Average | Motility | 2 ± 1[b] | 2 ± 2[ab] | 1 ± 1[b] | 1 ± 0[b] | 4 ± 2[a] |
| | Fertilization | 29 ± 35[a] | 27 ± 36[a] | 13 ± 15[a] | 9 ± 10[a] | 12 ± 11[a] |

Numbers in rows sharing the same superscript were not significantly different at $P = 0.05$.

MeOH for male CG04M96. The treatment effects of different cryoprotectants were found to be significant for post-thaw motility ($P < 0.001$) despite their low values, but were found to be non-significant for percent fertilization ($P = 0.540$), which may be due to the significant difference ($P < 0.001$) observed for the two males used in this experiment.

Positive controls using fresh sperm without addition of cryoprotectant yielded 99% fertilization, and controls using fresh sperm equilibrated with the same cryoprotectants for the same equilibration time all yielded >90% fertilization (Table 6), indicating cryoprotectants at these concentrations were only mildly toxic or non-toxic to sperm and eggs. Fresh eggs without sperm addition yielded 0% fertilization, indicating that eggs were not contaminated with extraneous sperm.

*Experiment 11: evaluation of cooling methods*

To directly compare the controlled-rate freezer (CRF) and commercial freezing method (CFM), these two methods were evaluated with same males (Table 8). Samples frozen with the CRF were found to be significantly higher in post-thaw motility ($P < 0.001$) and marginally different in percent fertilization ($P = 0.050$) than those frozen with CFM. No differences were observed within cryoprotectants for post-thaw motility ($P = 0.393$) or for percent fertilization ($P = 0.702$). Interactions between cooling methods and cryoprotectants were found to be significant for post-thaw motility ($P = 0.003$), but not for percent fertilization ($P = 0.550$). The two males used in this experiment showed significant differences in post-thaw

Table 8
Post-thaw motility (mean ± SD), percent fertilization and hatch of sperm samples suspended in 2% PEG/4% MeOH, 6% MeOH, and 5% E-glycol, and cooled at 5 °C/min using a controlled-rate freezer (CRF) or a commercial freezing method (CFM) developed for dairy bulls

| Male | Criterion (%) | PEG/MeOH | | MeOH | | E-glycol | | Average (CPA) | |
|---|---|---|---|---|---|---|---|---|---|
| | | CRF | CFM | CRF | CFM | CRF | CFM | CRF | CFM |
| Control | Fertilization | 95 | | 94 | | 96 | | 95 ± 1 | |
| CG04M105 | Motility | 24 ± 5 | 20 ± 0 | 24 ± 8 | 14 ± 3 | 24 ± 3 | 20 ± 0 | 24 ± 5 | 18 ± 4 |
| | Fertilization | 95 | 90 | 96 | 91 | 93 | 87 | 95 ± 2 | 89 ± 2 |
| | Hatch | — | — | — | — | 55 | 43 | — | — |
| CG04M106 | Motility | 6 ± 3 | 2 ± 2 | 8 ± 3 | 1 ± 0 | 4 ± 2 | 4 ± 2 | 6 ± 3 | 2 ± 2 |
| | Fertilization | 84 | 61 | 93 | 12 | 73 | 31 | 83 ± 10 | 35 ± 25 |
| | Hatch | — | — | — | — | 54 | — | — | — |
| Average (males) | Motility | 15 ± 10 | 8 ± 9 | 16 ± 10 | 8 ± 7 | 14 ± 11 | 12 ± 9 | 15 ± 10[a] | 10 ± 8[b] |
| | Fertilization | 90 ± 8 | 76 ± 21 | 95 ± 2 | 52 ± 56 | 83 ± 14 | 59 ± 40 | 89 ± 9[a] | 62 ± 34[b] |

Numbers in rows sharing the same superscript were not significantly different at $P = 0.05$. —, values not collected.

motility ($P < 0.001$) and percent fertilization ($P = 0.025$). Positive controls using fresh sperm without addition of cryoprotectant yielded 92% fertilization, and controls using fresh sperm equilibrated with the same cryoprotectants for the same equilibration time all yielded >94% fertilization (Table 8), indicating cryoprotectants at these concentrations were not toxic for sperm and eggs. Again, fresh eggs without any sperm addition yielded 0% fertilization.

## Discussion

A wide range of cooling rates has been reported for oyster sperm, from 1 °C/min [26] to immediate plunging in liquid nitrogen [27]. In the present study, direct comparison of 0.5, 5, 16, 30, 45, 50 °C/min did not show significant differences although the highest post-thaw motility was obtained with 5 and 30 °C/min. However, subsequent experiments with optimized cryoprotectants and concentrations showed that motility of samples cooled at 5 °C per min was significantly higher than those cooled at 0.5, 16, and 30 °C/min. Previous studies with oyster sperm have reported that cooling rates of ~5 °C/min [28,58] and 50 °C/min [52] have yielded fertilization success. Optimized cooling rates could depend on the type of cryoprotectant and concentrations, as a significant interaction was observed between cryoprotectant and cooling rate in the present study. Optimized cooling rates can also depend on other factors involved in the cryopreservation process such as the type of extender [3] or the type of cooling methods. In this study with the controlled-rate freezer and the cryoprotectants studied, more consistent results were observed with samples cooled at 5 °C/min. In contrast to the controlled-rate freezer, results from the present study showed lower post-thaw motility and fertilization resulted from the commercial protocols. Despite that, high fertilization (>90%) was obtained with the commercial protocols (e.g., for male CG04M105). Unlike cooling rate, cooling method rarely receives much attention in controlled studies. It would be informative to evaluate the differences among various cooling methods with the same nominal cooling rates.

Choosing an appropriate cryoprotectant has been a primary focus in gamete cryopreservation for mammals and aquatic species [38,55]. Non-permeating cryoprotectants such as sugars, proteins, and polymers have been found to confer protection by permitting a reversible influx and efflux of solutes during freezing and thawing, and thus enabling the cells to avoid the otherwise irreversible effects of excessive osmotic gradients [39]. A classic formula of combining sugars (e.g., glucose) or proteins (e.g., egg yolk) with permeating cryoprotectants such as DMSO (usually at a concentration of

5–10%) has been applied in fish species and with variable success (e.g., [16,43,51]). Contrary to that, few studies have been conducted to evaluate the combined effects of polymers (e.g., polyethylene glycol or polyvinyl pyrrolidone) with permeating compounds. The present study tested combinations of polyethylene glycol with MeOH, P-glycol, and DMSO and found that a low concentration (2%) of polyethylene glycol (FW 200) was effective in retaining post-thaw motility and fertilizing capability for sperm of diploid Pacific oysters when combined with permeating compounds. However, polyethylene glycol alone was not as effective as permeating compounds such as MeOH, DMSO, and P-glycol when using the same methods.

Previous studies with oyster sperm have excluded methanol from the list of suitable cryoprotectants (e.g. [52]). However, consistently higher post-thaw motility was obtained with MeOH or the PEG/MeOH combination in the present study, and the highest post-thaw motility (70%) and percent fertilization (98%) were obtained for samples cryopreserved with 6% MeOH. Other than DMSO, methanol is one of the most widely used cryoprotectants for sperm from aquatic species [55] with successful semen cryopreservation reported in a variety of species such as zebrafish *Danio rerio* [23], tilapia *Oreochromis* spp. [45], channel catfish *Ictalurus punctatus* [56], Siberian sturgeon *Acipenser baeri* [21], rainbow trout *Oncorhynchus mykiss* [35], and common carp *Cyprinus carpio* [40]. Controversies concerning the effectiveness of cryoprotectants for particular species frequently occur. For example, 20% glycerol was found to be better than 10% DMSO for semen cryopreservation of Arctic charr *Salvelinus alpinus* [43], but the opposite was reported for the same species in another study [46].

Despite the fact that MeOH and the PEG/MeOH combination yielded the highest percent fertilization in the present study, it is difficult to conclude that MeOH is a better overall cryoprotectant than the others tested for oyster sperm. Similar to the optimization of cooling rate, optimization of cryoprotectant and its concentration is interlinked with the other factors involved in the overall process of cryopreservation. Given optimized conditions for all other aspects (e.g., extender composition, cooling rate, and thawing method), several cryoprotectants could provide adequate protection for a given species. For example, DMSO and MeOH were each found to be equally efficient in sperm cryopreservation of Northern pike, *Esox lucius* [4,37] and paddlefish, *Polydon spathula* [5]. In the case of Pacific oyster sperm, high rates of fertilization (>90%) and development to D-stage larvae (>50%) have also been reported for samples cryopreserved with DMSO, P-glycol, and E-glycol [19,28]. Compared with the wide concentration range reported for DMSO, methanol may have a narrower effective range in terms of cryoprotection and toxic effects [34]. In the present study, methanol at 10% was found to be less effective in retaining post-thaw motility than was 5%. All told, protocols that provide equal protection for a specific cell population may vary with the type of cryoprotectant. Therefore, the effectiveness of different cryoprotectants for a specific species can be only evaluated through comparisons across different protocols.

Various sizes of straws or cryovials have been used for different purposes in previous studies of aquatic species. In general, large volume containers were studied for the purpose of scaling up production for hatchery application (e.g. [8,41,46,47]), and smaller volume containers such as 0.25-ml straws were used for species with limited sperm volume (e.g. [24,25]). Higher post-thaw motility or fertility was obtained with smaller volume straws in sperm cryopreservation of rainbow trout (among 0.5, 1.8, and 5-ml straws) [8], yellowtail flounder *Pleuronectes ferrugineus* (0.25-ml vs. 1.7-ml straws) [47], and channel catfish (0.25-ml vs. 0.5-ml straws) [13]. In the present study, straw sizes of 0.25- and 0.5-ml did not show significant differences in retaining post-thaw motility although higher post-thaw motility was obtained with 0.25-ml straws. In the eastern oyster *C. virginica*, 5-ml straws were found to be especially effective in preserving larvae [41]. The discrepancy of the effects of straw size may due to interactions of cooling rates, cryoprotectants, and thawing methods as indicated above. The better results associated with smaller straw volumes in general may result from their higher surface area-to-volume ratio, and a consequent enhanced uniformity of

heat transfer during freezing and thawing processes [42].

Optimal equilibration time before freezing is necessary to allow permeating cryoprotectants to penetrate the sperm while minimizing toxicity. In general, equilibration times of 10–20 min or shorter are considered to be better for oyster and fish semen cryopreservation (e.g. [7,19,29]). However, semen of sea bass, *Decentrarchus labrax*, equilibrated for 6 h with 10% ethylene glycol at 0–2 °C yielded motility when thawed comparable to that of fresh semen [50], and sperm of channel catfish, equilibrated for 10 days with 5% methanol at 4 °C showed increased motility compared to that of fresh sperm [12]. The present study showed higher post-thaw motility with longer equilibration time (30–60 min). Although long equilibration (60 min) was suspected to have a detrimental effect on larval development of Pacific oysters [29], it could be beneficial when the cryoprotectant concentration is low and the solution is added in a step-wise fashion at low temperatures. Post-thaw motility of 70% with sperm from diploid Pacific oysters was obtained when equilibration time was extended to 1 h with step-wise addition at 5 °C and cooling using commercial dairy protocols (Dong, unpublished data). Indeed, a wide range of equilibration times (30 min to 6 h) with stepwise addition of 7–11% glycerol at 5 °C are practiced in routine commercial-scale cryopreservation of dairy bull sperm [10].

Differences in post-thaw sperm quality (e.g., motility or percent fertilization) among individual males were evident in this study. Some males were found to have high percent fertilization (e.g., CG04M87 in Table 6), regardless of the choice of cryoprotectants, while others (e.g., CG04M95 in Table 7) were found to have poor fertilization (<5%), which suggested a difference in the tolerance to cryopreservation processes of sperm from individual males. The term "tolerance" used here is defined as the relative capacity of spermatozoa to fertilize eggs when subjected to unfavorable environmental factors. For example, the fertility of fresh sperm from different males could be same (e.g., >90% for all controls in this study), but varied when thawed sperm from those same males were compared, which was confirmed with findings in the eastern oyster where the fertility of fresh and thawed sperm from five males were compared [41]. To illustrate this phenomenon, a generalized classification is introduced here: males can be identified as individuals with broad, intermediate, or narrow tolerances to cryopreservation. Individuals with broad tolerance can accommodate various protocols available in practice and yield good results. On the contrary, individuals with a narrow tolerance can accommodate a more narrow range across protocols, and thus considerable research efforts are required before success could be observed. Those individuals with intermediate tolerance, however, can accommodate a moderate range of practical protocols, and good results will be obtained with some protocols but not others. This same concept could be equally applied to strain or species differences in tolerances of the cryopreservation process. For protocol optimization, the problems associated with individuals either of broad (in a sense of superior males) or narrow tolerance is the difficulty to differentiate treatment effects because the post-cryopreservation sperm quality for treatments would be all good (superior males) or all poor (narrow tolerance).

Factors that affect sperm tolerance can be biotic (e.g., genetic, physiological), abiotic (e.g., environmental factors), or arise from interactions among these. Variation in tolerance among males could be due to difference in their genetic tolerance to cryopreservation, genetic tolerance to stress (e.g., shipping), or genetic tolerance to the combinations of cryopreservation and stress. Findings in a recent study on boar sperm cryopreservation supported the hypothesis that there is a genetic basis for variation in post-thaw sperm quality among individuals [54]. In that study, analogous to the classification proposed in this study, boars were grouped into poor, average, and good based on post-thaw recovery assessed from a variety of sperm quality evaluation techniques such as motility, membrane integrity, acrosome integrity, and active motility (CASA: computer-assisted sperm analysis). Subsequent genomic analysis using amplified restriction fragment length polymorphism (AFLP) technology revealed sixteen candidate molecular markers linked to genes controlling semen response to cryopreservation [54].

Successful sperm cryopreservation involves many steps, not only those steps involved in the freezing and thawing processes, but can also include sample shipping [19], broodstock rearing and handling, egg quality, and fertilization methods [49]. Abiotic factors involved in activities prior to cryopreservation could possibly alter the tolerances of sperm from individual males, such as nutrient availability [32], rearing temperature [31], confinement [30], transportation [2], and invasive gonad sampling for sex or ploidy identification [19]. In the present study, sperm samples or intact oysters were shipped from the hatchery to the cryopreservation laboratory. The low post-thaw motility (<5%) reported in some treatments of this study could be due to the effects of shipping stress on sperm samples. Future research is necessary to differentiate these factors and their interactions.

Differences in sperm tolerance could have a profound impact on cryopreservation outcome (Fig. 8). For example, for a male or a species with a narrow tolerance, either due to biotic (genetic) or abiotic (stress) factors, sperm cryopreservation could be less effective when non-optimized or sub-optimized procedures are used. It is reasonable to conclude that male, strain, or species variation can be important factors responsible for the inconsistency observed in sperm cryopreservation of oysters and aquatic species in general. Besides protocol standardization, optimization of cryopreservation protocols and recognition of male (strain or species) variation in experiments would facilitate the process of reducing inconsistency and controversy. Although this study classified sperm tolerance in theory into three categories (broad, intermediate, and narrow), male oysters in reality displayed tolerance to cryopreservation procedures in a continuous fashion. However, this categorical classification of sperm tolerance may help to establish standardized cryopreservation technologies. For example, cryopreservation protocols could be developed and optimized separately for

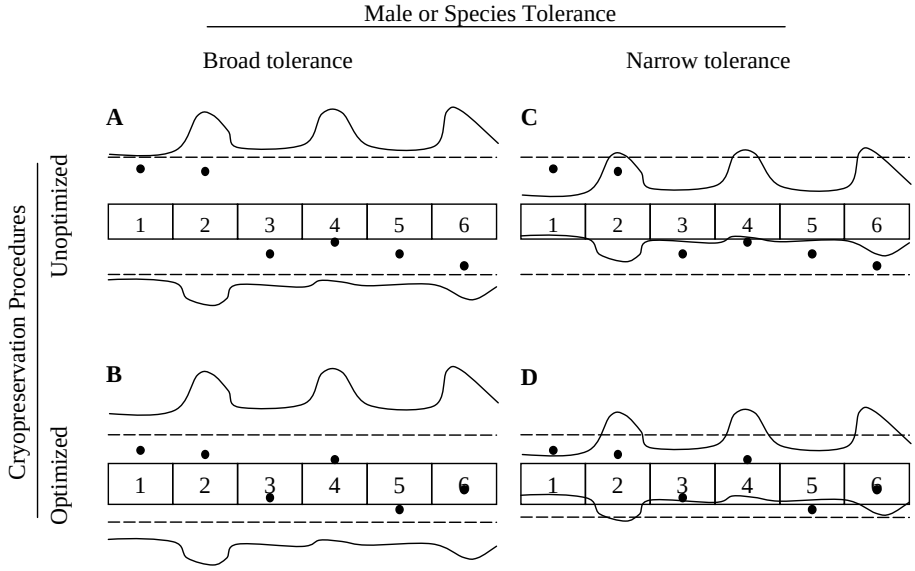


Fig. 8. Diagrammatic representation of interactions between male or species tolerance and cryopreservation procedures. The rectangles identified with the numbers 1–6 represent six presumptive critical factors involved in cryopreservation at a central facility (e.g., shipping, cryoprotectant toxicity, and cooling rate). Dotted lines indicate the range within which the practical protocols are included. Solid lines indicate the range that the individual males can tolerate, and black dots represent a protocol used in practice. Males with broad tolerance (e.g., genetically more tolerant to cryopreservation or stress, or males that were not subjected to stress) will survive cryopreservation well because all protocols are within the solid lines (A and B). Males with a narrow tolerance (e.g., genetically less tolerant to cryopreservation or stress, or males that have been stressed by unfavorable conditions) can accommodate a narrower range and some protocols fall outside the solid lines (C). However, the number of protocols falling outside the solid lines can be reduced when optimized cryopreservation procedures are used (D).

males with broad, intermediate, and narrow tolerances. As a result, assays that can differentiate sperm tolerance among different males prior to freezing are necessary in future studies including quantitative assessment tools.

Previous studies of sperm cryopreservation from oysters have rarely involved shipping [17]. The present study demonstrated that oyster sperm could be collected and shipped chilled to another facility for cryopreservation, and that it can also be shipped back to the hatchery for fertilization performed at a production scale yielding live larvae with >90% fertilization. Given the existence of facilities for commercial-scale cryopreservation of dairy bull sperm, the methods developed in the present study for oysters provide a template for the potential commercialization of cryopreserved sperm in aquatic species.

## Acknowledgments

We thank J. Chenevert, S. Pelitz, C. Jeansonne, and B. Schexnayder of Genex Custom Collection, for assistance with cryopreservation, K. Little and L. Nelson of Taylor Resources Quilcene Shellfish Hatchery for the assistance with fertilization trials, J. Geaghan at the LSU Department of Experimental Statistics for statistical consulting, and S. Allen, Jr., J. Jenkins, S. Leibo, R. Romaire, F. Sheldon, and J. Supan for critical review. The manuscript was approved for publication by the Director of the Louisiana Agricultural Experiment Station as number 05-11-0299.

## References

[1] S.K. Allen Jr., Flow cytometry: assaying experimental polyploidy fish and shellfish, Aquaculture 33 (1983) 317–328.

[2] M.L. Allyn, R.J. Sheehan, C.C. Kohler, The effects of capture and transportation stress on white bass semen osmolarity and their alleviation via sodium chloride, Tran. Am. Fish. Soc. 130 (2001) 706–711.

[3] I. Babiak, L. Fraser, S. Dobosz, K. Goryczko, H. Kuzminski, J. Strzezek, Computer-controlled freezing of rainbow trout *Oncorhnchus mykiss* (Walbaum) spermatozoa for routine programmes, Aquacult. Res. 30 (1999) 707–710.

[4] I. Babiak, J. Glogowski, M.J. Luczynski, D. Kucharczyk, M. Luczynski, Cryopreservation of the milt of the northern pike, J. Fish Bio. 46 (1995) 819–828.

[5] W.B. Bean, L. Tsvetkova, B. Gomelsky, S. Mims, Hatching rates of paddlefish *Polyodon spathula* milt using dimethyl sulfoxide and methanol as cryoprotectants, Book of Abstracts: Annual Meeting of the World Aquaculture Society. January 2005. New Orleans, Louisiana.

[6] R. Billard, T. Zhang, Techniques of genetic resource banking in fish, in: P.F. Walson, W.V. Holt (Eds.), Cryobanking the Genetic Resource: Wildlife Conservation for the Future, Taylor & Francis, New York, 2001, pp. 143–170.

[7] J.H.S. Blaxter, Sperm storage and cross-fertilization of spring and autumn spawning herring, Nature 172 (1953) 1189–1190.

[8] E. Cabrita, V. Robles, R. Alvarez, M.P. Herraez, Cryopreservation of rainbow trout sperm in large volume straws: application to large scale fertilization, Aquaculture 201 (2001) 301–314.

[9] G.M. Centola, The art of donor gamete cryobanking: current considerations, J. Andrology 23 (2002) 174–179.

[10] J.E. Chandler, Cryopreservation of sperm of dairy bulls, in: T.R. Tiersch, P.M. Mazik (Eds.), Cryopreservation in Aquatic Species, World Aquaculture Society, Baton Rouge, LA, 2000, pp. 84–90.

[11] J.E. Chandler, C.F. Ruiz, R.W. Adkinson, K.L. Koonce, Relationship between final temperature, thaw rate, and quality of bovine semen, J. Dairy Sci. 67 (1984) 1806–1812.

[12] J.M. Christensen, T.R. Tiersch, Refrigerated storage of channel catfish sperm, J. World Aquacult. Soc. 3 (1996) 340–346.

[13] J.M. Christensen, T.R. Tiersch, Cryopreservation of channel catfish spermatozoa: effect of cryopreservation, straw size, and formulation of extender, Theriogenology 47 (1997) 639–645.

[14] J.K. Crister, Current status of semen banking in the US, Human Reprod. 13 (Suppl. 2) (1998) 55–66.

[15] M.R. Curry, Cryopreservation of semen from domestic livestock, Rev. Reprod. 5 (2000) 46–52.

[16] J.D. DeGraaf, D.L. Berlinsky, Cryogenic and refrigerated storage of Atlantic cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) spermatozoa, Aquaculture 234 (2004) 527–540.

[17] Q. Dong, Comparative studies of sperm cryopreservation of diploid and tetraploid Pacific oysters. Baton Rouge, LA: Louisiana State University, Dissertation, 2005, pp. 11–33.

[18] Q. Dong, B. Eudeline, S.K. Allen Jr., T.R. Tiersch, Factors affecting sperm motility of tetraploid Pacific oysters, J. Shellfish Res. 21 (2002) 719–723.

[19] Q. Dong, B. Eudeline, C. Huang, S.K. Allen Jr., T.R. Tiersch, Commercial-scale sperm cryopreservation of diploid and tetraploid Pacific oysters, *Crassostrea gigas*, Cryobiology 50 (2005) 1–16.

[20] Q. Dong, B. Eudeline, C. Huang, T.R. Tiersch, Standardization of photometric measurement of sperm concentration from diploid and tetraploid Pacific oysters, *Crassostrea gigas* (Thunberg), Aquacult. Res. 36 (2005) 86–93.

[21] J. Glogowski, R. Kolman, M. Szczepkowski, A. Horvath, B. Urbanyi, P. Sieczynski, A. Rzemieniecki, J. Domagala, W. Demianowicz, R. Kowalski, A. Ciereszko, Fertilization rate of Siberian sturgeon (*Acipenser baeri*, Brandt) milt cryopreserved with methanol, Aquaculture 211 (2002) 367–373.
[22] J.C. Gwo, Cryopreservation of aquatic invertebrate semen: a review, Aquacult. Res. 31 (2000) 259–271.
[23] B. Harvey, R.N. Kelley, M.J. Ashwood-Smith, Cryopreservation of zebrafish spermatozoa using methanol, Can. J. Zool. 60 (1982) 1867–1870.
[24] C. Huang, Q. Dong, T.R. Tiersch, Sperm cryopreservation of a live-bearing fish, the platyfish *Xiphophorus couchianus*, Theriogenology 62 (2004) 971–989.
[25] C. Huang, Q. Dong, R.B. Walter, T.R. Tiersch, Sperm cryopreservation of green swordtail *Xiphophorus helleri*, a fish with internal fertilization, Cryobiology 48 (2004) 295–308.
[26] J.B. Hughes, An examination of eggs challenged with cryopreserved spermatozoa of the American oyster, *Crassostrea virginica*, Cryobiology 10 (1973) 342–344.
[27] S.W. Hwang, H.P. Chen, Fertility of male oyster gametes after freeze-thawing, Chin.-Am. Jt. Comm. Rural Reconstr. Fish. Ser. 15 (1973) 1–5.
[28] S. Ieropoli, P. Masullo, M. Do Espirito Santo, G. Sansone, Effects of extender composition, cooling rate and freezing on the fertilization viability of spermatozoa of the Pacific oyster (*Crassostrea gigas*), Cryobiology 49 (2004) 250–257.
[29] N. Iwata, H. Kurokura, R. Hirano, Cryopreservation of Pacific oyster, *Crassostrea gigas*, sperm, Suisanzoshoku 37 (1989) 163–166 (our translation from Japanese with English abstract).
[30] K. Kubokawa, T. Watanabe, M. Yoshioka, M. Iwata, Effects of acute stress on plasma cortisol, sex steroid hormone and glucose levels in male and female sockeye salmon during the breeding season, Aquaculture 172 (1999) 335–349.
[31] C. Labbe, G. Maisse, Influence of rainbow trout thermal acclimation on sperm cryopreservation: relation to change in the lipid composition of the plasma membrane, Aquaculture 145 (1996) 281–294.
[32] C. Labbe, G. Maisse, K. Muller, A. Zachowski, S. Kaushik, M. Loir, Thermal acclimation and dietary lipids alter the composition, but not fluidity, of trout sperm plasma membrane, Lipids 30 (1995) 23–33.
[33] R.P. Lang, K.L. Riley, J.E. Chandler, T.R. Tiersch, The use of dairy protocols for sperm cryopreservation of blue catfish *Ictalurus furcatus*, J. World Aquacult. Soc. 34 (2003) 66–75.
[34] F. Lahnsteiner, B. Berger, T. Weismann, R.A. Patzner, Changes in morphology, physiology, metabolism, and fertilization capacity of rainbow trout semen following cryopreservation, Prog. Fish-Cult. 58 (1996) 149–159.
[35] F. Lahnsteiner, N. Mansour, T. Weismann, A new technique for insemination of large egg batches with cryopreserved semen in the rainbow trout, Aquaculture 209 (2002) 359–367.
[36] F. Lahnsteiner, T. Weismann, R.A. Patzner, A uniform method for cryopreservation of semen of the salmonid fishes, *Oncorhynchus mykiss* (Walbaum), *Salmo trutta* f. fario L., *Salmo trutta* f. lacustris L., *Coregonus* sp., Aquacult. Res. 26 (1995) 801–807.
[37] F. Lahnsteiner, T. Weismann, R.A. Patzner, An efficient method for cryopreservation of testicular sperm from the Northern pike, *Esox lucius* L, Aquacult. Res. 29 (1998) 341–347.
[38] S.P. Leibo, Sources of variation in cryopreservation, in: T.R. Tiersch, P.M. Mazik (Eds.), Cryopreservation in Aquatic Species, World Aquaculture Society, Baton Rouge, LA, 2000, pp. 75–83.
[39] H.T. Meryman, Cryoprotective agents, Cryobiology 8 (1971) 173–183.
[40] E. Miskolczi, B. Urbanyi, A. Horvath, Cryopreservation of common carp sperm, Book of Abstracts: Annual Meeting of the World Aquaculture Society, January 2005, New Orleans, LA.
[41] C.G. Paniagua-Chavez, T.R. Tiersch, Laboratory studies of cryopreservation of sperm and trochophore larvae of the eastern oyster, Cryobiology 43 (2001) 211–223.
[42] B.W. Pickett, W.E. Berndton, Preservation of bovine spermatozoa by freezing in straws: a review, J. Dairy Sci. 57 (1974) 1287–1301.
[43] J. Piironen, Cryopreservation of sperm from brown trout (*Salmon trutta m. lacustris* L.) and Arctic charr (*Salvelinus alpinus* L.), Aquaculture 116 (1993) 275–285.
[44] K.J. Rana, Cryopreservation of fish spermatozoa, in: J.G. Day, M.R. McLellan (Eds.), Methods in Molecular Biology: Cryopreservation and Freeze-Drying Protocols, vol. 38, 1995, pp. 151–165.
[45] K.J. Rana, B.J. McAndrew, The viability of cryopreserved tilapia spermatozoa, Aquaculture 76 (1989) 335–345.
[46] G.F. Richardson, T.L. Miller, M.A. McNiven, Cryopreservation of Arctic charr, *Salvelinus alpinus* (L.), semen in various extenders and in three sizes of straw, Aquacult. Res. 31 (2000) 307–315.
[47] G.F. Richardson, C.E. Wilson, L.W. Crim, Z. Yao, Cryopreservation of yellowtail flounder (*Pleuronectes ferrugineus*) semen in large straws, Aquaculture 174 (1999) 89–94.
[48] K.L. Riley, C.G. Holladay, E.J. Chesney, T.R. Tiersch, Cryopreservation of sperm of red snapper (*Lutjanus campechanus*), Aquaculture 238 (2004) 183–194.
[49] E. Rurangwa, D.E. Kime, F. Ollevier, J.P. Nash, The measurement of sperm motility and factors affecting sperm quality in cultured fish, Aquaculture 234 (2004) 1–28.
[50] G. Sansone, A. Fabbrocini, S. Ieropoli, A. Langellotti, M. Occidente, D. Matassino, Effects of extender composition, cooling rate, and freezing on the motility of sea bass (*Dicentrarchus labrax*, L.) spermatozoa after thawing, Cryobiology 44 (2002) 229–239.
[51] P.D. Scheerer, G.H. Thorgaard, Improved fertilization by cryopreserved rainbow trout semen treated with theophylline, Prog. Fish-Cult. 51 (1989) 179–182.
[52] J.F. Smith, P.A. Pugh, H.R. Tervit, R.D. Roberts, A.R. Janke, H.F. Kaspar, S.L. Adams, Cryopreservation of shellfish sperm, eggs and embryos, Proc. N.Z. Soc. Anim. Prod. 61 (2001) 31–34.

[53] R.R. Sokal, F.J. Rohlf, Biometry: The Principles and Practice of Statistics in Biological Research, 3rd ed., W.H. Freeman and Company, New York, 1995 , pp. 419–422.

[54] L.M. Thurston, K. Siggins, A.J. Mileham, P.F. Watson, W.V. Holt, Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation, Biol. Reprod. 66 (2002) 545–554.

[55] T.R. Tiersch, Introduction, in: T.R. Tiersch, P.M. Mazik (Eds.), Cryopreservation in Aquatic Species, World Aquaculture Society, Baton Rouge, LA, 2000, pp. xix–xxvi.

[56] T.R. Tiersch, C.A. Goudie, G.J. Carmichael, Cryopreservation of channel catfish sperm: storage in cryoprotectants, fertilization trials, and growth of channel catfish produced with cryopreserved sperm, Tran. Am. Fish. Soc. 123 (1994) 580–586.

[57] T.R. Tiersch, W.R. Wayman, D.P. Skapura, C.L. Neidig, H.J. Grier, Transport and cryopreservation of sperm of the common snook, *Centropomus undecimalis* (Bloch), Aquacult. Res. 35 (2004) 278–288.

[58] K. Yankson, J. Moyse, Cryopreservation of the spermatozoa of *Crassostrea tulipa* and three other oysters, Aquaculture 97 (1991) 259–267.