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# Commercial-scale sperm cryopreservation of diploid and tetraploid Pacific oysters, *Crassostrea gigas*☆

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**Abstract**

Cryopreservation of sperm from tetraploid organisms (the possession of four chromosome sets) is essentially unexplored. This is the first cryopreservation study to address sperm from tetraploid Pacific oysters, *Crassostrea gigas*, and addresses the commercial production of triploid oysters (three chromosome sets). Initial motility, refrigerated storage of undiluted sperm, osmolality of extender solutions, sperm concentrations, equilibration time, and cryoprotectants of propylene glycol and dimethyl sulfoxide were evaluated with sperm from diploid and tetraploid oysters. Unlike most teleost fishes, in which the duration of active motility is typically brief, the motility of sperm from oysters lasts for hours. The present study showed that responses to treatment effects by sperm from tetraploids were different from diploids. The majority of tetraploid experiments resulted in less than 10% motility after thawing and less than 5% fertilization. The highest fertilization obtained for thawed sperm was 96% for sperm from diploid oysters and 28% for sperm from tetraploid oysters. Differential responses to treatments by sperm from tetraploid and diploid oysters may be due to differences in gonadal development. However, the use of cryopreserved sperm from tetraploid Pacific oysters produced 100% triploid offspring by fertilization of eggs from diploid females as determined by flow cytometry of larvae. This study demonstrates that sperm from tetraploid oysters can be collected, frozen, and stored for production of triploid offspring.


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

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Polyploidy (the possession of more than the normal two chromosome sets) has played an important role in genomic evolution, especially in plants [6,55]. Although it is relatively rare in animals and is lethal in mammals, polyploidy reports include the occurrence of triploidy (three chromosome sets) in domestic chickens, *Gallus gallus domesticus* [43] and western cottonmouth snake, *Agkistrodon piscivorus leucostoma* [62]. In lower vertebrates such as fish, polyploidy occurs naturally and is not lethal. For example, spontaneous triploidy was found in a cyprinid fish [24], and within the salmonids (a group characterized by ancestral tetraploidization) adult triploids of rainbow trout, *Oncorhynchus mykiss*, have been reported [17,61]. Polyploidization events characterize evolution in sturgeon, for example, the genus *Acipenser*, where species with ploidy levels of tetraploid, octoploid, dodecaploid, and hexaploid have been reported [12,72]. Although most marine bivalves are sexual diploids without evidence of recent polyploidization, they have been found to tolerate polyploidy well [8].

The induction of triploidy in bivalves is useful for aquaculture because of reduced gamete output (functional reproductive sterility) and improved meat quality and growth [4,66]. Triploidy is usually induced by carefully timed application of chemical treatments [5], physical stress such as heat shock [47,68,69], or hydrostatic pressure [3,14,51] to prevent release of the second polar body in fertilized eggs. Unfortunately, none of these methods of induction are completely reliable and the chemical methods are costly and potentially dangerous to the operator [27,41,42]. Neither physical nor chemical methods can guarantee 100% triploidy, and often high mortality is associated with the treatment stress. However, the crossing of gametes from tetraploids (four chromosome sets) and normal diploids offers reliable triploidy production and high survival rates [21,22,28,29,67].

The induction of tetraploidy in oysters was first reported 10 years ago [27]. Tetraploid oysters create opportunities for genetic improvement including the direct production of triploid (sterile) seedstocks by crossing with diploids as described above. Refrigerated and frozen storage of tetraploid oyster sperm will be a critical tool for commercial-scale application of tetraploid stocks, and for developing tetraploid breeding programs. Currently, cryopreservation of sperm from diploid oysters is limited to experimental stages (e.g. [13,45,54,63,65]), while cryopreservation of sperm from tetraploid oysters is unexplored. Development of protocols for cryopreservation of sperm from tetraploid Pacific oysters, *Crassostrea gigas*, will provide a model for cryopreservation of sperm from other tetraploid organisms. And comparisons between the sperm from diploid and tetraploid oysters could enhance our understanding of the principles of sperm cryobiology.

Our goal was to develop protocols for commercial-scale cryopreservation of sperm from diploid and tetraploid Pacific oysters. The specific objectives of this study were to: (1) evaluate refrigerated storage of undiluted sperm; (2) identify the appropriate osmolality of extender solutions; (3) identify optimal sperm concentrations; (4) evaluate equilibration time with cryoprotectants; and (5) identify effective cryoprotectant and concentration combinations.

## Materials and methods

### *Broodstock source and certification*

Tetraploid Pacific oysters (~3 years old) were collected from Totten Inlet (Puget Sound, 47° 09′ 017″ N, 122° 57′ 908″ W) and diploids (~2 years old) were collected from Willapa Bay (46° 29′ 885″ N, 124° 01′ 810″ W) in Washington State. They were shipped chilled by overnight delivery to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS) from Whiskey Creek Shellfish Hatchery (WCSH) in Tillamook, Oregon during April and August of 2002.

Tetraploid males were obtained from a group of putative tetraploids. Because the chemical process of producing tetraploids is imperfect, diploid and triploid oysters can be present among the tetraploids. To verify tetraploidy, we screened oysters individually by flow cytometry [1]. A non-destructive sampling method was employed, drilling the top shell and using a 26-G needle to biopsy a gonad sample (<10 μm). The biopsied oysters were given an individual temporary label using marked

rubber bands wrapped around the shell. Sex was identified by microscopic examination of the biopsy samples and when males were found, the sperm were placed in staining solution containing 10 μg/ml of 4′,6-diamidino-2-phenylindole and 10% dimethyl sulfoxide (DAPI/DMSO) (146 mM NaCl, 10 mM Tris, adjusted to pH 7.4, 2 mM $CaCl_2$, 22 mM $MgCl_2$, 0.05 g BSA, and 0.1% Triton X-100) in microcentrifuge tubes and held on ice. Samples were frozen at −80 °C for at least 30 min to assure liberation of sperm nuclei [1] and were thawed and kept on ice until analysis. For each sample, a cell suspension was prepared by aspirating the biopsies with a syringe through a 26-G needle. The cell suspensions were screened on 25-μm mesh to remove cellular debris and 10,000 cells were analyzed for each sample by use of a Partec CA-II flow cytometer and sperm of normal diploids used as an external reference. The proportion of cells in each ploidy class was calculated as a percentage of the total number of cells analyzed per sample after curve fitting with Modfit (Verity Software House, Topsham, ME, USA) [2].

*Sperm collection and motility estimation*

Upon arrival of the oysters, sperm were collected by dry stripping of the gonad [2] and were placed in 1.5-mL centrifuge tubes and weighed. Calcium-free Hanks' balanced salt solution (C-F HBSS) was added based on the gonad tissue weight to obtain the desired concentration and the sperm suspensions were filtered through 40-μm nylon mesh before use. The concentrations of sperm suspensions were adjusted using readings at 581 nm from a spectrophotometer (Genesys 20, Thermo Spectronic, Rochester, New York) and standard curves [19]. All chemicals used for preparation of solutions (diluted v/v) were of reagent grade (Sigma Chemical, St. Louis, Missouri).

Sperm motility was estimated at 200× magnification using darkfield microscopy (Optiphot 2, Nikon, Garden City, New York) and was expressed as the percentage of cells actively moving in a forward direction. Oyster sperm retain continuously motile after activation for hours to days, facilitating motility estimates. Initial motility was determined by equilibrating undiluted non-motile sperm in 30 μl of C-F HBSS at 1000 mOsmol/kg at 23 °C for 2 min [18]. Osmolality was measured with a vapor pressure osmometer (model 5500, Wescor, Logan, Utah). Motilities of sperm after suspension, before and after adding cryoprotectant (before freezing), and after thawing were evaluated by equilibrating 2 μl of sperm suspension in 30 μl of C-F HBSS at 1000 mOsmol/kg at 23 °C for 2 min.

*Freezing procedures*

To meet our goal of developing protocols for commercial-scale cryopreservation, existing commercial freezing methods developed for dairy bulls were evaluated in this study. Sperm samples were cryopreserved at the Louisiana State University T.E. Patrick Dairy Improvement Center in Baton Rouge. Sperm samples were mixed with the appropriate cryoprotectant and allowed 25–30 min to equilibrate in a walk-in cooler held at 5 °C before freezing, except for the experiment addressing equilibration time. The sperm solutions were placed into pre-labelled 0.5-mL French cryopreservation straws using an automated straw filler (model MRS 1, IMV Int., Minneapolis, Minnesota). 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 [15]. Once frozen, the samples were removed and placed under liquid nitrogen for sorting and preparation for long-term storage. After 2 days, two straws from each male were thawed at 40 °C water bath for 7 s to estimate the post-thaw motility. Ten straws of each treatment were shipped in a shipping dewar (CP35, Taylor-Wharton, Theodore, Alabama) to the WCSH for fertilization trials within 2 months after sample collection. The appearance of sperm samples after thawing was documented before use for fertilization.

*Fertilization and larval evaluation*

Diploid females were used for fertilization trials. Eggs from individual females were obtained by dissection, sieved, washed on 25-μm mesh, and suspended in filtered seawater at 25 °C. Fecundity of each female was determined by direct count using a Sedgwick–Rafter chamber. After counting, the eggs were held in seawater at 25 °C for at least 30 min to observe germinal vesicle breakdown at 100× magnification using microscopy. Unfertilized eggs (fresh) were pooled 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 2000–12,000 eggs (fresh) held in 200 ml of seawater. The gametes were incubated at 25 °C and percent fertilization was calculated by counting developing embryos at 2 h after insemination. Percent hatching was calculated by counting normal straight-hinge larvae at 24 h after fertilization. For the negative control, eggs were monitored after treatment as described above but without addition of sperm.

For the evaluation of fresh (non-frozen) sperm from tetraploid control males, the above techniques were used for egg collection and the sperm were washed through a 70-μm mesh and added to fresh eggs to obtain about 20 spermatozoa per egg. 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 separated in different containers to avoid unintended fertilization.

Approximately 10,000 24- to 48-hour-old larvae were sampled from each culture for flow cytometric analysis to determine the ploidy of the larvae produced. In preparation for flow cytometry, larvae were concentrated into a 1-ml suspension and decanted. Supernatant seawater was withdrawn and 0.5 ml of DAPI/DMSO stain solution (described above) were added. Larvae were resuspended by vortex and were disaggregated by repeated aspiration with a 1-ml syringe fitted with a 26-G needle. Samples were analyzed by flow cytometry (described above) and triploid larvae had approximately three times the cellular DNA content of sperm (Fig. 1).

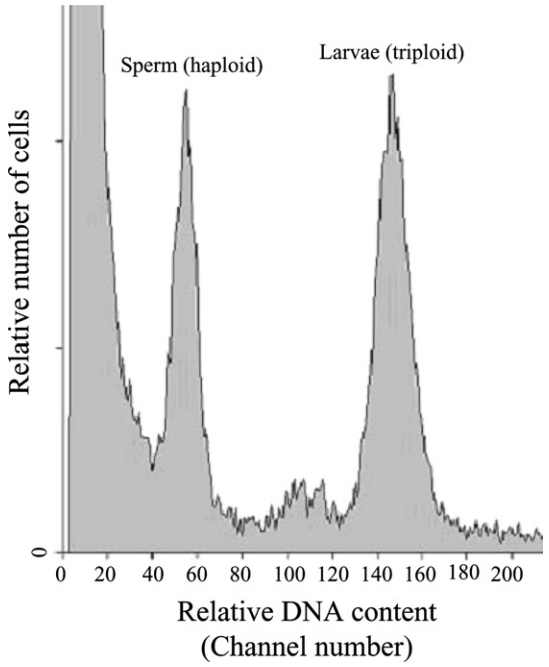


Fig. 1. Representative nuclear DNA fluorescence distribution from flow cytometric analysis of sperm from a diploid Pacific oyster (channel 50), and cells from a triploid larvae (channel number 150). Channel number is directly proportional to cellular DNA content.

*Refrigerated storage of undiluted sperm*

The initial motility of sperm from diploid ($n = 53$) and tetraploid ($n = 53$) Pacific oysters was estimated. Undiluted sperm from diploid ($n = 15$) and tetraploid ($n = 18$) Pacific oysters were stored in a refrigerator at 4 °C for 7 days. Motility was checked every 24 h and samples were allowed to warm to room temperature before assessment. Shipments of diploid and tetraploid oysters were received on July 3, July 10, July 16, and July 23, 2002.

*Effect of osmolality of C-F HBSS*

Sperm from diploid ($n = 4$) and tetraploid ($n = 4$) Pacific oysters were suspended in C-F HBSS at 1000 and 671 mOsmol/kg. Ten percent of propylene glycol (PG) was used as the cryoprotectant and samples were equilibrated for 30 min before freezing. Initial motility and motilities

before and after adding cryoprotectant, and after thawing were estimated, as well as percent fertilization. Diploid and tetraploid oysters were received on July 3 and July 10, 2002.

*Effect of sperm concentration with PG as the cryoprotectant*

Sperm from diploid ($n = 4$) and tetraploid ($n = 4$) Pacific oysters were suspended in C-F HBSS at 1000 mOsmol/kg and were adjusted to provide final freezing concentrations of $1 \times 10^6$, $1 \times 10^7$, $1 \times 10^8$ and $1 \times 10^9$ cells/ml. Samples were mixed to provide the final concentration of cryoprotectant (10% PG) and were equilibrated for 30 min before freezing. Motility after thawing and fertilization were estimated. Diploid and tetraploid oysters were received on June 19, 2002.

*Effect of equilibration time*

Sperm from diploid ($n = 3$) and tetraploid ($n = 3$) Pacific oysters were suspended in C-F HBSS at 1000 mOsmol/kg and were adjusted to $2 \times 10^9$ cells/ml. Sperm suspensions were mixed with 20% PG to obtain the final concentration of $1 \times 10^9$ cells/ml and 10% PG. Each sperm suspension was divided into two equal subsamples and one set of subsamples was equilibrated for 10 min and the other was equilibrated for 20 min before freezing. Initial motility and motilities before and after adding cryoprotectant, and after thawing were estimated, as well as percent fertilization. Diploid and tetraploid oysters were received on July 16, 2002.

*Effect of PG concentration*

Sperm from diploid ($n = 3$) and tetraploid ($n = 3$) Pacific oysters were suspended in C-F HBSS at 1000 mOsmol/kg and were adjusted to $2 \times 10^9$ cells/ml. Each sperm suspension was divided into three subsamples, which were mixed with PG at 10, 20, and 30% to obtain the final concentrations of $1 \times 10^9$ cells/ml and 5, 10, and 15% PG. Samples were equilibrated for 25 min before freezing. Initial motility and motilities before and after adding cryoprotectant, and after thawing were estimated, as well as percent fertilization. Diploid and tetraploid oysters were received on July 23, 2002.

*Effect of cryoprotectant*

Sperm from diploid ($n = 7$) and tetraploid ($n = 9$) Pacific oysters were suspended in C-F HBSS at 1000 mOsmol/kg and were adjusted to $2 \times 10^9$ cells/ml. Each sperm suspension was divided into two equal subsamples and one set of subsamples was mixed with 16% DMSO and the other was mixed with 20% PG to obtain final concentrations of $1 \times 10^9$ cells/ml and 8% DMSO and 10% PG. Samples were equilibrated for 30 min before freezing. Initial motility and motilities before and after adding cryoprotectant, and after thawing were estimated, as well as percent fertilization. Diploid and tetraploid oysters were received on July 10 and July 30, 2002.

*Effect of sperm concentration with DMSO as the cryoprotectant*

Sperm from diploid ($n = 8$) and tetraploid ($n = 8$) Pacific oysters were suspended in C-F HBSS at 1000 mOsmol/kg and were adjusted to provide the final freezing concentrations of $1 \times 10^6$, $1 \times 10^7$, $1 \times 10^8$, and $1 \times 10^9$ cells/ml. They were mixed to provide the final concentration of cryoprotectant (8% DMSO). Samples were equilibrated for 30 min before freezing. Initial motility and motilities before and after adding cryoprotectant, and after thawing were estimated, as well as percent fertilization. Diploid and tetraploid oysters were received on August 13, 2002.

*Data analysis*

Data were analyzed using the independent-sample $t$ test (comparisons between two means), one-way or two-way (fixed model) analysis of variance (ANOVA). When significance ($\alpha = 0.05$) was observed among treatments, Tukey's honestly significant difference procedure was used for pairwise comparisons. Results were presented as means $\pm$ SD, and probability values of $P < 0.05$ were considered to be significant. Data for sperm

motility and percent of fertilization and hatch were arcsine transformed prior to analysis.

## Results

### *Gonad morphology and initial sperm motility after shipment*

A total of 106 Pacific oysters were transported in 13 shipments from April 23 to August 20, 2002. Ripe gonads from diploid oysters were characterized by the presence of prominent genital canals and a creamy white appearance (Figs. 2A1, 2A2 and 2B). Although gonads from the tetraploid oysters used in this study were larger due to older age, they lacked the prominent genital canals and were more firm and dense (Figs. 2A3 and 2C). Sperm collected from tetraploid oysters had lower ($P = 0.006$) initial motility ($45 \pm 18\%$) than that of diploid oysters ($57 \pm 27\%$). The highest motility observed for sperm from tetraploid oysters was 80%, while greater than 95% motility was observed for sperm from diploid oysters (Fig. 3).

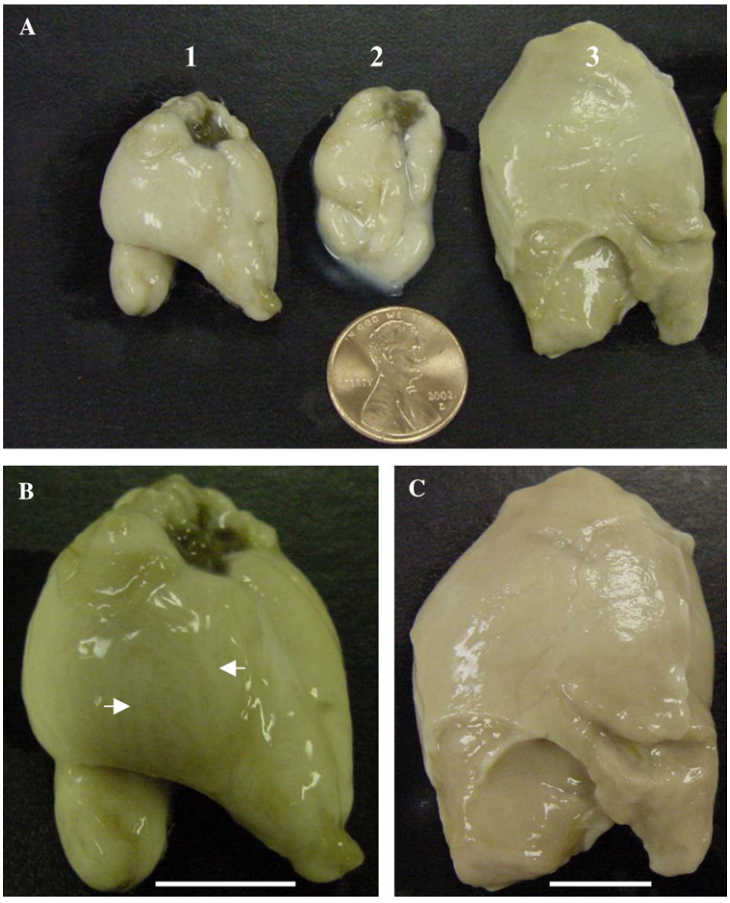


Fig. 2. Photographs of gonads from diploid oysters (A1: shell height, 72.2 mm; length, 46.5 mm; and wet weight, 54.6 g; A2: shell height, 78.2 mm; length, 39.8 mm; and wet weight, 34.7 g) and tetraploid oysters (A3: shell height, 132.0 mm; length, 75.0 mm; and wet weight, 209.6 g); A1 was enlarged to show the appearance of prominent genital canals (arrows in B); A3 was enlarged to show the firm and dense appearance of the gonad from tetraploids (C). The diameter of a US one-cent coin is 19.05 mm. The scale bars in (B and C) are each 1 cm.

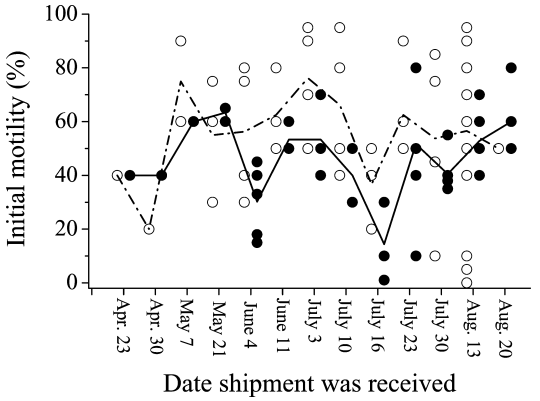


Fig. 3. The motility of sperm from diploid (open circles; dotted line) ($n_d = 53$) and tetraploid (filled circles; solid line) ($n_t = 53$) oysters upon arrival at ARS after shipment from Whiskey Creek Shellfish Hatchery (WCSH) in Tillamook, Oregon in 2002 on April 23 ($n_d = 3$, $n_t = 2$), April 30 ($n_d = 1$, $n_t = 3$), May 7 ($n_d = 2$, $n_t = 2$), May 21 ($n_d = 3$, $n_t = 3$), June 4 ($n_d = 4$, $n_t = 5$), June 11 ($n_d = 4$, $n_t = 3$), July 3 ($n_d = 4$, $n_t = 3$), July 10 ($n_d = 4$, $n_t = 4$), July 16 ($n_d = 3$, $n_t = 5$), July 23 ($n_d = 4$, $n_t = 6$), July 30 ($n_d = 4$, $n_t = 6$), August 13 ($n_d = 16$, $n_t = 7$), and August 20 ($n_d = 1$, $n_t = 4$). The points connected with lines represent the mean values for each shipment.

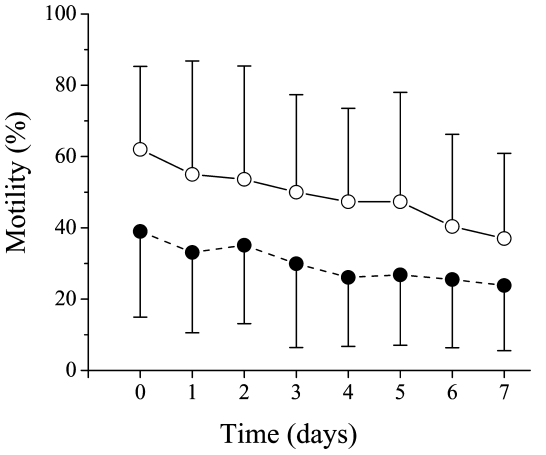


Fig. 4. Motility (mean ± SD) of refrigerated storage of undiluted sperm from diploid (open circles) and tetraploid (filled circles) Pacific oysters over 7 days. Diploid ($n = 15$) and tetraploid ($n = 18$) oysters were received on July 3, July 10, July 16, and July 23 of 2002.

### *Refrigerated storage of undiluted sperm*

Refrigerated storage of undiluted sperm did not reveal a significant decrease in motility over 7 days for sperm from diploid oysters ($P = 0.281$) and tetraploid oysters ($P = 0.337$) (Fig. 4). But motility decreased from 62 ± 23% at the beginning to 37 ± 24% at day 7 for sperm from diploid oysters, and from 39 ± 24% to 24 ± 18% at day 7 for sperm from tetraploid oysters. Standard deviations as large as 32% were observed among the samples.

### *Fertilization trials with fresh sperm*

For fertilization trials, treatments using fresh sperm generally showed fertilization above 91% except for the last trial (October 7), in which fertilization for sperm from diploid oysters was 74% and for tetraploid oysters was 72% (Fig. 5). The percent of hatch showed a range of 52–70% for diploid oysters, and a range of 48–66% for tetraploid oysters (Fig. 5). This indicated that high quality eggs from diploid oysters were used for crosses with cryopreserved sperm and that egg quality was consistent from batch to batch. No significant difference in fertilization rate ($P = 0.382$) or hatching rate ($P = 0.288$) was found between the sperm from diploid and tetraploid oysters, which also indicated that the quality of eggs within batches was consistent.

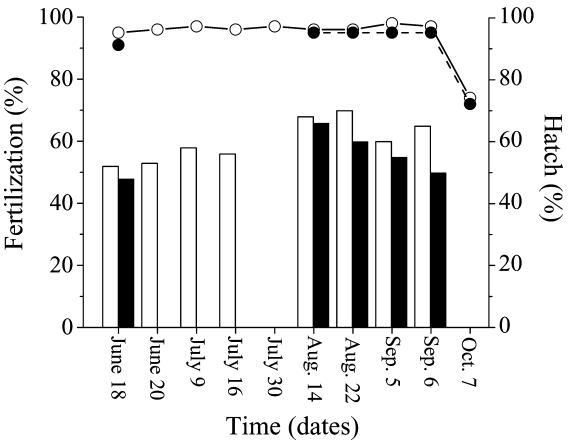


Fig. 5. Percent fertilization (line) and hatch (histogram) for fertilization trials with fresh sperm of diploid (open symbols) and tetraploid (filled symbols) Pacific oysters from June 18 to October 7 of 2002. The points and bars each represent individual samples.

*Effect of osmolality of C-F HBSS*

Comparisons of C-F HBSS osmolalities showed that sperm had higher motility and fertilization when suspended in 1000 mOsmol/kg than in 671 mOsmol/kg (Fig. 6). Significant differences were found between C-F HBSS at 1000 and 671 mOsmol/kg for motility after addition of cryoprotectant (before freezing) ($P = 0.029$), motility after thawing ($P = 0.033$) and for percent fertilization ($P = 0.034$). There was no significant difference between sperm from diploid and tetraploid oysters for motility after thawing ($P = 0.164$) and percent fertilization ($P = 0.120$), although differences were found for the initial motility ($P = 0.013$) and motilities before ($P = 0.015$) and after addition of cryoprotecant ($P = 0.014$). For sperm from diploid oysters (Fig. 6), the motility after addition of cryoprotectant, motility after thawing and percent fertilization were significantly higher in C-F HBSS at 1000 mOsmol/kg than at 671 mOsmol/kg ($P < 0.050$). For sperm from tetraploid oysters (Fig. 6), no significant difference was found between the two osmolalities for all motility evaluations and fertilization. For all treatments, motility of sperm from diploid and tetraploid oysters decreased with successive steps in the cryopreservation procedures, but the largest single decrease occurred after addition of the cryoprotectant.

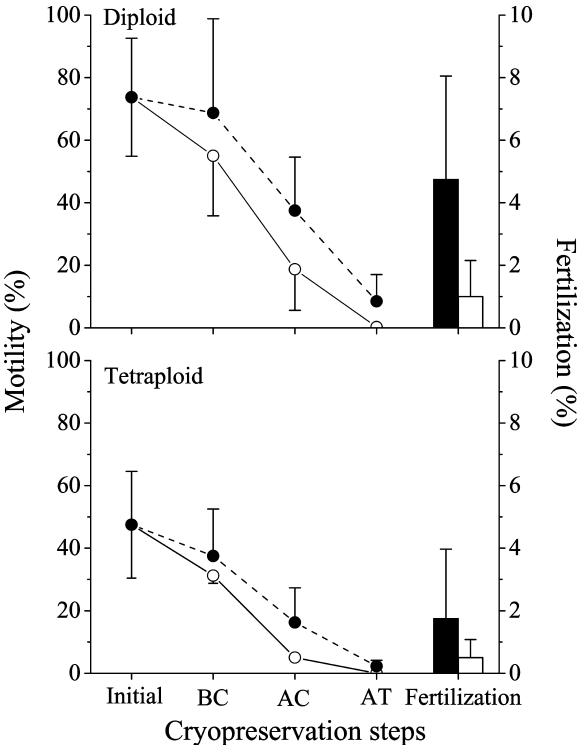


Fig. 6. Cryopreservation of sperm, from diploid ($n = 4$) and tetraploid ($n = 4$) Pacific oysters, suspended in calcium-free Hanks' balanced salt solution (C-F HBSS) at 671 (open symbols) and 1000 mOsmol/kg (filled symbols). Ten percent of propylene glycol was used as the cryoprotectant and samples were frozen using commercial dairy methods after 30 min equilibration. Initial motility, motilities before (BC) and after (AC) adding cryoprotectant, motility after thawing (AT) were estimated, as well as percent fertilization. Error bars indicate standard deviation.

*Effect of sperm concentration with PG as the cryoprotectant*

Sperm from diploid oysters at $1 \times 10^9$ cells/ml with 10% PG yielded the highest post-thaw motility (20%) and fertilization rate (37%) among sperm frozen at different cell concentrations (Fig. 7). For diploid oysters, motility after thawing at a sperm concentration of $1 \times 10^9$ cells/ml was $11 \pm 11\%$, and for $1 \times 10^8$ cells/ml was $8 \pm 8\%$. No motile sperm were found at concentrations of $1 \times 10^6$ and $1 \times 10^7$ cells/ml, but a low percentage of fertilization (2–3%) was obtained. Percent fertilization of $23 \pm 13\%$ was observed with a cell concentration of $1 \times 10^9$ cells/ml, and no significant difference in percent fertilization was found among $1 \times 10^8$, $1 \times 10^7$, and $1 \times 10^6$ cells/ml. Sperm agglutination was observed at a cell concentration of $1 \times 10^9$ cells/ml after thawing. Thawed sperm samples yielding the highest fertilization (37%) at this concentration typically showed the formation of elongated noodle-like sperm aggregates. However, samples at the other cell concentrations were homogenous suspensions after thawing. For sperm from tetraploid oysters (Fig. 7), low motility after thawing (<10%) and low percent fertilization (<3%) were observed and no significant difference was found in these parameters among the four concentrations ($P > 0.050$). There was no significant difference ($P = 0.422$) between sperm from diploid and tetraploid oysters for motility after thawing.

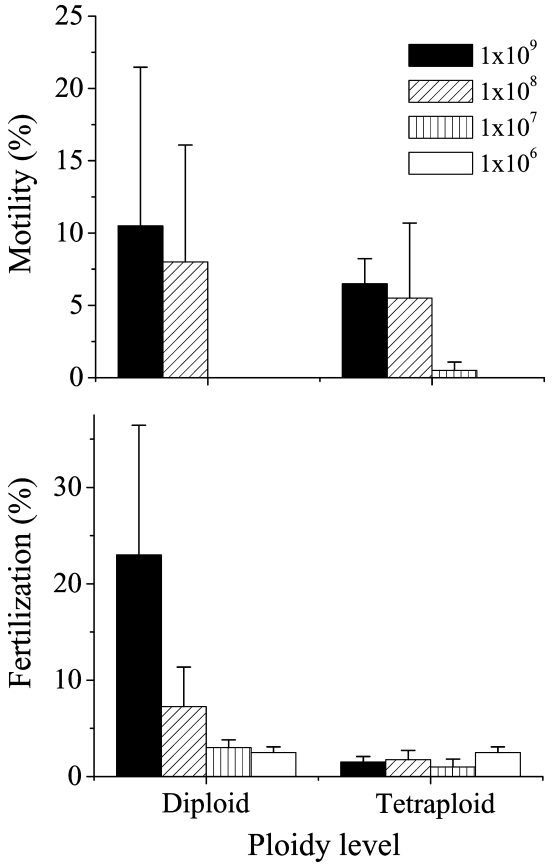


Fig. 7. Cryopreservation of sperm from diploid ($n = 4$) and tetraploid ($n = 4$) Pacific oysters with concentrations of $1 \times 10^6$, $1 \times 10^7$, $1 \times 10^8$, and $1 \times 10^9$ cells/ml using 10% propylene glycol. Sperm were suspended in C-F HBSS at 1000 mOsmol/kg and samples were frozen using commercial dairy methods after 30 min equilibration. Motility after thawing and percent fertilization were estimated. Error bars indicate standard deviation.

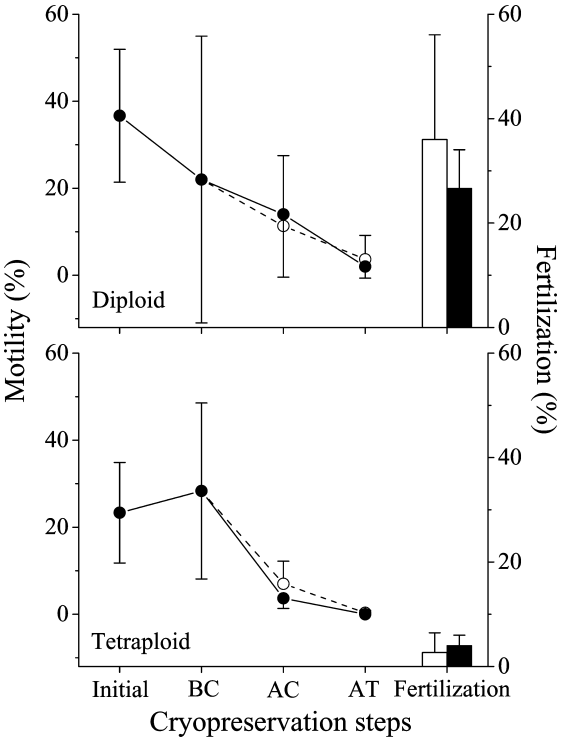


Fig. 8. Cryopreservation of sperm from diploid ($n = 3$) and tetraploid ($n = 3$) Pacific oysters with equilibration time at 10 (open symbols) and 20 min (filled symbols) using 10% propylene glycol. Sperm were suspended in C-F HBSS at 1000 mOsmol/kg. Initial motility, motilities before (BC) and after (AC) adding cryoprotectant, motility after thawing (AT) were estimated, as well as percent fertilization. Error bars indicate standard deviation.

*Effect of equilibration time*

No significant difference in motility or percent fertilization ($P > 0.050$) was found between equilibration times of 10 and 20 min for sperm from diploid oysters (Fig. 8). The highest motility after thawing (10%) and percent fertilization (50%) were found with 10-min equilibration, in which thawed sperm were found to agglutinate and form elongated noodle-like masses. For diploid oysters (Fig. 8), motility after thawing was $4 \pm 6\%$ for a 10-min equilibration and was $2 \pm 3\%$ for a 20-min equilibration. No significant difference was found for percent fertilization, in which equilibration for 10 min yielded $36 \pm 20\%$ and 20 min yielded $27 \pm 7\%$. For tetraploid oysters (Fig. 8), a large decrease of motility occurred after addition of cryoprotectant, and motility after thawing was less than 1% for both equilibration times. Fertilizations of <5% were obtained at each equilibration time. No significant difference was found between sperm from diploid and tetraploid oysters for initial motility, motilities before and after addition of cryoprotectant, and after thawing. But a significant difference was found for percent fertilization, in which sperm from diploid oysters were on average 33% higher than sperm from tetraploid oysters for 10-min equilibration and 23% higher for 20-min equilibration.

*Effect of PG concentration*

The highest post-thaw motility (15%) was found with 5% PG for sperm of tetraploid oysters, and the highest fertilization rate (17%) was found with 5% PG for sperm of diploid oysters (Fig. 9). No significant difference was observed among the three PG concentrations or between sperm from diploid and tetraploid oysters for motility after thawing ($P > 0.05$). However, the highest motility after addition of cryoprotectant before freezing was at 5% PG, followed by 10% PG, with 15% PG as the lowest. For diploid oysters (Fig. 9), fertilization with 5% PG ($9 \pm 7\%$) was significantly higher than those at 10% PG ($1 \pm 1\%$) and 15% PG ($2 \pm 2\%$). Motility decreased with each step in the cryopreservation procedure and the largest single decrease occurred after sperm suspension in C-F HBSS. For tetraploid oysters (Fig. 9), fertilization with 5% PG ($5 \pm 5\%$) was higher than those at 10% PG ($4 \pm 3\%$) and 15% PG ($1 \pm 0\%$), but they were not significantly different from each other, nor did they differ from those of sperm from diploid oysters. The largest decreases in motility occurred after addition of cryoprotectant and after the freezing and thawing phases.

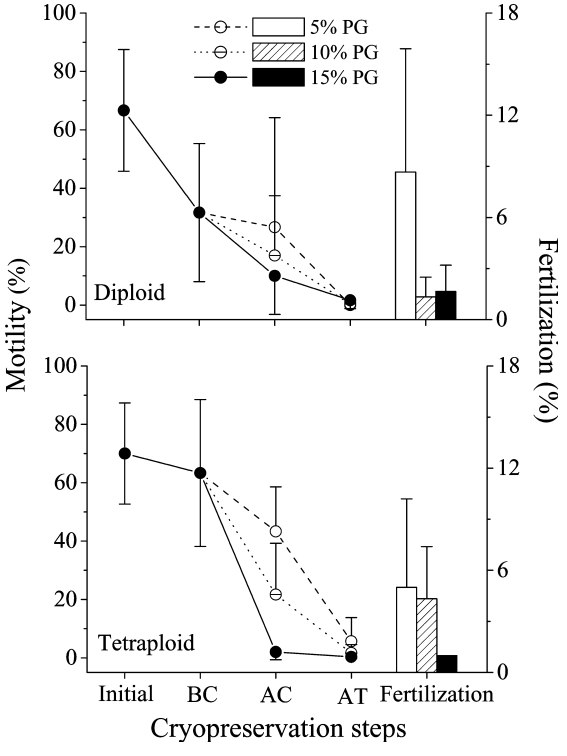


Fig. 9. Cryopreservation of sperm from diploid ($n = 3$) and tetraploid ($n = 3$) Pacific oysters with propylene glycol (PG) at 5, 10, and 15%. Samples were frozen using commercial dairy methods with 25 min equilibration. Initial motility, motilities before (BC) and after (AC) adding cryoprotectant, motility after thawing (AT) were estimated, as well as percent fertilization. Error bars indicate standard deviation.

*Effect of cryoprotectant*

The highest fertilization rates for sperm from diploid (26%) and tetraploid (22%) oysters were found with 8% DMSO (Fig. 10). Motilities after adding cryoprotectant ($P = 0.022$) and after thawing ($P = 0.004$) were significantly higher with 8% DMSO than with 10% PG. For diploid oysters (Fig. 10), a significant difference in percent fertilization was found between 8% DMSO ($15 \pm 5\%$) and 10% PG ($5 \pm 3\%$). For tetraploid oysters (Fig. 10), the percent fertilization with 8% DMSO ($3 \pm 2\%$) and 10% PG ($6 \pm 7\%$) were not significantly different from each other.

*Effect of sperm concentration with DMSO as the cryoprotectant*

For sperm frozen at different cell concentrations using 8% DMSO, the highest motility after thawing (30%) and percent fertilization (96%) were obtained with $1 \times 10^9$ cells/ml for diploid oysters (Fig. 11). A significant difference was found among the four sperm concentrations for motility before adding cryoprotectant ($P < 0.001$) and after thawing ($P < 0.001$). There was no significant difference between $1 \times 10^8$ and $1 \times 10^7$ cells/ml but significant differences ($P < 0.050$) were found among the other cell concentrations for the motility before addition of cryoprotectant. Sperm frozen at $1 \times 10^9$ cells/ml had significantly higher motility after thawing than did the other concentrations. There was a significant difference between sperm from diploid and tetraploid oysters for the initial motility ($P = 0.0007$) and motility before adding cryoprotectant ($P = 0.003$), but no significant difference was found for motility after thawing

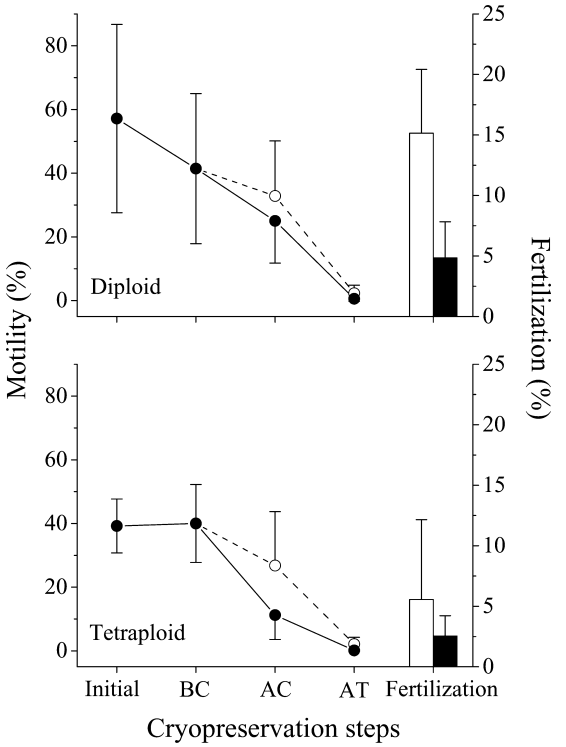


Fig. 10. Cryopreservation of sperm from diploid ($n = 7$) and tetraploid ($n = 9$) Pacific oysters with 8% dimethyl sulfoxide (open symbols) and 10% propylene glycol (filled symbols) as the cryoprotectants. Samples were frozen using commercial dairy methods with 30 min equilibration. Initial motility, motilities before (BC) and after (AC) adding cryoprotectant, motility after thawing (AT) were estimated, as well as percent fertilization. Error bars indicate standard deviation.

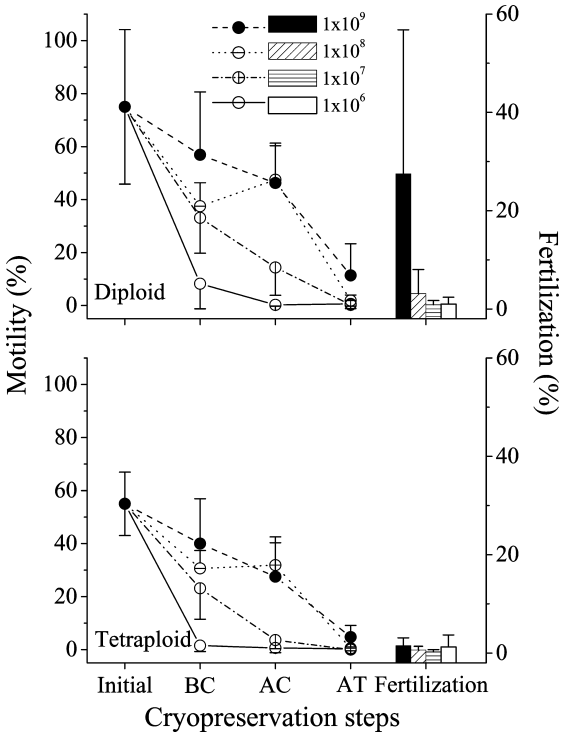


Fig. 11. Cryopreservation of sperm from diploid ($n = 8$) and tetraploid ($n = 8$) Pacific oysters with concentrations of $1 \times 10^6$, $1 \times 10^7$, $1 \times 10^8$, and $1 \times 10^9$ cells/ml using 8% dimethyl sulfoxide as the cryoprotectant. Sperm were suspended in C-F HBSS at 1000 mOsmol/kg and samples were frozen using commercial dairy methods after 30 min equilibration. Initial motility, motilities before (BC) and after (AC) adding cryoprotectant, motility after thawing (AT) were estimated, as well as percent fertilization. Error bars indicate standard deviation.

($P = 0.067$). For diploid oysters (Fig. 11), the motility before adding cryoprotectant, motility after thawing, and fertilization were significantly higher with the concentration at $1 \times 10^9$ cells/ml than with other concentrations ($P < 0.05$). For tetraploid oysters (Fig. 11), motility before and after adding cryoprotectant was significantly higher with the freezing concentration at $1 \times 10^9$ cells/ml than with $1 \times 10^6$ or $1 \times 10^7$ cells/ml, but no significant difference was found among them in fertilization. Motility of sperm from diploid and tetraploid oysters decreased with each step in the cryopreservation procedure, but the largest decrease occurred after freezing and thawing for concentrations at $1 \times 10^8$ and $1 \times 10^9$ cells/ml, and occurred after suspension in C-F HBSS for concentrations at $1 \times 10^6$ and $1 \times 10^7$ cells/ml.

## Discussion

Genetically, tetraploid and diploid oysters can be produced from the same parents, although morphologically their gametes are different. The present study showed that responses to treatment effects by sperm from tetraploids were different from those of diploids. The majority of tetraploid experiments resulted in less than 10% motility after thawing and less than 5% fertilization. Differential responses to cryopreservation by sperm from

tetraploid and diploid oysters may be due to the differences in gonadal development. Ripe oyster gonads were usually characterized by the presence of prominent genital canals and a creamy white appearance [58]. However, the gonads from tetraploid oysters usually lacked the prominent genital canals and were more firm and dense. The lower initial motility of sperm from tetraploid oysters compared to diploids found in this study also suggests differences in spermatogenesis. Unfortunately, the study of spermatogenesis in tetraploid oysters is unexplored at present.

The effect of shipping or shell drilling may also have reduced sperm quality because temperatures as high as 23 °C were measured inside the shipping box upon arrival for shipments in July 2002. In practice, it is difficult to maintain constant low temperatures (4 °C) when shipping whole oysters instead of sperm suspensions. Interactions between shipping or handling stress and gamete quality may have greater effects on the quality of sperm from tetraploid oysters than diploid ones. The highest motility observed for sperm from tetraploid oysters was 80% in this study, however, most previous oyster studies (e.g. [13,45,54,63,65]) specified motility above 95% for experiments with diploids, a value which may not be practical for the sperm from tetraploid Pacific oysters. Thus, strategies such as reducing handling stress before freezing, or improving motility after thawing with the addition of caffeine [18], should be evaluated for cryopreservation of weakly motile sperm such as for tetraploid Pacific oysters.

Dilution of samples could reduce sperm motility by removing protective components of seminal plasma, as suggested for mammals and other fishes [9,30,33,49]. A previous study of sperm from the eastern oyster, *Crassostrea virginica*, showed that sperm maintained motility best in less diluted samples, and the highest motility was obtained with undiluted samples [44]. Although the present study did not evaluate the effect of dilution on sperm motility, refrigerated storage at 4 °C for 7 days of undiluted sperm from diploid and tetraploid oysters did not show significant decreases in motility. Thus, storage or shipping of undiluted sperm at low temperature is recommended if there is to be a delay between sperm collection and freezing. Whether the refrigerated storage will affect the motility after thawing or percent fertilization will require future studies.

The osmotic pressure of the extender is important in the storage and activation of sperm from aquatic species [38,56], and is often the first factor to be evaluated before sperm cryopreservation is attempted [7,18,33,40]. Sperm motility of tetraploid Pacific oysters was found to increase with osmotic pressure, and the highest motility was observed at 1000 mOsmol/kg C-F HBSS [18]. The evaluation of C-F HBSS at 671 mOsmol/kg was designed to see whether the inhibition of sperm motility before freezing would improve survival after thawing due to the conserved energy from less active swimming. However, the results in the present study showed lower motility and fertilization of sperm samples suspended in C-F HBSS 671 than in C-F HBSS 1000, which may indicate an initial pre-freezing damage from sperm swelling under hypotonic conditions [20,39]. Ultrastructural examination of sperm cells suspended in extenders at different osmolalities will assist the understanding of osmotic effects on sperm morphology and behavior.

Determination of sperm cell concentration is necessary to standardize methods for toxicity studies of cryoprotectants before freezing, fertilization studies of sperm after thawing, and the ''freezing unit'' (number of sperm per freezing container) for commercial-scale production. Most previous reports [34–36,70,71] for diploid oysters did not identify sperm concentrations. Instead sperm volumes were presented, which hinders or prevents the reproducibility of experimental conditions. The present study showed that a sperm concentration of $1 \times 10^9$ cells/ml yielded the highest motility after thawing and fertilization regardless of using 10% PG or 8% DMSO as the cryoprotectant. The higher fertilization obtained by using sperm samples cryopreserved at $1 \times 10^9$ cells/ml may be due simply to the higher number of sperm available compared to those at lower concentrations. However, in practice a 10- to 1000-fold increase in numbers of thawed sperm is typically required in oysters to achieve fertility comparable to fresh sperm [52,64]. The sperm agglutination associated with high cell concentrations may be due to initia-

tion of the acrosomal reaction upon freezing and thawing, which releases the bindin molecules that coagulate cells with each other, however, the high fertilization (50%) associated with agglutinated sperm samples merits further study. Increased survival after thawing of sperm frozen at high cell concentrations was also found in mammalian species such as horses and dogs [37,46]. Freezing of sperm at high concentrations also reduces the number of holding units required, but whether this is practical for commercial production in oysters needs further evaluation. However, standardization of cell concentration would facilitate future research on sperm cryopreservation. Such practices would also improve the utility of cryopreservation through establishment of optimal sperm-to-egg ratios [71].

Optimal equilibration time before freezing is necessary to allow permeating cryoprotectants to penetrate the sperm while minimizing toxicity. Equilibration times of 10–20 min are most commonly used for fish semen [11], although 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 [48], and sperm of channel catfish, *Ictalurus punctatus*, equilibrated for 10 days with 5% methanol at 4 °C showed increased motility compared to that of fresh sperm [16]. The present study showed higher fertilization with a 10-min equilibration than with 20 min. Equilibration time may not affect motility after thawing, per se, but the effect of toxicity of the cryoprotectant associated with longer equilibration should be considered with regard to fertilization and subsequent larval development. Toxicity may be especially important when dealing with sperm that has low initial motility, such as sperm from tetraploid oysters, which may be more vulnerable than sperm from diploid oysters.

The present study evaluated PG and DMSO as cryoprotectants. The former cryoprotectant was extensively evaluated with the eastern oyster [45], and the latter is the most commonly used cryoprotectant in previous studies of oyster sperm cryopreservation (e.g. [13,36,64,70]). The evaluation of 5, 10, and 15% PG showed highest fertilization was obtained with 5% PG while no significant difference was found among the concentrations for the motility after thawing, which suggested that cryoprotectant toxicity may not affect motility, but could have a negative effect on fertilization. Comparisons indicated that 8% DMSO was better than 10% PG in terms of motility and fertilization for sperm from diploid and tetraploid Pacific oysters. However, it is difficult to identify which is more effective at this moment without extensive comparisons among different concentrations for these two cryoprotectants.

Motility is a simple and rapid estimator of sperm quality, and has been commonly used to assess thawed sperm despite variable correlations to fertility in invertebrates, fishes, and mammals [25,53,57]. The present study showed weak correlations between motility after thawing and fertilization. That is, sperm samples that were non-motile after thawing yielded fertilization as high as 45%. Computer-aided sperm analysis (CASA) could be used in the future to objectively estimate a variety of components beyond percent motility for the sperm from oysters because motility lasts for hours [18], unlike most teleost fishes, in which the duration of active motility is typically brief, lasting no longer than 1 or 2 min [10,23,31,59]. Factors other than motility such as membrane integrity, acrosomal integrity, and mitochondrial function can also affect fertilization [26,60]. With proper protocols, flow cytometric assays can be rapid and relatively simple to perform, and could be utilized to assess the quality of sperm from oysters and other aquatic species [50].

Overall, during the present study, motility decreased with each handling and cryopreservation step, and the highest fertilization rate obtained was 28% for thawed sperm from tetraploid oysters and 96% for thawed sperm from diploid oysters. Future research should address practical problems such as standardization of shipping and handling of sperm samples, development of a rapid and reliable method to estimate sperm concentration, selection of indicators for fertilization success, standardization of freezing units by optimization of egg-to-sperm ratio, and scaling up of existing procedures for commercial application. Of special importance to improve the protocols for cryopreservation of sperm from tetraploid Pacific oysters would be the understanding of spermatogenesis

and the selection of optimal cooling rates. Although similar optimal cooling rates (40–70 °C/min) were predicted for sperm from diploid and tetraploid oysters in a recent study of membrane transport properties using differential scanning calorimetry [32], the same study also indicated differences in membrane permeability parameters between these two types of sperm cells. Future studies should validate with empirical values the range of optimal cooling rates predicted using the water transport models.

In summary, the present study addresses for the first time cryopreservation of sperm from tetraploid Pacific oysters, and it is also the first detailed report for sperm cryopreservation for any tetraploid organism. As an initial attempt, we were able to cryopreserve the sperm from diploid and tetraploid Pacific oysters by use of commercial protocols used within the dairy bull sperm industry. Flow cytometry also confirmed that larvae produced from thawed sperm of two tetraploid males were triploid. This demonstrates that commercial-scale cryopreservation of sperm from tetraploid Pacific oysters for distribution to hatcheries for production of triploid offspring is possible. Further work as indicated above will be necessary to establish practical procedures.

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