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Theriogenology
Theriogenology 66 (2006) 387–403
www.journals.elsevierhealth.com/periodicals/the

Systematic factor optimization for sperm cryopreservation of tetraploid Pacific oysters, *Crassostrea gigas*

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Received 26 August 2005; accepted 2 December 2005

Abstract

The availability of tetraploid Pacific oysters provides a unique opportunity for comparative studies of sperm cryopreservation between diploids and tetraploids. In parallel to studies with sperm from diploid oysters, this study reports systematic factor optimization for sperm cryopreservation of tetraploid oysters. Specifically, this study evaluated the effects of cooling rate, single or combined cryoprotectants at various concentrations, equilibration time (exposure to cryoprotectant), and straw size. Similar to sperm from diploids, the optimal cooling rate was 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 showed that a combination of the cryoprotectants 6% polyethylene glycol/4% propylene glycol and 6% polyethylene glycol/4% dimethyl sulfoxide yielded consistently high post-thaw motility. A long equilibration (60 min) yielded higher percent fertilization, and confirmed that extended equilibration could be beneficial when low concentrations of cryoprotectant are used. There was no significant difference in post-thaw motility between straw sizes of 0.25 and 0.5 mL. Despite low post-thaw fertilization (<10%) in general for sperm from tetraploids, optimized protocols in the present study effectively retained post-thaw motility for sperm from tetraploid oysters. This study confirmed that sperm from tetraploid Pacific oysters were more negatively affected by cryopreservation than were those of diploids. One possible explanation is that sperm from these two ploidies are different in their plasma membrane properties (e.g., structure, permeability, and elasticity), and the plasma membrane of sperm from tetraploids is more sensitive to cryopreservation effects. The fact that combinations of non-permeating and permeating cryoprotectants improved post-thaw motility in sperm from tetraploids provided presumptive evidence for this interpretation.

Keywords: Cryopreservation; Oyster; *Crassostrea gigas*; Sperm; Tetraploid

1. Introduction

Polyploidy (possession of more than the normal two chromosome sets) has played an important role in bivalve aquaculture due to immediate commercial interest. The benefits associated with triploid oysters (three chromosome sets), such as reduced gamete output, improved meat quality and growth, and year-round marketability

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0093-691X/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2005.12.004

have enabled this technique to be applied commercially in Pacific oysters, *Crassostrea gigas* [1]. Until the advent of tetraploid oysters (four chromosome sets) 10 years ago [2], triploidy induction in oysters was primarily achieved by blocking the release of the second polar body with cytochalasin B [3]. Compared to the use of chemical methods, the crossing of gametes from tetraploids and normal diploids offers reliable triploidy production and high survival rates [4–6]. In the past 5 years, techniques used to produce tetraploid Pacific oysters have improved substantially, such that several generations of tetraploids have been produced and maintained in commercial hatcheries [7,8]. As a consequence, triploid production can be facilitated by the use of tetraploid breeding lines, rather than the continued chemical production of tetraploids. Refrigerated and frozen storage of tetraploid oyster sperm will be a critical tool for developing tetraploid breeding programs, especially for commercial-scale application of tetraploid stocks in the future.

Although sperm cryopreservation has been reported with some 200 fish species and 30 invertebrates [9–11], unlike normal haploid sperm, studies of cryopreservation of diploid sperm from tetraploid aquatic organisms are rare. Except for our previous attempts with sperm of tetraploid Pacific oysters [12], the only study associated with the use of cryopreserved diploid sperm was from rainbow trout for the purpose of producing androgenetic progeny [13]; protocols used to cryopreserve sperm from tetraploid rainbow trout were adopted from protocols developed for diploid Atlantic salmon [14], and no attempts were made to develop protocols specifically for sperm from tetraploids (despite the low quality of thawed sperm). In our previous studies, dimethyl sulfoxide at 8%, propylene glycol at 5, 10, and 15%, and a commercial dairy freezing protocol, were used to cryopreserve sperm of tetraploid Pacific oysters; the results suggested that sperm of tetraploids were more sensitive to cryopreservation damage than were the sperm of diploids [12]. Subsequent ultrastructural studies found that sperm from tetraploid oysters were 25% larger in linear dimensions (lengths and widths), and 53% of the sperm cells studied had five mitochondria, compared to four in diploids [15]. Differences between these two types of sperm of the same species also provide unique opportunities for basic research in sperm cryobiology.

The present study continued efforts, using a systematic approach, to improve methods to cryopreserve sperm. The goal of this study was to optimize protocols used for sperm cryopreservation of tetraploid Pacific oysters. 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; and (6) straw size (0.25 mL versus 0.5 mL).

2. Materials and methods

2.1. Sperm collection and motility estimation

Tetraploid Pacific oysters were obtained within the peak spawning season (June 10–July 7, 2004) from Taylor Resources Quilcene Shellfish Hatchery (TRQSH; www.taylorshellfish.com) in Quilcene, WA, USA (47°49′133″N, 122°49′523″W). Oysters were conditioned in a flow-through system at 30 ppt, 20 °C, and fed with a mixture of microalgae. For Experiments 1 and 2, intact oysters were shipped and sperm were collected by dry stripping of the gonad upon arrival [16]. For other experiments, sperm samples were collected with the same method and placed separately into a 15-mL centrifuge tube for each male, and each week, 8–10 samples (undiluted) were shipped chilled at 5–10 °C by overnight delivery from TRQSH to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS). Because this project also included study of sperm from diploid oysters (reported in [17]), the ploidy level of individual oysters was verified by flow cytometry [12,18]. Sperm samples were placed in 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 mOs mol/kg [19] and suspensions were filtered through a 40-μm cell strainer (BD Biosciences Discovery Labware, Bedford, MA, USA). The concentrations of sperm suspensions were adjusted to 2×10^9 cells/mL using readings at 581 nm from a spectrophotometer (Genesys^{TM} 20, Thermo Spectronic, Rochester, NY, USA) and standard curves established for oysters of this population [20]. A total of 29 males were used in this study, and each oyster or sperm sample was identified by a code (e.g., CG04M02) with the species designation (CG: *C. gigas*), year (04: 2004), sex

(M: male), and the order in which it was processed (02: the second oyster of 2004). Motility was evaluated by equilibrating 2 μL of sperm suspension in 30 μL of C-F HBSS at 1000 mOs mol/kg on a two-well glass slide at 23 °C for 2 min, and estimated visually at 200× magnification using darkfield microscopy (Optiphot 2, Nikon Inc., Garden City, NY, USA). Sperm motility was expressed as the percentage of cells actively moving in a forward direction, and was recorded in increments of 5% (samples with motility below 5% but greater than 0% were recorded as 1%, and samples with motility below 100% but greater than 95% were recorded as 99%) [16]. For post-thaw motility, four straws were thawed for each treatment and the average values were used for data analysis.

2.2. 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 mOs mol/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, MO, USA).

Table 1
Abbreviations for cryoprotectants used to cryopreserve sperm from tetraploid Pacific oysters

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.

2.3. Freezing and thawing procedures

Freezing with a controlled-rate freezer (Kryo 10 Series II; Planer Products, Sunbury-on-Thames, UK) followed the methods described in [17]. 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 to −80 °C. For cooling rates of 45 and 50 °C/min, samples were cooled in a single step from 5 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. Existing commercial freezing methods developed for dairy bulls were described in [12,17]. In brief, 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 [21]. Thawing for motility estimation and fertilization trials was conducted as described in [17]. In general, samples were stored for at least 12 h before thawing, and 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.

2.4. Fertilization and larval evaluation

The procedures for fertilization and larval evaluation are described in detail in [17]. Briefly, 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 from diploid females) held in 250 mL of seawater, yielding approximately 10,000 spermatozoa/egg. Percent fertilization was calculated by counting developing embryos 2 h after insemination, and percent hatching was calculated by counting normal straight-hinge larvae 24 h after insemination (with a dissecting microscope). For a negative control, eggs were monitored after treatment as described above, without addition of sperm.

2.5. Experimental design and data analysis

A total of 11 experiments (Fig. 1) were conducted. 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 on 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

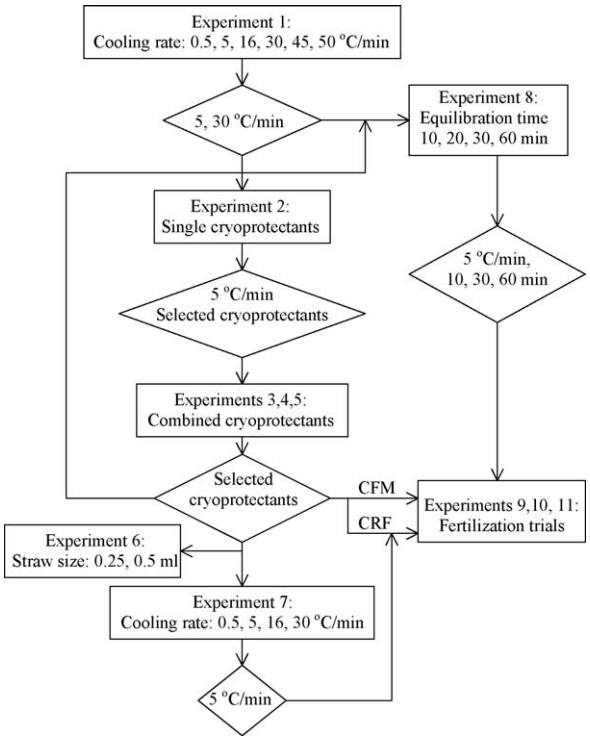

Fig. 1. Design of experiments for optimization of sperm cryopreservation from tetraploid Pacific oysters. Post-thaw motility was used as the main criterion for procedure optimization and percent fertilization and hatch were used to test the results of optimized procedures. CRF: cooled at 5 °C/min using 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 and rhomboids indicate decisions made based on experiments.

various concentrations. Among 8 single cryoprotectants tested in Experiment 2, 6 were selected to form 16 single or combined treatment levels to test possible combinations between different cryoprotectants (Experiment 3). Subsequently, three combination groups (PEG/MeOH, PEG/P-glycol, and PEG/DMSO) were selected for optimal concentration combination evaluation (Experiment 4). Separate males were used for each combination because of the large number of treatments (for each combination: 16 treatments × 4 straws/treatment/male × 2 males = 128 straws). Therefore, in Experiment 5 the same males were used to test 16 single or combined cryoprotectants selected from Experiment 4. Single or cryoprotectant combinations with consistently higher post-thaw motility from Experiments 2–5 were selected to evaluate straw size (Experiment 6), equilibration time (Experiment 8), and re-evaluate cooling rate (Experiment 7). For all eight 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 or hatch was used to evaluate the equilibration time and selected cryoprotectants (Experiment 8), selected cryoprotectants and cooling rate with the controlled-rate freezer (Experiment 10), and selected cryoprotectants with commercial freezing method (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. 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 with only two males (Table 2). However, the experimental design outlined above (Fig. 1) actually allowed factors such as cooling rate, cryoprotectant, and equilibration time to be repeatedly evaluated in successive experiments (Table 2), and in some cases the same treatment (e.g., 6% PEG/4% P-glycol) was tested on as many as 13 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, two-way (mixed model), or three-way (mixed model) analysis of variance (ANOVA) (SAS 9.0; SAS Institute Inc., Cary, NC, USA). When a significant difference ($\alpha = 0.05$) was detected among treatments, Tukey's Honestly Significant Difference Procedure was used for pair-wise comparisons. Results were presented as means ± S.D., and probability values of $P < 0.05$ were considered significant. Data for sperm motility, percent fertilization, and percent hatch were arcsine-square root transformed prior to analysis [22].

2.6. Experiment 1: effect of cooling rate

Sperm from four males (CG04M35, 36, 37, 38) were used to evaluate six cooling rates: 0.5, 5, 16, 30, 45, and 50 °C/min. Five percent MeOH, 10% E-glycol, 10% P-glycol, and 10% DMSO were used as individual cryoprotectants and sperm suspensions were frozen in 0.5-mL straws. Motilities were estimated after suspension and after thawing.

2.7. Experiment 2: effect of single cryoprotectant

Sperm from three males were used to test eight cryoprotectants each at 5 and 10%: MeOH, P-glycol, DMA, DMSO, E-glycol, glycerol, PEG200, and

Table 2
Experimental design and model statement for the 11 experiments for cyropreservation of sperm from tetraploid Pacific oysters

Experiment	Factor (treatment level)	Male code[a]	ANOVA	Model statement[b]
1	Cooling rate (6); cryoprotectant (4)	35, 36, 37, 38	Two way	$y = \mu + CR + cpa + cr \times cpa + male + \varepsilon$
2	Cryoprotectant (8); concentration (2) cooled at 5 °C/min	56, 57	Two way	$y = \mu + CPA + con + cpa \times con + male + \varepsilon$
	Cryoprotectant (8); concentration (2) cooled at 30 °C/min	56, 58	Two way	$y = \mu + CPA + con + cpa \times con + male + \varepsilon$
3	Single or combined cryoprotectants (16)	64, 65	One way	$y = \mu + CPA + male + \varepsilon$
4	PEG/MeOH combination (16)	74, 75	One way	$y = \mu + CPA + male + \varepsilon$
	PEG/P-glycol combination (16)	71, 72	One way	$y = \mu + CPA + male + \varepsilon$
	PEG/DMSO combination (16)	72, 73	One way	$y = \mu + CPA + male + \varepsilon$
5	Selected cryoprotectants (16)	85, 86	One way	$y = \mu + CPA + male + \varepsilon$
6	Straw size (2); cryoprotectant (5)	99, 100	Three way	$y = \mu + STRAW + cpa + straw \times CPA + male + \varepsilon$
7	Cooling rate (4); cryoprotectant (5)	99, 100	Two way	$y = \mu + CR + CPA + CR \times CPA + male + \varepsilon$
8	Equilibration time (4); cryoprotectant (2); cooling rate (2)	101, 102	Three way	$y = \mu + TIME + cpa + cr + time \times cpa + time \times cr + cpa \times cr + time \times cpa \times cr + male + \varepsilon$
9	Equilibration time (3); combined cryoprotectant (3)	107, 108	Two way	$y = \mu + TIME + cpa + time \times cpa + male + \varepsilon$
	Equilibration time (3); single cryoprotectant (3)	109, 110	Two way	$y = \mu + TIME + cpa + time \times cpa + male + \varepsilon$
10	Selected cryoprotectant (7) with CRF[c]	89, 90, 91	One way	$y = \mu + CPA + male + \varepsilon$
11	Selected cryoprotectant (5) with CFM[d]	93, 94	One way	$y = \mu + CPA + male + \varepsilon$

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

PEG600. Based on the results of Experiment 1, sperm suspensions were placed in 0.5-mL straws and were cooled at 5 °C/min (CG04M56, 57) and 30 °C/min (CG04M56, 58). Motilities were estimated after suspension and after thawing.

2.8. Experiment 3: effect of combined cryoprotectants (first selection)

Sperm from two males (CG04M64, 65) 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.

2.9. 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

Table 3
Post-thaw motility (mean ± S.D.) of sperm samples (from tetraploid Pacific oysters) suspended in 16 single or combined cryoprotectants, and cooled at 5 °C/min (males: CG04M64 and CG04M65)

Cryoprotectant (%)		Post-thaw motility (%)		
First	Second	CG04M64	CG04M65	Average
PEG200 (4)	MeOH (6)	1 ± 1	20 ± 15	17 ± 12^{ab}
PEG200 (4)	P-glycol (6)	4 ± 2	23 ± 10	19 ± 9^{a}
PEG200 (4)	E-glycol (6)	2 ± 2	12 ± 8	10 ± 6^{abc}
PEG200 (4)	DMSO (6)	2 ± 2	15 ± 10	13 ± 8^{ab}
PEG600 (4)	MeOH (6)	2 ± 2	7 ± 4	6 ± 4^{abcd}
PEG600 (4)	P-glycol (6)	1 ± 0	4 ± 4	3 ± 3^{bcd}
PEG600 (4)	E-glycol (6)	1 ± 1	7 ± 4	6 ± 4^{abcd}
PEG600 (4)	DMSO (6)	1 ± 0	2 ± 2	2 ± 2^{bcd}
DMSO (4)	MeOH (4)	1 ± 1	0 ± 1	0 ± 0^{d}
DMSO (4)	P-glycol (4)	2 ± 2	2 ± 2	2 ± 2^{bcd}
DMSO (4)	E-glycol (4)	1 ± 1	2 ± 2	2 ± 2^{bcd}
P-glycol (4)	E-glycol (4)	1 ± 1	4 ± 2	3 ± 2^{bcd}
–	MeOH (8)	0 ± 1	2 ± 2	2 ± 2^{cd}
–	P-glycol (8)	1 ± 1	2 ± 2	2 ± 2^{cd}
–	E-glycol (8)	1 ± 1	1 ± 0	1 ± 0^{cd}
–	DMSO (8)	3 ± 2	–	2 ± 1^{bcd}

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

Table 4
Post-thaw motility (mean ± S.D.) of sperm samples (from tetraploid Pacific oysters) suspended in single or combined cryoprotective agents (CPA), and cooled at 5 °C/min

PEG200 (%)	Second CPA (%)	Second cryoprotectant (CPA)		
		MeOH (CG04M74, 75)[a]	P-glycol (CG04M71, 72)	DMSO (CG04M72, 73)
0	4	5 ± 0^{ef}	1 ± 0^{h}	1 ± 0^{g}
	6	11 ± 2^{bcde}	5 ± 0^{g}	4 ± 2^{efg}
	8	10 ± 2^{cde}	31 ± 8^{bc}	11 ± 8^{bcd}
2	0	1 ± 0^{f}	1 ± 0^{h}	1 ± 0^{g}
	4	14 ± 2^{bcd}	12 ± 4^{ef}	7 ± 5^{def}
	6	18 ± 7^{bc}	45 ± 5^{a}	9 ± 5^{cde}
	8	11 ± 2^{bcde}	42 ± 4^{a}	18 ± 11^{ab}
4	0	5 ± 0^{ef}	10 ± 0^{efg}	2 ± 0^{fg}
	4	13 ± 9^{bcde}	31 ± 6^{bc}	14 ± 9^{abc}
	6	20 ± 0^{ab}	29 ± 4^{bc}	14 ± 7^{abc}
	8	9 ± 2^{de}	28 ± 8^{bc}	16 ± 7^{ab}
6	0	11 ± 3^{bcde}	18 ± 3^{de}	5 ± 0^{def}
	4	29 ± 4^{a}	36 ± 5^{ab}	21 ± 14^{a}
	6	13 ± 3^{bcd}	17 ± 5^{de}	10 ± 5^{bcd}
	8	11 ± 6^{cde}	8 ± 3^{fg}	7 ± 4^{def}
8	0	11 ± 6^{cde}	23 ± 9^{cd}	9 ± 3^{bcd}

Numbers in columns sharing the same superscript were not different at $P = 0.05$.
[a] Male numbers.

Table 5
Post-thaw motility (mean ± S.D.) of sperm samples (from tetraploid Pacific oysters) suspended in 16 single or combined cryoprotectants, and cooled at 5 °C/min (males: CG04M85 and CG04M86)

PEG200 (%)	Second cryoprotectant (%)	Post-thaw motility (%)
0	MeOH (6)	8 ± 2^{abcd}
0	MeOH (8)	6 ± 2^{cd}
0	P-glycol (8)	4 ± 2^{d}
0	DMSO (8)	5 ± 2^{cd}
2	MeOH (6)	13 ± 3^{ab}
2	P-glycol (6)	9 ± 4^{abcd}
2	P-glycol (8)	10 ± 4^{abc}
2	DMSO (8)	9 ± 4^{abcd}
4	MeOH (6)	15 ± 6^{a}
4	P-glycol (4)	8 ± 4^{abcd}
4	DMSO (4)	9 ± 2^{abcd}
4	DMSO (6)	13 ± 3^{ab}
6	MeOH (4)	14 ± 8^{ab}
6	P-glycol (4)	15 ± 7^{a}
6	DMSO (4)	15 ± 4^{a}
8	–	7 ± 2^{bcd}

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

total of 48 combinations (Table 4). Based on the availability of sperm volume, sperm from five males were allocated to these three groups: PEG/MeOH (CG04M74, 75), PEG/P-glycol (CG04M71, 72), and PEG/DMSO (CG04M72, 73). Sperm suspensions were placed in 0.25-mL straws, and cooled at 5 °C/min. Motilities were estimated after suspension and after thawing.

2.10. 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 (CG04M85, 86) 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.

2.11. Experiment 6: effect of straw size (0.25 mL versus 0.5 mL)

Sperm from two males (CG04M99, 100) were used to evaluate the two straw sizes. Based on previous experiments, the combined and single cryoprotectants used were 6% PEG/4% MeOH, 6% PEG/4% P-glycol, 6% PEG/4% DMSO, 6% MeOH, and 8% PEG200, and the samples were cooled at 5 °C/min. Motilities were estimated after suspension and after thawing.

2.12. Experiment 7: effect of interactions between cooling rate and cryoprotectant

Sperm from two males (CG04M99, 100) 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: 6% PEG/4% DMSO, 6% PEG/4% MeOH, 6% PEG/4% P-glycol, 6% MeOH, and 8% PEG200. Sperm suspensions were placed in 0.25-mL straws and motilities were estimated after suspension and after thawing.

2.13. Experiment 8: effect of equilibration time

Sperm from two males (CG04M101, 102) were used to evaluate the equilibration times of 10, 20, 30, and 60 min. Six percent PEG/4% P-glycol and 6% PEG/4% DMSO were used as combined cryoprotectants. Sperm suspensions were placed in 0.25-mL straws and cooled at 5 and 30 °C/min. Motilities were estimated after suspension and after thawing.

2.14. Experiment 9: evaluation of equilibration time on percent fertilization

Sperm from four males were used to evaluate the interactions between cryoprotectants and equilibration times of 10, 30, and 60 min. Sperm samples were suspended with 6% PEG/4% MeOH, 6% PEG/4% P-glycol, 6% PEG/4% DMSO (CG04M107, 108) or 6% MeOH, 8% DMSO, and 8% PEG200 (CG04M109, 110), and were placed in 0.5-mL straws and cooled at 5 °C/min. Motilities were estimated after suspension and after thawing, and percent fertilization were estimated as described above.

2.15. Experiment 10: evaluation of selected cryoprotectants on percent fertilization

Sperm from three males (CG04M89, 90, 91) 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 6% PEG/4% MeOH, 6% MeOH, 6% PEG/4% P-glycol, 8% P-glycol, 6% PEG/4% DMSO, 8% DMSO, and 8% PEG200. Motilities were estimated after suspension and after thawing, and percent fertilization and hatch were estimated as described above.

2.16. Experiment 11: evaluation of selected cryoprotectants with commercial-scale freezing method

Sperm from two males (CG04M93, 94) were used to test commercial-scale freezing protocols developed for dairy bulls. Sperm samples were suspended in 6% PEG/4% MeOH, 6% MeOH, 6% PEG/4% P-glycol, 6% PEG/4% DMSO, and 8% PEG200, equilibrated for 15 min prior to freezing. Motilities were estimated after suspension and after thawing, and percent fertilization were estimated as described above.

3. Results

3.1. Initial sperm motility after shipment

A total of 29 Pacific oysters were transported in 6 shipments from June 10 to July 7, 2004. The initial motility ranged from 5 to 95% with the average of 64 ± 25%. The median value was 70%, and the mode was 90% (Fig. 2). The initial motility of sperm samples collected from shipped oysters (44 ± 25%) had lower

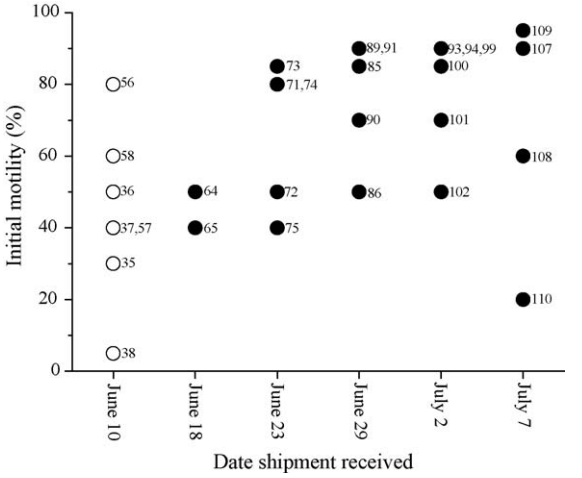

Fig. 2. The motility of sperm from 29 tetraploid oysters transported in 6 shipments from June 10 to July 7, 2004. Intact oysters (open circles) were transported in the first shipment ($n = 7$), and undiluted sperm (filled circles) were transported in the other shipments ($n = 22$). The numbers identified each individual oyster at the order of their usage in experiments (the full coding would include the designation ''CG04M'' preceding each number).

($P = 0.009$) initial motility than did shipped sperm samples (70 ± 22%).

3.2. Experiment 1: effect of cooling rate

Post-thaw motility of sperm cooled at rates ranging from 0.5 to 50 °C/min were different from one another ($P = 0.032$), with the highest post-thaw motility obtained with the rates between 0.5 and 30 °C/min (Fig. 3). There was a significant difference among the four cryoprotectants ($P = 0.007$) with 10% DMSO and P-glycol yielding higher post-thaw motility than did 10% E-glycol and 5% MeOH. A significant interaction was detected between the cryoprotectant and cooling rate ($P < 0.001$). Post-thaw motility was different among males ($P = 0.032$), with the lowest post-thaw motility found for male CG04M36 (<5% for all treatments). After thawing, motility was highest (8 ± 10%) for samples suspended in 10% DMSO and cooled at 5 °C/min. Cooling rates of 5 and 30 °C/min were chosen for subsequent experiments.

3.3. Experiment 2: effect of single cryoprotectants

For samples cooled at 5 °C/min (Fig. 4), there were differences among the eight cryoprotectants ($P = 0.015$); post-thaw motility of samples suspended in DMSO was significantly higher than the motility of those suspended in glycerol or PEG600. There were no

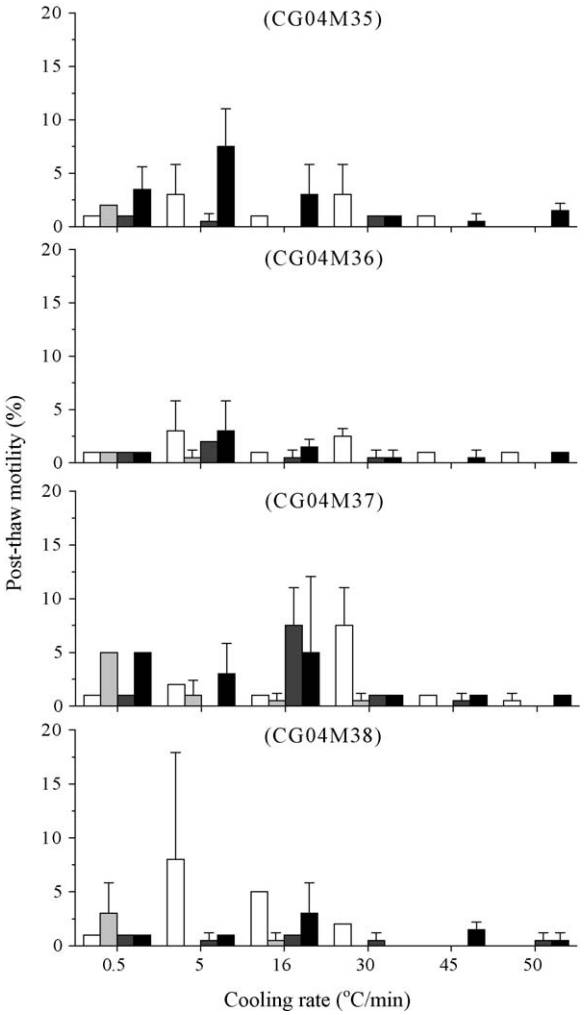

Fig. 3. Post-thaw motility (mean ± S.D.) of sperm samples suspended in 10% DMSO (white bars), 10% E-glycol (light gray bars), 5% MeOH (dark gray bars), and 10% P-glycol (black bars), and cooled at 0.5, 5, 16, 30, 45, and 50 °C/min. Four males were used (CG04M35, CG04M36, CG04M37, and CG04M38).

differences between the cryoprotectant concentrations of 5 and 10% ($P = 0.161$), and there was no interaction between cryoprotectant and concentration ($P = 0.231$). However, there were differences among males ($P < 0.001$), with CG04M58 yielding the lowest overall post-thaw motility. The highest post-thaw motility (15 ± 0%) was found in samples from CG04M56 when the sperm were suspended either in 10% DMSO or in 5% PEG200, followed by samples suspended in 5% methanol (13 ± 4%).

For samples cooled at 30 °C/min (Fig. 4), there were no differences in post-thaw motility among the eight cryoprotectants ($P = 0.231$). However, there was a difference between 5 and 10% ($P = 0.037$), with 5% yielding higher motility. There was no interaction between cryoprotectant and concentration ($P = 0.930$), and post-thaw motility of sperm from the two males was not different ($P = 0.250$). Post-thaw motility was highest (13 ± 4%) in samples from CG04M56 suspended in 5% DMSO, followed by samples suspended in 5% E-glycol (8 ± 10%) and 5% MeOH (8 ± 4%). Due to the better sperm morphology (sperm with most tails attached) for samples cryopreserved with PEG200 and PEG600, subsequent experiments employed polyethylene glycol as the non-permeating agent and combined it with MeOH, P-glycol, E-glycol, and DMSO at various concentrations.

3.4. Experiment 3: effect of combined cryoprotectants (first selection)

There were differences ($P < 0.001$) among the 16 single and combined cryoprotectants, with the combined cryoprotectants of PEG/P-glycol (23 ± 10%), PEG/MeOH (20 ± 15%), and PEG/DMSO (15 ± 10%) having the highest post-thaw motility (Table 3). Post-thaw motilities of sperm from the two males were different ($P < 0.001$). Combinations of non-permeating agent (PEG200) and the permeating agents MeOH, P-glycol, and DMSO were chosen for further optimization in the subsequent experiments.

3.5. Experiment 4: effect of combined cryoprotectants (second selection)

There were differences ($P < 0.001$) for various concentration combinations within each group of combined cryoprotectants (Table 4). Of the single cryoprotectants and cryoprotectant combinations of PEG200 and MeOH, the highest post-thaw motility was found in samples suspended in 6% PEG/4% MeOH (29 ± 4%), followed by samples suspended in 4% PEG/6% MeOH (20 ± 0%). The two males used to test this combination were not different ($P = 0.877$). 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 (45 ± 5%), followed by 2% PEG/8% P-glycol (42 ± 4%) and 6% PEG/4% P-glycol (36 ± 5%), which were not different from one another ($P > 0.050$). However, post-thaw motility differed between the two males ($P < 0.001$). Of the single cryoprotectants and cryoprotectant combinations of PEG200 and DMSO, post-thaw motility was highest in samples suspended in 6% PEG/4% DMSO (21 ± 14%), followed by samples suspended in 2% PEG/8% DMSO

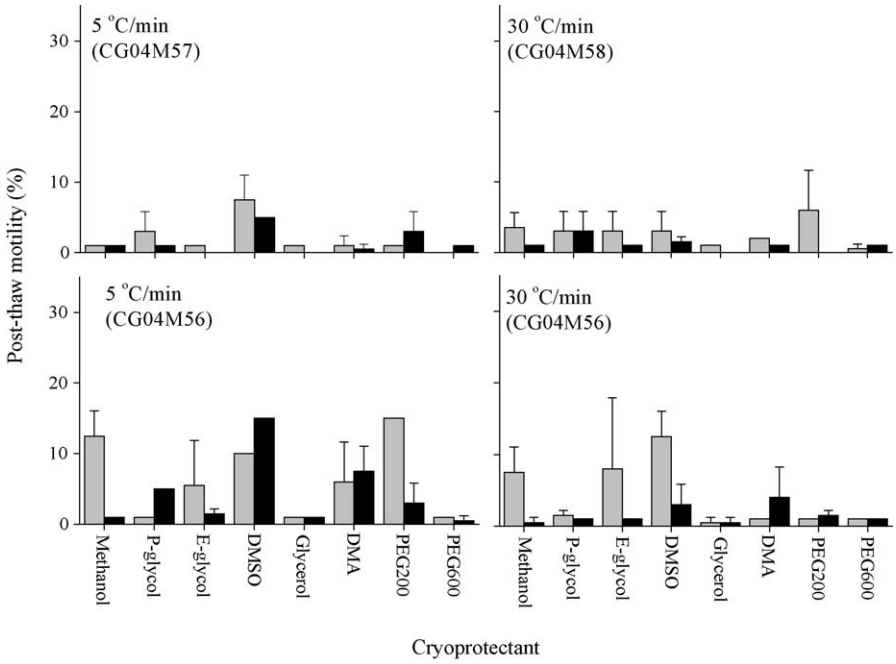

Fig. 4. Post-thaw motility (mean ± S.D.) of sperm samples suspended in eight cryoprotectants: MeOH, P-glycol, DMA, DMSO, E-glycol, glycerol, PEG200, and PEG600 each at 5% (light gray bars) and 10% (black bars), and cooled at 5 and 30 °C/min. Three males were used (CG04M56, CG04M57, and CG04M58).

(18 ± 11%) and 4% PEG/8% DMSO (16 ± 7%). Post-thaw motility differed between the two males ($P < 0.001$). 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.

3.6. Experiment 5: effect of combined cryoprotectants (final selection)

There were differences ($P < 0.001$) among the re-grouped 16 single or combined cryoprotectants with the highest post-thaw motility observed in samples suspended in 6% PEG/4% P-glycol (15 ± 7%), followed by 4% PEG/6% MeOH (15 ± 6%), 6% PEG/4% DMSO (15 ± 4%), and 6% PEG/4% MeOH (14 ± 8%) (Table 5). The two males used in this experiment differed ($P = 0.004$). Based on the combined results of Experiments 3–5, 6% PEG/4% MeOH, 6% PEG/4% P-glycol, and 6% PEG/4% DMSO were selected for subsequent experiments, and 6% MeOH, 8% P-glycol, 8% DMSO, and 8% PEG200 were also included as a single-cryoprotectant comparison.

3.7. Experiment 6: effect of straw size (0.25 mL versus 0.5 mL)

To evaluate possible effects from straw size differences, 0.25- and 0.5-mL straws were compared in this experiment (Fig. 5). There was no difference between the two straws in post-thaw motility ($P = 0.281$) and there was no interaction between straw size and cryoprotectant ($P = 0.104$). There were differences observed among the five cryoprotectants ($P = 0.002$) and the two males ($P < 0.001$). Similar to the previous experiment, samples suspended in 6% PEG/4% P-glycol yielded the highest post-thaw motility (40 ± 0% in 0.25-mL straws and 38 ± 3% in 0.5-mL straws), followed by 6% PEG/4% DMSO (33 ± 5% in 0.25-mL straws and 33 ± 3% in 0.5-mL straws).

3.8. Experiment 7: effect of interactions between cooling rate and cryoprotectant

Samples cooled at 5 °C/min yielded the highest post-thaw motility, which was different ($P < 0.001$) from that observed for cooling at 0.5, 16, and 30 °C/min (Fig. 6). Samples cooled at 16 and 30 °C/min were not different from one another ($P = 0.936$), but these cooling rates yielded higher post-thaw motility than

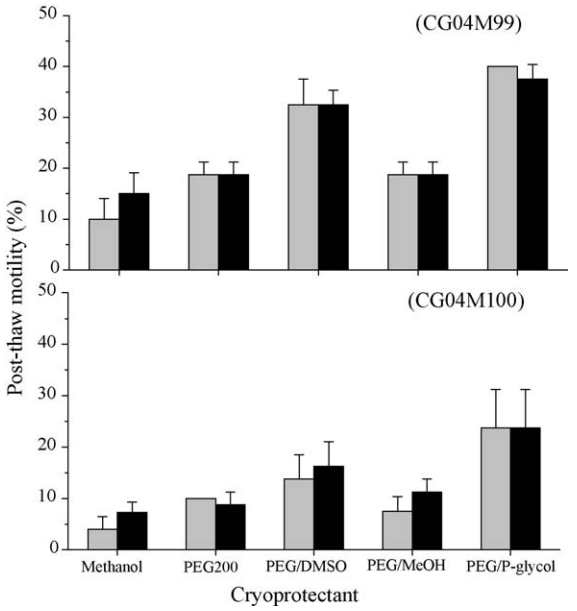

Fig. 5. Post-thaw motility (mean ± S.D.) of sperm samples suspended in 6% MeOH, 8% PEG200, 6% PEG/4% DMSO, 6% PEG/4% MeOH, and 6% PEG/4% P-glycol, and equilibrated for 12 min in 0.25-mL (light gray bars) or 0.5-mL straws (black bars). Samples were cooled at 5 °C/min and thawed in a water bath at 40 °C (6 and 10 s for 0.25- and 0.5-mL straws, respectively). Two males were used (CG04M99 and CG04M100).

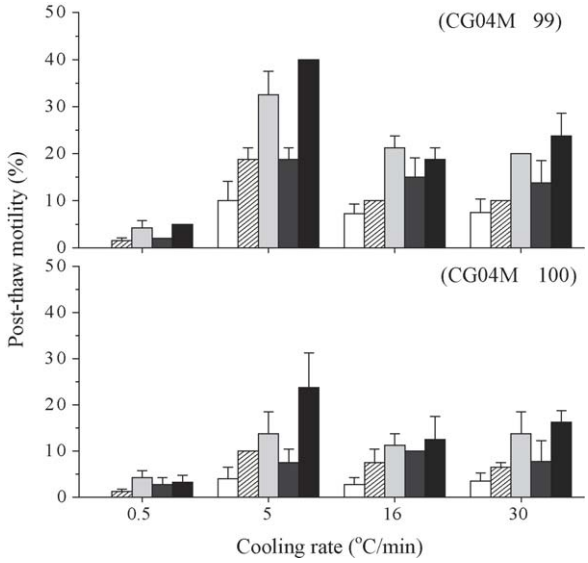

Fig. 6. Post-thaw motility (mean ± S.D.) of sperm samples suspended in 6% MeOH (white bars), 8% PEG200 (hatched bars), 6% PEG/4% DMSO (light gray bars), 6% PEG/4% MeOH (dark gray bars), and 6% PEG/4% P-glycol (black bars), and equilibrated for 12 min in 0.25-mL straws. Samples were cooled at 0.5, 5, 16, and 30 °C/min, and thawed in a 40 °C water bath for 6 s. Two males were used (CG04M99 and CG04M100).

did samples cooled at 0.5 °C/min ($P < 0.001$). There were also effects for the interaction of cooling rate and cryoprotectant ($P < 0.001$), the cryoprotectant ($P < 0.001$), and the two males ($P < 0.001$) used in this experiment. The cryoprotectant combinations of PEG/P-glycol and PEG/DMSO yielded higher post-thaw motilities than did the others ($P < 0.001$). The highest post-thaw motilities were found with samples from male CG04M99 suspended in 6% PEG/4% P-glycol (40 ± 0%) and 6% PEG/4% DMSO (33 ± 5%), and cooled at 5 °C/min. Consequently, a cooling rate of 5 °C/min was chosen for subsequent experiments.

3.9. 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 ($P = 0.919$) (Fig. 7). All interactions among equilibration time, cooling rate, and cryoprotectant were found to be non-significant

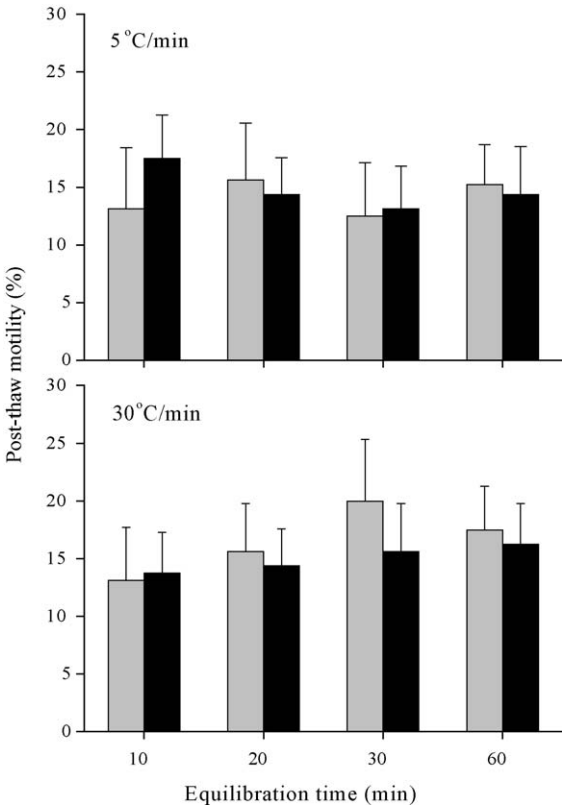

Fig. 7. Post-thaw motility (mean ± S.D.) of sperm samples suspended 6% PEG/4% DMSO (light gray bars) and 6% PEG/4% P-glycol (black bars), equilibrated for 10, 20, 30, and 60 min, and cooled at 5 and 30 °C/min. Two males were used (CG04M101 and CG04M102).

($P > 0.100$). Cooling rates of 5 and 30 °C/min were not different in this experiment ($P = 0.598$); neither were the cryoprotectant combinations of PEG/P-glycol and PEG/DMSO ($P = 0.865$). Post-thaw motility was also not significant for the two oysters used in this experiment ($P = 0.560$).

3.10. Experiment 9: evaluation of equilibration time on percent fertilization

Equilibration times of 30 and 60 min did not affect post-thaw motility in the previous experiment. To evaluate whether longer equilibration might affect percent fertilization, this experiment compared 10, 30, and 60 min with different cryoprotectants (Table 6). For three combined cryoprotectants, there were no differences among equilibration times for post-thaw motility ($P = 0.121$) or for percent fertilization ($P = 0.229$). However, higher percent fertilization was observed with a longer equilibration time, with the highest (30%) found in samples suspended with 6% PEG/4% DMSO with 60 min equilibration. Within cryoprotectants, there were differences for post-thaw motility ($P = 0.007$), but not for percent fertilization ($P = 0.608$). There were no interactions between equilibration time and cryoprotectants for post-thaw motility ($P = 0.864$) and percent fertilization ($P = 0.792$). For three single cryoprotectants, although there were no differences among the equilibration times for post-thaw motility ($P = 0.112$), the percentage of fertilization were higher ($P = 0.033$) in samples with longer equilibration, with the highest value (48%) in samples suspended in 6% MeOH for 60 min (Table 6). The interaction between equilibration time and cryoprotectants were not significant for post-thaw motility ($P = 0.118$) and percent fertilization ($P = 0.962$).

Sperm from the same male were used for controls of both combined and single cryoprotectants in this experiment, but eggs were from two different batches. Positive controls using fresh sperm without addition of cryoprotectant yielded 99% for the egg batch used for combined cryoprotectants and 100% fertilization for the egg batch used for single cryoprotectants. Controls using fresh sperm equilibrated with the same cryoprotectants for the same equilibration time yielded fertilization ranged from 51 to 99% with egg batch used for combined cryoprotectants, but yielded consistent high fertilization (>96%) with egg batch used for single cryoprotectants (Table 6). Fresh eggs without any sperm addition yielded 0% fertilization.

Table 6
Post-thaw motility (mean ± S.D.) and percent fertilization of sperm samples (from tetraploid Pacific oysters) suspended in various cryoprotectants, equilibrated for 10, 30, and 60 min, and cooled at 5 °C/min using a controlled-rate freezer

Male	Criterion	6% PEG/4% MeOH			6% PEG/4% P-glycol			6% PEG/4% DMSO		
		10 min	30 min	60 min	10 min	30 min	60 min	10 min	30 min	60 min
Control	Fertilization (%)	72	51	99	94	90	88	97	92	63
CG04M107	Motility (%)	10 ± 0	10 ± 0	18 ± 4	20 ± 0	20 ± 0	18 ± 11	10 ± 0	10 ± 0	15 ± 0
	Fertilization (%)	8	24	21	13	23	–	8	10	30
CG04M108	Motility (%)	5 ± 0	5 ± 0	5 ± 0	2 ± 0	2 ± 0	4 ± 2	1 ± 0	1 ± 0	1 ± 0
	Fertilization (%)	1	4	5	9	2	7	6	3	8
Average	Motility (%)	8 ± 3^{ab}	8 ± 3^{ab}	11 ± 8^{a}	11 ± 10^{a}	11 ± 10^{a}	11 ± 10^{a}	6 ± 5^{b}	6 ± 5^{b}	8 ± 8^{ab}
	Fertilization (%)	5 ± 5^{a}	14 ± 14^{a}	13 ± 11^{a}	11 ± 3^{a}	13 ± 15^{a}	–	7 ± 1^{a}	7 ± 5^{a}	19 ± 16^{a}
		6% MeOH			8% DMSO			8% PEG		
		10 min	30 min	60 min	10 min	30 min	60 min	10 min	30 min	60 min
Control	Fertilization (%)	97	96	96	98	98	99	99	97	99
CG04M109	Motility (%)	5 ± 0	8 ± 4	10 ± 0	6 ± 1	5 ± 4	10 ± 0	7 ± 0	9 ± 2	9 ± 2
	Fertilization (%)	7	17	48	4	5	13	0	2	2
CG04M110	Motility (%)	4 ± 2	5 ± 0	6 ± 1	5 ± 0	4 ± 2	5 ± 0	5 ± 0	5 ± 0	7 ± 0
	Fertilization (%)	0	1	1	1	3	3	0	0	1
Average	Motility (%)	4 ± 2^{bc}	6 ± 3^{a}	8 ± 2^{a}	6 ± 1^{ab}	4 ± 2^{b}	8 ± 3^{a}	6 ± 1^{ac}	7 ± 2^{a}	8 ± 2^{a}
	Fertilization (%)	4 ± 5^{a}	9 ± 11^{a}	25 ± 33^{a}	3 ± 2^{a}	4 ± 1^{a}	8 ± 7^{a}	0 ± 0^{b}	1 ± 1^{b}	2 ± 1^{a}

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

Table 7
Post-thaw motility (mean ± S.D.), and percent fertilization and hatch for sperm samples (from tetraploid Pacific oysters) suspended in 6% PEG/4% MeOH, 6% MeOH, 6% PEG/4% P-glycol, 8% P-glycol, 6% PEG/4% DMSO, 8% DMSO, and 8% PEG200, and 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[a]	Fertilization (%)	95	97	99	93	96	98	97
	Hatch (%)	65	55	72	72	73	75	66
CG04M89	Motility (%)	20 ± 0	15 ± 0	18 ± 4	9 ± 2	15 ± 0	10 ± 0	18 ± 4
	Fertilization (%)	3	6	1	2	1	3	0
CG04M90	Motility (%)	10 ± 0	5 ± 0	15 ± 0	5 ± 0	18 ± 4	4 ± 2	8 ± 4
	Fertilization (%)	21	2	3	9	2	5	3
CG04M91	Motility (%)	13 ± 4	8 ± 4	18 ± 4	10 ± 0	15 ± 0	6 ± 1	6 ± 0
	Fertilization (%)	7	15	18	–	8	15	7
Average	Motility (%)	14 ± 5^{ab}	9 ± 5^{bc}	17 ± 3^{a}	8 ± 2^{c}	16 ± 2^{a}	7 ± 3^{c}	10 ± 6^{bc}
	Fertilization (%)	10 ± 9^{a}	8 ± 7^{a}	7 ± 9^{a}	6 ± 5^{a}	4 ± 4^{a}	8 ± 6^{a}	3 ± 4^{a}

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

3.11. Experiment 10: evaluation of selected cryoprotectants on percent fertilization

For a cooling rate of 5 °C/min with the controlled-rate freezer (Table 7), the highest post-thaw motilities were obtained with samples suspended in 6% PEG/4% P-glycol (17 ± 3%) and 6% PEG/4% DMSO (16 ± 2%), which were higher than those of the other cryoprotectants, although there was variation ($P < 0.001$) among the three males used. The highest percentages of fertilizations were found in samples suspended in 6% PEG/4% MeOH (21%) with male CG04M90, followed by 6% PEG/4% P-glycol (18%) with male CG04M91. However, there was no difference in percent fertilization among all cryoprotectants ($P = 0.810$).

3.12. Experiment 11: 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, however, low values were obtained for post-thaw motility and fertilization (Table 8). There were treatment effects ($P < 0.001$) of different cryoprotectants for post-thaw motility (despite the low values), but no difference for percent fertilization ($P = 0.504$), perhaps due to differences between the two males ($P = 0.002$).

For Experiments 10 and 11, positive controls using fresh sperm without addition of cryoprotectant yielded 100% fertilization, and controls using fresh sperm equilibrated with the same cryoprotectants for the same equilibration time all yielded >90% fertilization and >55% hatch (Table 7), which were comparable with the fresh controls without addition of cryoprotectant (56% hatch), indicating cryoprotectants at these concentrations were not toxic to sperm and eggs. Fresh eggs without sperm addition yielded 0% fertilization, indicating that eggs were not contaminated.

4. Discussion

Similar to previous findings [12], initial motility for sperm of tetraploid oysters after shipment was low (64 ± 25%) compared with sperm samples from diploid oysters (82 ± 22%) collected and shipped at the same

Table 8
Post-thaw motility (mean ± S.D.), percent fertilization of sperm samples (from tetraploid Pacific oysters) suspended in 6% PEG/4% MeOH, 6% MeOH, 6% PEG/4% P-glycol, 8% P-glycol, and 6% PEG/4% DMSO, equilibrated for 15 min, cooled using a commercial freezing method developed for dairy bulls (males: CG04M93 and CG04M94)

Criterion	PEG/MeOH	MeOH	PEG/P-glycol	PEG/DMSO	PEG200
Motility (%)	6 ± 2^{a}	1 ± 1^{b}	2 ± 2^{b}	2 ± 0^{b}	2 ± 1^{b}
Fertilization (%)	1 ± 1^{a}	6 ± 4^{a}	2 ± 1^{a}	3 ± 4^{a}	3 ± 2^{a}

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

time (Table 9). For the limited number of fertilization trials tested, percent fertilization was low in general (the highest value was 48%). However, post-thaw motility was improved compared to earlier studies [12], in which motility rarely exceeded 10% after thawing (only two males were found to have the highest post-thaw motility of 15% among 31 males tested). In the present study, motility after thawing ranged from 5 to 50%, with an average of 20% for 29 males that yielded the highest post-thaw motility in various experiments. In light of the low initial motility, optimized protocols in the present study were found to be effective in retaining post-thaw motility for sperm from tetraploid oysters. The low percent fertilization could be due to the low initial sperm motility with sperm from tetraploids. Ultrastructural studies found differences between sperm from diploid and tetraploid Pacific oysters [15]. Instead of the four mitochondria always found in sperm from diploid oysters, 44% of sperm from tetraploid oysters had four mitochondria, 53% had five, and 3% had six. None had eight, which would be predicted on a simple doubling of sperm components based on the haploid–diploid comparison. This may partially explain the low initial motility associated with sperm from tetraploids.

Due to the larger linear dimensions (lengths and widths) of sperm from tetraploids compared to diploids [15], and the consequent smaller surface area-to-volume ratio in sperm from tetraploids that allows water or permeating cryoprotectants to move across the cell membrane more slowly, a slower optimal cooling rate could be predicted for sperm from tetraploids. However, neither theoretical prediction from a differential scanning calorimetric (DSC) analysis [23], nor empirical data in this study showed a difference in the optimal cooling rate for sperm from the two ploidies. In the first experiment, comparison of cooling rates of 0.5, 5, 16, 30, 45, and 50 °C/min indicated an optimal range of 0.5–30 °C/min; subsequent attempts with optimized cryoprotectants and concentrations showed that 5 °C/min was significantly better than other rates in retaining post-thaw motility, which agreed with the findings for sperm from diploid oysters (Table 9). It seems that size and volume differences between sperm from diploids and tetraploids were not large enough to cause differences in observed optimal cooling rates, or the range or precision of cooling rates tested were not sufficient for differentiation between the ploidy levels. Various optimal cooling rates have been reported in previous studies of sperm from diploid oysters [16]. However, as observed for sperm from diploid oysters [17], optimal cooling rates for oyster sperm depend on the interactions of factors such as the choice of cryoprotectant and concentration, extenders, equilibration time, cooling rate, and thawing method.

Table 9
Summary of the studies of sperm cryopreservation from diploid and tetraploid Pacific oysters

Parameter	Diploid[a]	Tetraploid[b]
Oyster received dates (year 2004)	June 4–July 7	June 10–July 7
Number of males used	27	29
Initial motility (%)		
Range	5–95	5–95
Average (mean ± S.D.)	82 ± 22	64 ± 25
Cooling rate (°C/min)		
Range tested	0.5, 5, 16, 30, 45, 50	0.5, 5, 16, 30, 45, 50
Optimal	5	5
Optimal cryoprotectants	6% MeOH; 2% PEG/4% MeOH	6% PEG/4% P-glycol; 6% PEG/4% DMSO
Equilibration time (min)		
Tested time	10, 20, 30, 60	10, 20, 30, 60
Optimal	30, 60	60
Straw size (0.25 mL vs. 0.5 mL)	No significant difference	No significant difference
Cooling methods (CRF[c] vs. CFM[d])	Better with CRF	No direct comparison
Highest post-thaw motility (%)	70	50
Highest percent fertilization	98	48
Highest percent hatch	67	28

[a] Dong et al. [12,15,17,20].
[b] Present study.
[c] Cooled at 5 °C/min using a controlled-rate freezer.
[d] Cooled using a commercial freezing method developed for dairy bulls.

Sperm from tetraploids were found to be more negatively affected by the cryopreservation process than were sperm from diploids [12], and a more vulnerable plasma membrane of sperm from tetraploids than that of diploids was suspected. Non-permeating cryoprotectants such as sugars, proteins, and polymers have been found to confer protection by permitting a reversible influx and efflux of solute during freezing and thawing, and thus help to stabilize the cell membrane [24]. In the present study, polyethylene glycol was used alone or in combination with the permeating cryoprotectants MeOH, P-glycol, and DMSO, and the combined cryoprotectants were found to be more effective in retaining post-thaw motility than were permeating compounds alone. The highest post-thaw motility (50%) was obtained with 2% PEG/6% P-glycol. However, 6% PEG/4% P-glycol and 6% PEG/4% DMSO yielded consistently high post-thaw motility in various trials. Similar to the findings with sperm from diploids, polyethylene glycol alone was not as effective as it was in combination with the permeating cryoprotectants MeOH, DMSO, and P-glycol when using the same methods (Table 9). In the future, fertilization trials with more males are required to confirm the effectiveness of 6% PEG/4% P-glycol and 6% PEG/4% DMSO in retaining fertility for thawed sperm samples.

Although sperm from a large oyster (e.g., with a shell height $\sim$ 100 mm) could produce more than two hundred 0.5-mL straws with a sperm concentration of 1×10^9 cells mL^{-1}, straws of small volume (e.g., 0.25 mL) may be necessary in some cases, especially for protocol optimization with multiple treatment factors for small oysters, for complicated breeding designs, or for oysters with poor gonadal development (such as tetraploids). *C. gigas* is a protandric species, in which at first maturation, oysters generally function as males and reverse sex as they grow. During the spawning season, females generally dominate a population with multiple-year classes and a higher percent of males is found in younger (smaller) animals, the sperm from which could produce fewer than ten 0.5-mL straws. By using 0.25-mL straws, the number of straws available for treatment factor evaluation could be doubled while maintaining the sperm concentrations suggested in previous studies [12]. Similar to findings for sperm from diploid oysters (Table 9), there was no significant post-thaw motility when sperm was stored in 0.25-mL straws versus 0.5-mL straws.

Long equilibration times of 30 and 60 min were found to yield higher post-thaw motility for sperm of diploid oysters (Table 9). The present study showed no difference in post-thaw motility for equilibration times of 10, 20, 30, and 60 min for sperm of tetraploids. However, higher percentages of fertilization were achieved with longer equilibration times. This confirmed that longer equilibration time could be beneficial when the cryoprotectant concentration is low ($<$10%). Controls with fresh sperm exposed to the same cryoprotectants, same concentrations, for the same period of time as that of thawed sperm but without freezing and thawing, had greater than 90% fertilization, which suggested that toxicity of those cryoprotectants and their concentrations used in this study was minimal. In practice, a wide range of equilibration times would allow sufficient time for manual straw filling. In addition, long equilibration times (30 min–6 h) with step-wise addition of cryoprotectants is a routine practice in freezing facilities for sperm of dairy bulls [25]. If the same method yielded satisfactory results with aquatic species, the use of specialized cryopreservation centers such as dairy facilities could assist the integration of cryopreservation of fish or shellfish sperm into hatchery operations. In oysters, future studies should evaluate such practices on percent fertilization as well as larval development.

Similar to sperm of diploid oysters, differences in post-thaw sperm quality (e.g., post-thaw motility, and percent fertilization) between individual males were evident in this study for sperm of tetraploid oysters (e.g., CG04M64 and CG04M65 in Table 3). Observations of male-to-male variation are not limited to sperm of Pacific oysters (this study and [17]) and eastern oysters *C. virginica* [26], but have been reported in other aquatic species such as zebrafish *Danio rerio* [27], rainbow trout *Oncorhynchus mykiss* [28], Atlantic halibut *Hippoglossus hippoglossus* [29], and sea urchin *Evechinus chloroticus* [30]. Furthermore, it is noteworthy that male-to-male variation is also well recognized in mammalian species such as dogs, bulls, boars, stallions, and humans [31,32]. Variation of freezing sensitivity among males observed in mammals has been hypothesized to be genetically determined, and was supported by a recent study on boar sperm cryopreservation using amplified restriction fragment length polymorphism (AFLP) technology [33]. Individual variations in fish sperm cryopreservation have been attributed to genetic variability, membrane quality, collection techniques (e.g., contamination with urine), and seasonality (for reviews, see [9,34,35]). As a result, pooling of milt from males has been practiced routinely in studies of fish semen cryopreservation to reduce individual variation [36]. Unlike the sperm collection techniques used in fish, which are usually done by

abdominal message, dry stripping of the oyster gonads used in this study can avoid contamination with other materials. Therefore, the male-to-male variation observed in sperm from diploid and tetraploid Pacific oysters could be genetically derived as well as environmentally influenced. In the near future, the use of molecular tools is necessary to address hypotheses that apply to oyster sperm or sperm from other aquatic species in general.

Compared to sperm from diploid oysters [17], fertilization with thawed sperm samples of tetraploids were generally low (<10%). This is in agreement with previous findings of sperm cryopreservation from diploid and tetraploid Pacific oysters [12]. It appears that sperm from tetraploids have a higher sensitivity to cryopreservation effects. In a sense, therefore, the response of sperm from diploid and tetraploid Pacific oyster is analogous to that of two species with different sensitivities to cryopreservation effects. According to the tolerance model proposed in our parallel studies with sperm of diploid oysters [17], sperm from tetraploids could be considered as having a narrow tolerance to the stresses of cryopreservation in comparison to sperm from diploids. Future efforts to minimize shipping effects, and continued optimization of current methods is necessary for further improvement of sperm cryopreservation in tetraploid oysters.

Sperm from different strains of mice have different sensitivity to freezing, and such differences were mainly attributable to their differential sensitivity to the osmotic shocks associated with the addition and removal of cryoprotectant [37]. Techniques involving gradual addition and removal of cryoprotectant in a series of decremental steps have been shown to increase the recovery of live spermatozoa in humans considerably by minimizing osmotic injury [38]. These observations suggested that the plasma membrane properties played an important role in post-thaw sperm quality. It is possible that the same principle also holds true with oyster sperm. If this is the case, the high sensitivity to cryopreservation observed with sperm from tetraploids in the present study may be reduced by applying serial addition and removal of cryoprotectants in the same fashion. In addition, given the doubled amount of DNA in sperm from tetraploid oysters compared to that of diploids, if the plasma membrane thickness or composition was not different between the ploidies, but cell size was, sperm from tetraploids could have a compromised membrane. This might explain why sperm from tetraploids were more negatively affected by cryopreservation than were sperm from diploids.

To obtain percent fertilization comparable to fresh sperm, a higher sperm-to-egg ratio is generally required for thawed sperm samples [39–41]. In diploid Pacific oysters, cryopreserved sperm are thought to be 30–100-fold less fertile than fresh sperm, and a sperm-to-egg ratio ranging from 1600 to 5000 was required to produce 50% fertilization for cryopreserved sperm [42]. In our project with sperm of diploid [17] and tetraploid oysters (present study), sperm suspensions were frozen at a concentration of 1×10^9 cells/mL for all treatments based on spectrophotometer readings [20]. For fertilization trials, ten 0.5-mL straws were used to fertilize 500,000 eggs. Assuming that all spermatozoa were capable of fertilization after thawing, the sperm-to-egg ratio would be 10,000:1. However, sperm cells were found to agglutinate after thawing, and the degree of agglutination corresponded to the number of free-swimming sperm available after thawing [16]. Thus, if only the number of motile sperm available for thawed samples were counted, based on the studies combining the agglutination level (0–5, corresponding to 30–100% sperm availability) [16] and the percentage of post-thaw motility for sperm from diploid oysters (2–70% with the average of 29%) [17], the actual sperm-to-egg ratio would be in a range of 60–7000 sperm/egg. Within this range, percentage fertilization as high as 98% were obtained with thawed samples in sperm from diploid oysters (Table 9). Considering the actual number of motile sperm available in thawed samples, cryopreserved sperm that survived the freezing and thawing processes may have fertility comparable to that of fresh samples. In terms of sperm from tetraploid oysters, if spermatozoa with different numbers of mitochondria respond to cryopreservation differently, a lower percentage of spermatozoa capable of fertilizing eggs may be expected for thawed samples compared with that of diploids. Thus, future fertilization trials should be tested with increased sperm-to-egg ratios for cryopreserved sperm from tetraploids.

In summary, compared to sperm from diploid Pacific oysters, sperm from tetraploids were more negatively affected by cryopreservation. Those combinations of non-permeating and permeating cryoprotectants were more effective in retaining post-thaw motility than were permeating compounds alone provided presumptive evidence for the interpretation that plasma membrane properties play an important role in post-thaw sperm quality. Thus, serial addition and removal of cryoprotectants may help to reduce osmotic shock and improve the post-thaw survival. A long equilibration time with step-wise addition of cryoprotectants at low concentrations might also help to reduce toxicity. Differences in

freezing sensitivity among males could be genetically determined. As stated above, future studies should also evaluate the shipping methods to minimize stress or other negative influences on sperm quality. Further optimization of the existing protocols, including sperm-to-egg ratio, is still required and may help to increase the percentage of fertilization.

Acknowledgements

This work was supported in part by funding from the USDA-SBIR program, 4Cs Breeding Technologies, Inc., and the Louisiana Sea Grant College Program. We thank J. Chenevert, S. Pelitz, C. Jeansonne, and B. Schexnayder of Genex Custom Collection, Inc., 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 consultation, and J. Jenkins, S. Leibo, R. Romaire, F. Sheldon, J. Supan for critical review. This manuscript was approved for publication by the Director of the Louisiana Agricultural Experiment Station as number 05-11-0575.

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