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Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: Evidence from sperm agglutination in oysters ☆

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Abstract

A lack of standardization in sperm cryopreservation of aquatic organisms is one of the main reasons for inconsistency observed among various studies. In particular, there have been few attempts to standardize sperm concentration during procedural optimization. This study was intended to call attention to sperm concentration standardization through research of sperm agglutination in Pacific oysters *Crassostrea gigas*. Sperm agglutination after thawing is a relatively frequent phenomenon observed for various aquatic species, especially when sub-optimal cryopreservation protocols are used; however, no systematic attempts have been made to explain this phenomenon. The present study evaluated various factors affecting sperm agglutination of thawed samples from diploid and tetraploid Pacific oysters, and is the first detailed report addressing the sperm agglutination phenomenon of thawed samples from any aquatic organism. Agglutination of oyster sperm was classified into six levels with a scale ranging from 0 (homogenous suspension) to 5 (well-developed "noodles"). It was found that agglutination in thawed samples was mainly due to the lack of sufficient cryoprotectant for a specific sperm concentration. Interestingly, high levels of agglutination did not necessarily lead to low fertilization. On the contrary, some sperm cells appeared to gain protection from the formation of peripheral agglutination within 0.5-ml French straws. The exact mechanism of sperm agglutination remains unclear. However, morphological examination of cross sections of the noodles (agglutination level 5) indicated at least two forms of agglutination (formed with and without cryoprotectant) which could be used as a tool to understand the cryopreservation process within the micro-environment of the straw. Furthermore, the fact that the level of sperm agglutination was directly determined by sperm concentration, in addition to the type of cryoprotectant, cryoprotectant concentration, and cooling and thawing methods emphasized the importance of procedural standardization and systematic optimization and integration of protocols involving multiple factors.

Keywords: Sperm agglutination; Standardization; Cryopreservation; Oyster; *Crassostrea gigas*

Sperm cryopreservation has been studied for more than 230 aquatic species since the first report with the Pacific herring, *Clupea harengus*, in 1953 [4,18,31]. These research efforts have yielded protocols with variable success with different application purposes. In addition, components of cryopreservation technology among and within studies, such as initial sperm quality, gamete collection, extender, cryoprotectant, cooling, thawing, and evaluation of post-thaw sperm quality, vary considerably [26,31]. For example, a review of the previous research of sperm cryopreservation in oysters indicated a systematic lack of standardization in procedures [11]. Consequently, for aquatic species, well

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integrated protocols that can be applied with consistent levels of success across laboratories are lacking. In fact, researchers are often confronted with successful protocols that cannot be repeated, or worse, contradictory findings within a single species [31]. This research was intended to call attention to sperm concentration standardization through the study of a frequently occurring but often neglected phenomenon—sperm agglutination—during cryopreservation.

Sperm agglutination in thawed samples has been observed in aquatic species such as common carp *Cyprinus carpio* [20,29], Atlantic croaker *Micropogonias undulates* [19], Pacific oyster *Crassostrea gigas* [1,5,22], and eastern oyster *Crassostrea virginica* [21]. In bivalves, sperm cryopreservation of tetraploid oysters was initiated for the purpose of expanding the market of triploid oyster production [13], and comparative studies of sperm from diploid and tetraploid Pacific oysters revealed differences in morphology [17] and tolerance to cryopreservation [11,15]. In addition, these studies also demonstrated a sperm agglutination phenomenon with the formation in extreme cases of elongated noodle-like structures [13]. Terms used to describe this phenomenon include "clumped", "coagulation", "jelly-like agglutination", "agglutinated", "agglutination", and "sperm aggregations". In the present study, "agglutination" was used to describe this phenomenon and "noodle" was chosen to describe the extreme form of sperm agglutination due to the appearance of agglutinated sperm samples cryopreserved in 0.5-ml French straws.

Despite the awareness of sperm agglutination in thawed samples of various aquatic species, no reports have systematically examined this phenomenon. In addition, agglutination is generally considered as a negative outcome, and often thought to be an indicator of failure in cryopreservation [5,22]. However, thawed sperm samples with the appearance of "noodles" yielded fertilization as high as 50% for sperm from diploid Pacific oysters in a previous study [13]. The fact that sperm agglutination did not necessarily indicate a reduction in fertility was also reported in common carp [20].

The goal of this study was to evaluate the phenomenon of sperm agglutination in thawed sperm from diploid and tetraploid Pacific oysters. The specific objectives were to: (1) classify the degree of agglutination after thawing (visual observation on a qualitative scale), and to evaluate the effect on sperm agglutination scale of: (2) interactions among cooling rate, cryoprotectant (type and concentration), and thawing rate; (3) interactions of thawing method and dimethyl sulfoxide (DMSO) concentration; (4) interactions of sperm concentration and DMSO concentration, and to compare (5) the morphology of sperm agglutination from samples frozen and thawed with and without addition of cryoprotectant. Our findings show that the formation of sperm agglutination is mainly due to the lack of sufficient cryoprotectant for a specific sperm concentration, which calls attention to the requirement to standardize sperm concentrations prior to cryopreservation.

Materials and methods

Sperm collection and motility estimation

Tetraploid and diploid Pacific oysters were obtained during July and August of 2003 and August of 2004 from Taylor Resources Quilcene Shellfish Hatchery (www.taylorshellfish.com) in Quilcene, Washinton (47°49′133″N, 122°49′ 523″W) and were shipped chilled at 5–10 °C by overnight delivery to the Louisiana State University Agricultural Center, Aquaculture Research Station (ARS). Ploidy level of individual oysters was verified by flow cytometry [2,13]. Sperm were collected by dry stripping of the gonad [3,16] and suspended in calcium-free Hanks' balanced salt solution (C-F HBSS) at 1000 mOsm/kg [12], and suspensions were filtered through a 40-μm cell strainer (BD Biosciences Discovery Labware, Bedford, Massachusetts). The concentrations of sperm suspensions (except for the experiment addressing sperm concentration) were adjusted to 2×10^9 cells/mL using readings at 581 nm with a spectrophotometer (Genesys™ 20, Thermo Spectronic, Rochester, NY) and standard curves derived specifically for this oyster population [14]. Sperm motility was estimated visually at 200× magnification using darkfield microscopy (Optiphot 2, Nikon Inc., Garden City, NY) and was expressed as the percentage of cells actively moving in a forward direction [13].

Freezing and thawing procedures

Two freezing methods were used in this study: controlled-rate freezing and commercial freezing methods developed for dairy bulls. Freezing with a controlled-rate freezer (Kryo 10 Series II; Planer Products, Sunbury-on-Thames, UK) followed the method described in [16]. For cooling rates of 1, 5, and 16 °C per min, samples were cooled in two steps, initially to −30 °C at these rates, followed by cooling at 45 °C per min from −30 °C to −80 °C. For cooling rates of 45 and 50 °C per min, samples were cooled in a single step from 5 °C to −80 °C 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 used without modification (described in [13,16]), and straws were filled with an automated straw filler (model MRS 1, IMV Int. Corp., Minneapolis, MN).

The thawing procedures are described in detail elsewhere [11]. Briefly, thawing at room temperature (23–25 °C) was performed by placing the straws on paper towels on the laboratory bench, and allowing the straws to warm in the air for approximately 4 min. Thawing in a water bath (Model 1141, VWR Scientific, Niles, IL) was performed at various temperatures for different time periods: 13 s at 20 °C, 7 s at 40 °C, 6 s at 60 °C, and 5 s at 80 °C. Samples were thawed after a minimum of 12 h of storage in liquid nitrogen, and two straws from each treatment were thawed.

Assessment of sperm agglutination scale

Samples in 0.5-ml French straws were transferred after thawing into 35 × 10 mm tissue culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ), and the degree of sperm agglutination was classified by visual observation into six levels based on the descriptive criteria for each level (Table 1). Examples of each level were photographed using a digital camera (Coolpix 5700, Nikon).

Sperm counts after thawing

For each agglutination classification, the number of individual sperm cells that were not agglutinated (e.g., groups of two or more agglutinated cells were not counted) was estimated with a hemacytometer (Hausser Scientific, Horsham, Pennsylvania) and the average of duplicate counts was used. For samples with the formation of elongated clumps (agglutination levels of 4 and 5), non-agglutinated sperm were suspected to be trapped within the noodles, and thus, sperm counts were performed before and after crushing of the aggregates. For crushing, aggregates were disrupted gently with the end of the French straw to the point that the samples appeared to be equivalent to an agglutination level of 2 or 3. Two batches of samples from tetraploid oysters were used in this experiment with the first batch of samples from a single male and the second batch from sperm samples pooled from five males. Oysters used in this experiment were received on July 25, 2003.

Effect of cooling, cryoprotectant and concentration, thawing, and sperm type

Interactions among multiple factors were evaluated in this experiment. Sperm samples were suspended in DMSO, ethylene glycol (E-glycol), methanol (MeOH), and propylene glycol (P-glycol) at 5 and 10%, cooled at 1, 5, 16, 45, and 50 °C per min with a controlled-rate freezer, and thawed at room temperature (RT, 23–25 °C) for 4 min, or in a 40 °C water bath for 7 s. Cryoprotectant solutions were prepared within 2 h of use with C-F HBSS at 1000 mOsm/kg as the diluent and were stored at 4 °C. All treatment combinations were evaluated with sperm samples from diploid (one male) and tetraploid (milt pooled from five males) oysters. To evaluate whether there was variation in the agglutination scale of sperm from diploid and tetraploid oysters, ploidy was considered as the block in this experiment. As a result, agglutination level did not show a significant difference between the two ploidies, that is to say, sperm agglutination was independent of the ploidy level. Thus, for simplicity, graphical presentation in the results is for pooled data from both ploidies. Oysters used in this experiment were received on July 10, 2003.

Table 1
Description for sperm agglutination on a scale of six levels

Agglutination level	Description
0	Homogeneous suspension
1	Few clumps discernable
2	Many clumps evident
3	Aggregation of clumps
4	Formation of elongated clumping ("noodles")
5	Formation of well-developed noodles

Effect of thawing and DMSO concentration

Sperm samples in this experiment were equilibrated with DMSO at 2%, 5%, 8%, 10%, 12%, 15%, and 20%, cooled with commercial freezing methods, thawed at RT in the air, and in a water bath at 20, 40, 60, and 80 °C as described above. Sperm samples from diploid (one male) and tetraploid (milt pooled from two males) oysters were used. Oysters used in this experiment were received on July 10, 2003.

Effect of sperm concentration, DMSO concentration, and thawing

In this experiment, sperm samples at different concentrations were equilibrated with DMSO at 0%, 2%, 5%, 8%, 10%, 12%, and 15%, cooled with commercial freezing methods, thawed at RT in the air for 4 min and in a 40 °C water bath for 7 s. For sperm from diploid oysters, sperm concentrations of 5×10^9, 2.5×10^9, 5×10^8, 2.5×10^8, 5×10^7, 2.5×10^7 cells mL^{-1} from one male were used. For sperm from tetraploid oysters, sperm concentrations of 2×10^9, 5×10^8, 2.5×10^8, 5×10^7, 2.5×10^7 cells mL^{-1} from one male were used. Oysters used in this experiment were received on August 1, 2003.

For the three experiments above, due to their complexity (multiple factors with high numbers of treatment levels for each factor), the individual experiments were not replicated at the animal level (males), but only at the sampling unit (straws). To ensure that the results derived from high quality samples from a single male or a single batch of pooled sperm was representative for all males, subsequent experiments with a simpler design (one factor) examined a series of randomly selected treatment factors on multiple males (4–5 males), and unlike other criteria, such as motility and percent fertilization, sperm agglutination showed minimal variation among males (data not shown). In addition, results from 4 years of research in this species [e.g.,11,13] were in complete agreement with findings generated in the present study.

Microscopy examination of sperm agglutination

In this experiment, sperm samples from one male (diploid) were frozen with combined cryoprotectants of 2% polyethylene glycol and 4% methanol [16] or without any addition of cryoprotectant. Samples were thawed at RT and fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (CB, pH 7.4) with the osmolality adjusted to 1000 mOsmol/kg [17]. The subsequent steps followed those described for sample preparation for transmission electron

microscopy in [17]. Cross sections (0.5 μm) were cut with a DuPont Sorvall microtome (MT-2, Ivan Sorvall Inc., Norwalk, CT), placed on glass slides, stained with 0.05% toluidine blue O (0.05 g toluidine blue O in 100 ml 2% sodium borate) (Sigma Chemical Corp., St. Louis, MO), covered with cover slips, and sealed with Permount (Fisher Scientific, Fairlawn, NJ). Slides were examined at 200× and 800× magnification by light microscopy. Digital images were captured through a diagnostic instrument CCD camera (SPOT RT Slider, SpectraCore, Inc. Webster, NY). The oyster used in this experiment was received on August 13, 2004.

Effect of sperm agglutination on percent fertilization

The classification of agglutination scale allowed sperm agglutination to serve as an independent criterion for thawed samples in addition to post-thaw motility and percent fertilization. Consequently, thawed sperm samples with the records of agglutination scale and fertilization data could be used to evaluate the effect of sperm agglutination on percent fertilization. In this study, a total of 82 data points from 22 diploid oysters and 99 data points from 19 tetraploid oysters were sorted into the six agglutination levels defined as above based on the sample appearance after thawing. These data points were derived from samples frozen with various cryoprotectants at different concentrations, but all fertilization trials were conducted during July 2004. The detailed fertilization procedures are described elsewhere [15,16]. Briefly, fertilization trials were conducted by mixing 5 ml of thawed sperm suspension (agglutinated samples were crushed immediately after thawing) with 500,000 eggs (fresh from diploid females) held in 250 ml of seawater, yielding approximately 10,000 spermatozoa per egg. Percent fertilization was calculated by counting developing embryos at 2 h after insemination.

Data analysis

For experiments with categorical response variables (e.g., the agglutination scale in this study), data can be analyzed with either non-parametric methods (e.g., using ranked data), or logistic regression (e.g. data with counts). However, in this particular case, either method would encounter problems such as multiple ties for analysis using ranked data, and only two observations (thawing from two straws) for each combination of treatment factors when analyzed using logistic regression. In addition, although the agglutination scale was reported as discrete numbers, they were ordinal variables (categorical variables having ordered scales), and can be viewed as corresponding to the percentage of non-agglutinated sperm available after thawing. Therefore, to facilitate the data analysis, the categorical response variable of agglutination levels from 0 to 5 were translated into the continuous variable of percentage from 0% to 100% with an interval of 20%.

Data were therefore analyzed using two-way (fixed factor), three-way (fixed factor) or four-way (fixed factor) analysis of variance (ANOVA) (SAS 9.0, SAS Institute Inc., Cary, NC). When a significant difference ($\alpha = 0.05$) was observed among treatments, Tukey's Honestly Significant Difference Procedure was used for pair-wise comparisons. For percent fertilization, data were analyzed by one-way ANOVA. The Tukey–Kramer method for unequal cell sizes was used to test for differences ($\alpha = 0.05$) among results. All data were arcsine-square root transformed prior to analysis [30].

Results

Agglutination scale

Sperm samples after thawing showed various degrees of agglutination (Table 1), ranging from homogenous suspensions (Fig. 1A) to the formation in extreme cases of elongated "noodles" (Fig. 1B).

Sperm counts after thawing

Sperm counts of thawed samples with an agglutination level of 0 were considered as baseline data for the maximum numbers of sperm available after thawing. The percentage of sperm available in samples with levels of 1 and above was estimated by dividing the sperm counts by the baseline number. Samples with levels from 1 to 5 showed a decreasing trend of percent of individual sperm available after thawing with only 8% available for samples with a level of 5 (Table 2). For agglutination levels of 4 and 5, sperm counts were increased after crushing of the aggregates, especially for samples with an agglutination level of 5, in which a 25% increase in sperm number was observed after crushing (Table 2).

Effect of cooling, cryoprotectant and concentration, thawing, and sperm type

The initial sperm motility was 90% for diploid oysters and 60% for pooled milt from tetraploid oysters. Except for the ploidy effect, the four-factor interaction term, and the three-factor interaction term of cooling rate, cryoprotectant and concentration, all other terms showed significant differences (Table 3). This suggested that cooling rate, the type and concentration of cryoprotectant, and thawing each has an effect on sperm agglutination. Generally, significantly higher levels of agglutination were observed with samples thawed at RT than with those thawed at 40 °C (Figs. 2 and 3). Cryoprotectants at a concentration of 5% (Fig. 2) were also found to yield higher levels of sperm agglutination than those at 10% (Fig. 3). Among the four cryoprotectants, the level of sperm agglutination was significantly lower ($P < 0.05$) in samples equilibrated with MeOH than in samples equilibrated with DMSO, E-glycol or P-glycol. For the five cooling rates, the highest level of sperm agglutination was found for samples cooled at 1 °C per min, which was significantly higher than for

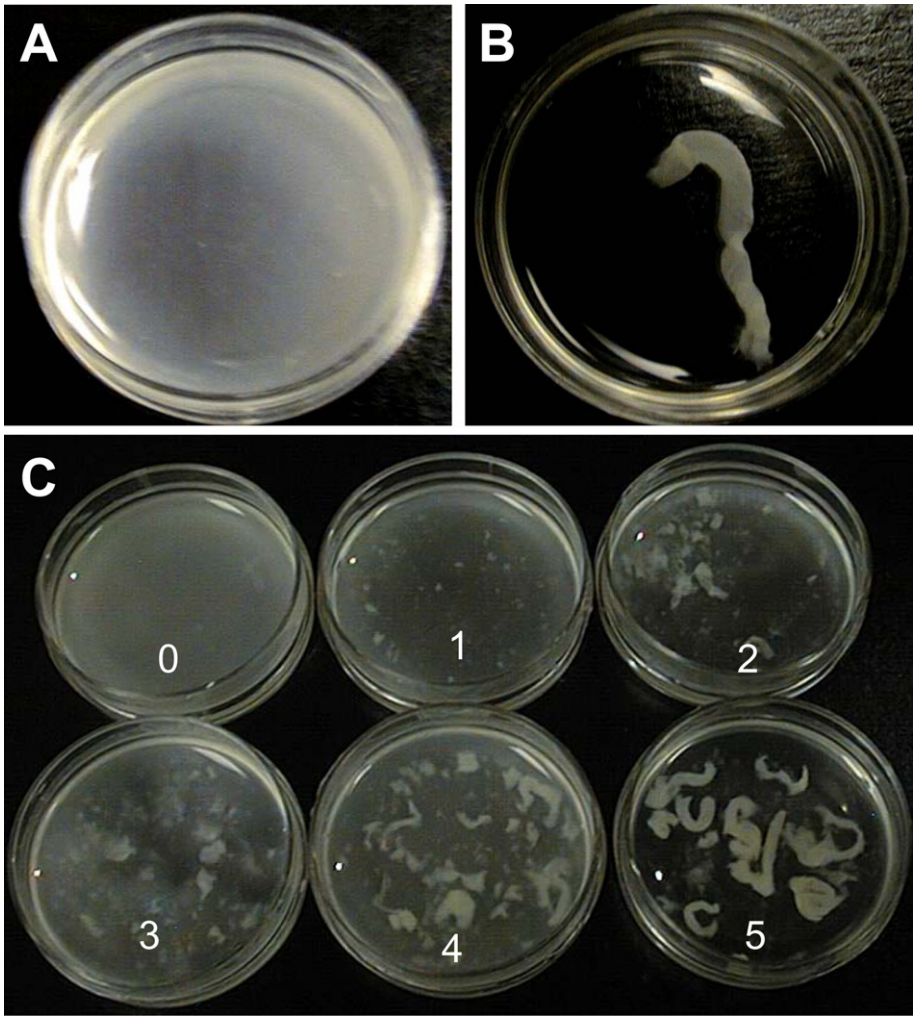

Fig. 1. The appearance of sperm samples after thawing. (A) homogenous suspension; (B) elongated "noodle"; (C) six levels of sperm agglutination: 0, homogeneous suspension; 1, few clumps discernable; 2, many clumps evident; 3, aggregation of clumps; 4, formation of elongated clumping ("noodles"); 5, formation of well-developed noodles (All tissue culture dishes were the same size with a diameter of 35 mm).

Table 2
Percentage of non-agglutinated sperm available after thawing for each agglutination level (cell counts for the agglutination level of zero were set at 100%)

Agglutination level	Before crushing (%)	After crushing (%)	Difference (%)
0	100	—[a]	—
1	74	—	—
2	75	—	—
3	55	—	—
4	40	45	5
5	8	33	25

[a] Not evaluated (only evaluated in elongated clumps).

samples cooled at other rates. The lowest level of agglutination was observed in samples cooled at 16 °C per min.

Effect of thawing and DMSO concentration

The initial sperm motility was 95% for diploid oyster and 80% for pooled milt from tetraploid oysters. For sperm samples from diploid and tetraploid oysters, the level of sperm agglutination was found to decrease significantly ($P < 0.0001$) with increased DMSO concentration, and all samples with DMSO concentrations above 12% showed agglutination levels of zero (Fig. 4). Significant differences were observed for the various thawing rates ($P < 0.0001$), and the level of agglutination decreased with increased thawing rate (from RT to 80 °C). Interactions between thawing rate and DMSO concentration were also significant ($P = 0.0035$), and samples from tetraploid oysters equilibrated with 2% DMSO consistently showed levels of 5 regardless of thawing rate (Fig. 4).

Effect of sperm concentration, DMSO concentration, and thawing

The initial sperm motility was 95% (diploid). Significant differences were found for sperm concentration ($P < 0.0001$), DMSO concentration ($P < 0.0001$) and the interactions between them ($P < 0.0001$). The levels of agglutination increased with increasing sperm concentration (Fig. 5). Agglutination levels of samples with sperm

Table 3
Statistical results for effect of cooling, cryoprotectant and concentration, thawing, and sperm type (ploidy)

Effects	Degrees of freedom	*P*-value
Cooling rate	4	<0.0001
Cryoprotectant	3	<0.0001
Concentration	1	<0.0001
Thawing	1	<0.0001
Cooling rate × Cryoprotectant	12	0.0001
Cooling rate × Concentration	4	0.0148
Cooling rate × Thawing	4	<0.0001
Cryoprotectant × Concentration	3	<0.0001
Cryoprotectant × Thawing	3	<0.0001
Concentration × Thawing	1	<0.0001
Cooling rate × Cryoprotectant × Concentration	12	0.2743
Cooling rate × Cryoprotectant × Thawing	12	0.0005
Cooling rate × Concentration × Thawing	4	<0.0001
Cryoprotectant × Concentration × Thawing	3	<0.0001
Cooling rate × Cryoprotectant × Concentration × Thawing	12	0.1223
Ploidy	1	0.0616
Error	159	

concentrations of 5×10^9, 2.5×10^9, and 5×10^8 cells mL^{-1} were not significantly different from one another ($P > 0.05$), but they were significantly higher than those with 2.5×10^8 cells mL^{-1} ($P < 0.05$). Agglutination levels of zero were observed for samples with 5×10^7 and 2.5×10^7 cells mL^{-1}. Similar to the previous experiment, the level of agglutination decreased significantly ($P < 0.0001$) with increased DMSO concentration for samples thawed at RT or 40 °C (Fig. 5). Sperm samples equilibrated with 2% DMSO yielded the same agglutination level as those without the addition of cryoprotectant, and again all samples with DMSO concentrations of above 12% showed a level of zero agglutination regardless of the sperm concentrations within the tested range. However, no significant differences were found between thawing at RT and 40 °C ($P = 0.0769$), or for the interactions between sperm concentration and thawing ($P = 0.6543$), DMSO concentration and thawing ($P = 0.0658$), and the three-factor interaction term ($P = 0.9809$).

The initial sperm motility of the tetraploid oysters was 95%. For sperm concentration and DMSO concentration, patterns similar to those of diploids were observed for sperm samples from tetraploid oysters (Fig. 6). Agglutination levels of samples with sperm concentrations of 2×10^9 and 5×10^8 cells mL^{-1} were not significantly different from one another ($P > 0.05$), but they were significantly higher than those with 2.5×10^8 cells mL^{-1} ($P < 0.05$). An agglutination level of zero were observed for samples with 2.5×10^7 cells mL^{-1} and which was significantly lower than for those with 5×10^7 cells mL^{-1} ($P < 0.05$). All terms, including treatment effects and interactions, were found to be significant ($P < 0.05$) in this experiment.

Microscopy examination of sperm agglutination

As seen from previous experiment, sperm agglutination levels of 5 were observed in samples frozen with or without cryoprotectants. The morphology of these two types of "noodles" was examined microscopically after they were

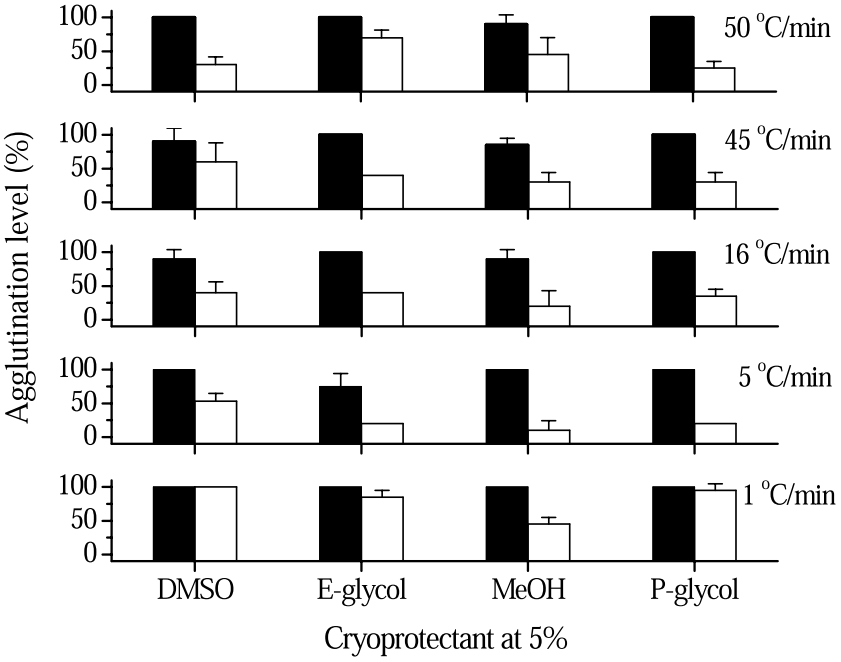

Fig. 2. Agglutination level (mean ± SD) of sperm samples equilibrated with dimethyl sulfoxide (DMSO), ethylene glycol (E-glycol), methanol (MeOH), and propylene glycol (P-glycol) at 5%, cooled at 1, 5, 16, 45, and 50 °C per min with a controlled-rate freezer, thawed in the air at room temperature for 4 min (black bars) or in a water bath of 40 °C for 7 s (white bars).

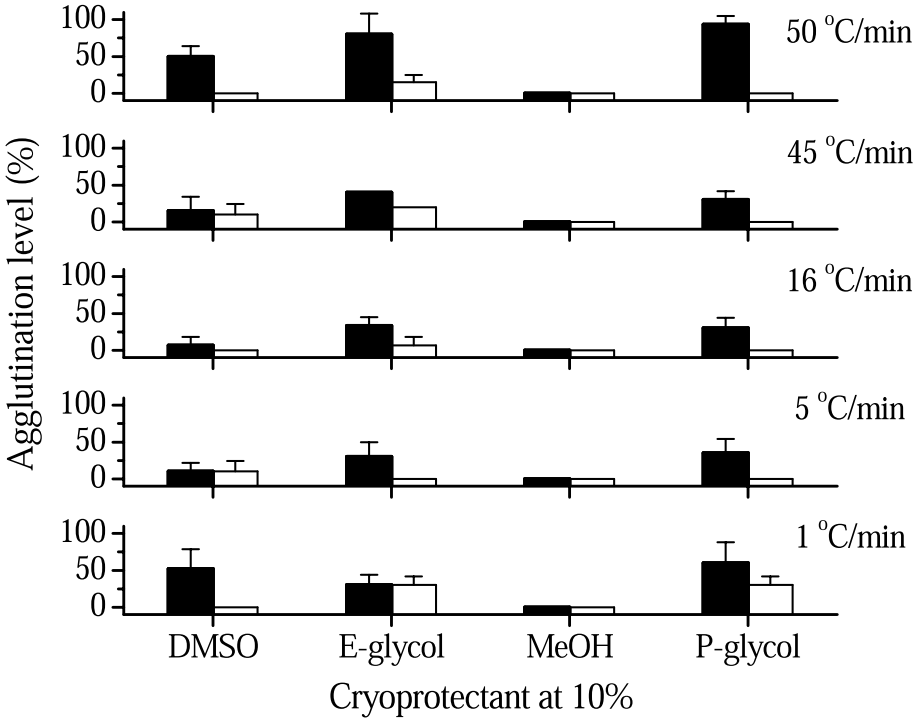

Fig. 3. Agglutination level (mean ± SD) of sperm samples equilibrated with dimethyl sulfoxide (DMSO), ethylene glycol (E-glycol), methanol (MeOH), and propylene glycol (P-glycol) at 10%, cooled at 1, 5, 16, 45, and 50 °C per min with a controlled-rate freezer, thawed in the air at room temperature for 4 min (black bars) or in a water bath of 40 °C for 7 s (white bars).

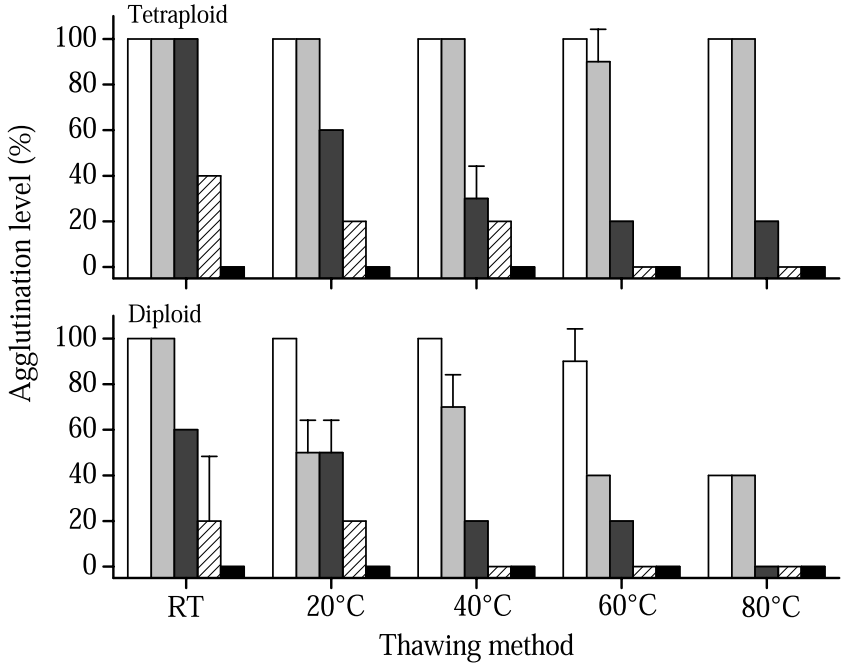

Fig. 4. Agglutination level (mean ± SD) of sperm samples from diploid and tetraploid oysters cryopreserved with dimethyl sulfoxide at 2% (white bars), 5% (light grey bars), 8% (dark grey bars), 10% (hatched bars), 12%, 15% and 20% (black bars), thawed in the air at room temperature for 4 min (RT), or in a water bath at 20 °C for 13 s (20 °C), 40 °C for 7 s (40 °C), 60 °C for 6 s (60 °C), and 80 °C for 5 s (80 °C).

fixed. The initial sperm motility was 50% (diploid) in this experiment. Morphological differences of the cross sections of "noodles" formed with (Fig. 7A and B) and without (Fig. 7C and D) the addition of cryoprotectant for samples from the same male were distinguishable. For "noodles" formed with the addition of cryoprotectant, few large empty areas were observed (arrows in Fig. 7A and B), and the majority of sperm heads retained their round shapes and appeared to be distributed randomly (Fig. 7B). On the contrary, "noodles" formed without the addition of

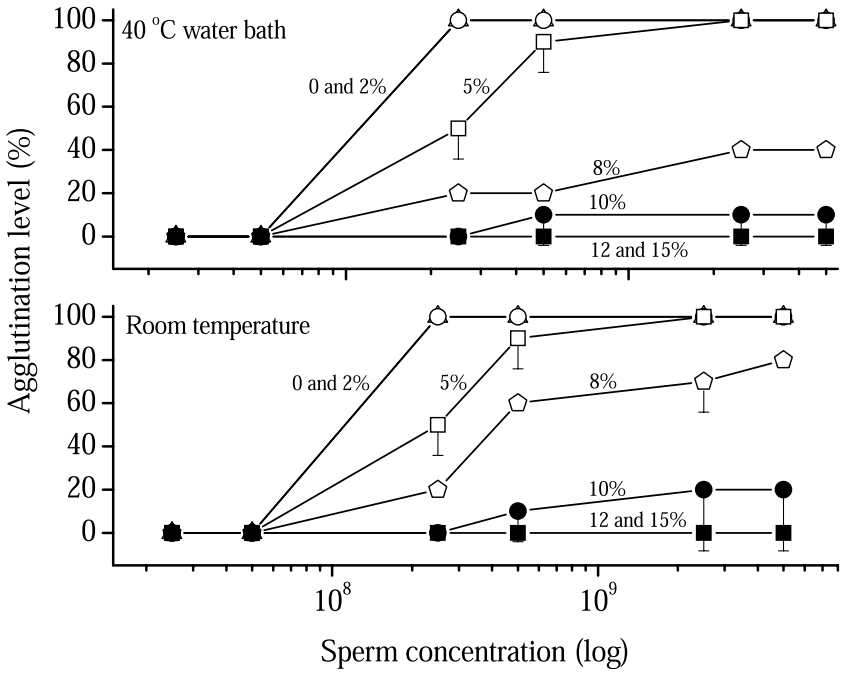

Fig. 5. Agglutination level (mean ± SD) of sperm samples from diploid oysters at sperm concentrations of 5×10^9, 2.5×10^9, 5×10^8, 2.5×10^8, 5×10^7, 2.5×10^7 cells mL^{-1}, cryopreserved with dimethyl sulfoxide at 0%, 2%, 5%, 8%, 10%, 12%, and 15%, thawed in the air at room temperature for 4 min, or in a water bath at 40 °C for 7 s.

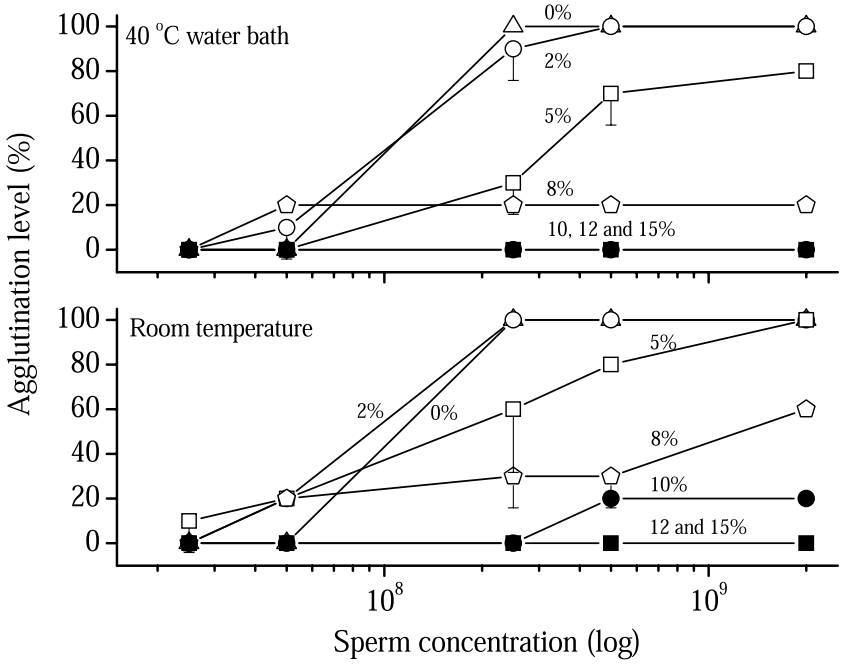

Fig. 6. Agglutination level (mean ± SD) of sperm samples from tetraploid oysters at sperm concentrations of 2×10^9, 5×10^8, 2.5×10^8, 5×10^7, 2.5×10^7 cells mL^{-1}, cryopreserved with dimethyl sulfoxide at 0%, 2%, 5%, 8%, 10%, 12%, and 15%, thawed in the air at room temperature for 4 min or in a water bath at 40 °C for 7 s.

cryoprotectant were characterized by the presence of large empty areas (arrows in Fig. 7C and D) and lysed cells with few discernable sperm heads (Figs. 7D). The empty areas observed in the cross sections (arrows in Fig. 7) could be due to formation of large ice crystals during cryopreservation.

Effect of sperm agglutination on percent fertilization

Samples with an agglutination level as high as 5 after thawing yielded fertilization as high as 96% in diploids and 48% in tetraploids (Table 4). For sperm from diploid oysters, there was no significant difference in percent fertilization among the

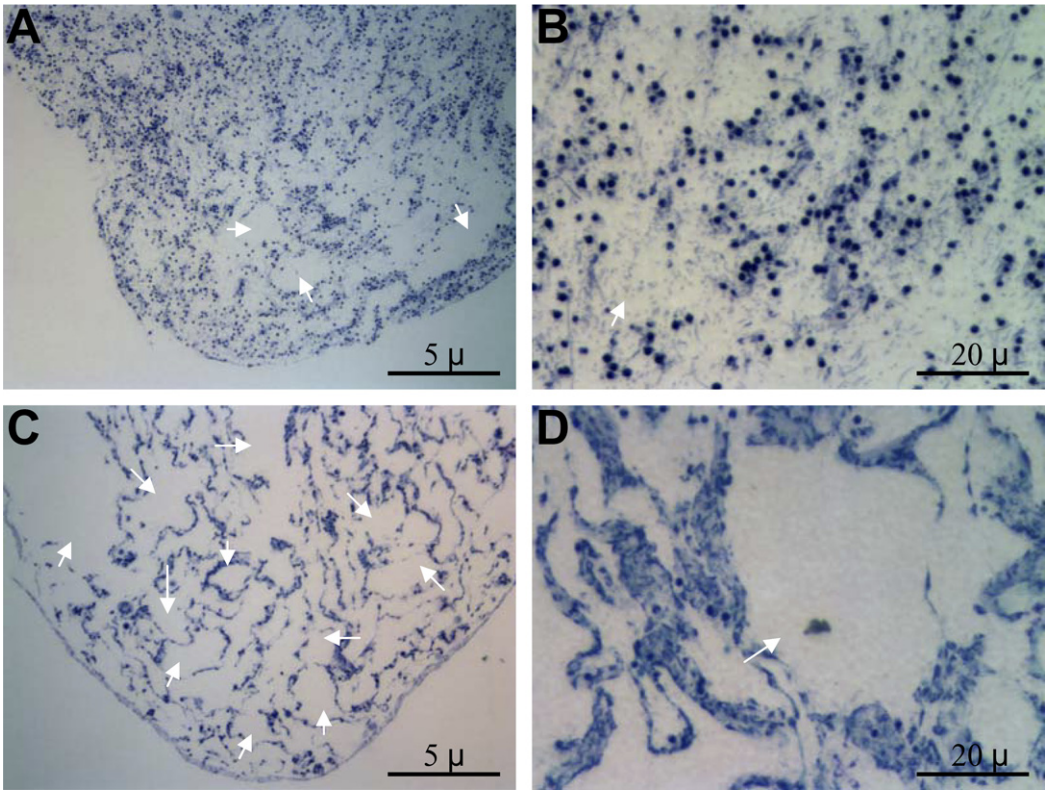

Fig. 7. Microscopic examination of diploid oyster sperm samples with "noodle" formation (agglutination level of 5) after thawing. Cross sections of "noodles" formed in samples with the combination of cryoprotectants (4% methanol and 2% polyethylene glycol) at 200× magnification (A) and at 800× (B), and "noodles" formed in samples without the addition of cryoprotectant at 200× (C), and 800× (D). Arrows indicate empty areas where ice crystals presumably formed during cryopreservation.

Table 4
Percentage fertilization of samples from diploid and tetraploid oysters with different agglutination levels after thawing

Level	Diploid			Tetraploid		
	Mean ± SD	Range	Sample size	Mean ± SD	Range	Sample size
0	27 ± 33[a]	1–86	11	6 ± 11[a]	0–45	35
1	40 ± 36[a]	1–90	8	7 ± 6[a]	0–21	20
2	42 ± 36[a]	1–98	11	10 ± 10[a]	0–30	16
3	59 ± 36[a]	4–93	13	3 ± 2[a]	0–5	10
4	30 ± 20[a]	2–62	17	14 ± 17[a]	1–44	5
5	44 ± 35[a]	0–96	22	13 ± 13[a]	2–48	13

There was no significant difference in percent fertilization among the six agglutination levels for sperm from diploids ($P = 0.1659$), and significant differences were found in sperm from tetraploids ($P = 0.0385$) though subsequent Tukey–Kramer analysis failed to differentiate a difference among the six levels.

[a] The superscript "a" indicates the result of the statistical testing by Tukey- Kramer analysis (all values received an "a" because there was no difference among them).

six agglutination levels ($P = 0.1659$). For sperm from tetraploid oysters, although a significant difference was observed for agglutination levels ($P = 0.0385$), subsequent Tukey–Kramer analysis failed to differentiate a difference among the six levels.

Discussion

The present study evaluates for the first time the various factors affecting sperm agglutination of thawed samples from diploid and tetraploid oysters, and it is also the first detailed report addressing the sperm agglutination phenomenon of thawed samples from any aquatic organism. Sperm agglutination was classified into six levels ranging from 0 (homogenous suspension) to 5 (well-developed "noodles"). The results clearly demonstrate that cryoprotectant at low concentrations, cooling and thawing at slow rates, and freezing samples at a sperm concentration of greater than 1×10^8 cells per mL led to the formation of higher levels of sperm agglutination. Based on these findings, the formation of sperm agglutination is concluded to be mainly due to the lack of sufficient cryoprotectant for a specific sperm concentration although sub-optimal cooling and thawing rates could cause the effect as well, which was further evidenced by the fact that sperm samples frozen without the addition of cryoprotectant always resulted in the highest level of agglutination. These findings were in agreement with agglutination of thawed semen of Atlantic croaker observed in samples of undiluted semen and in samples that contained either 3% sodium citrate or 1% NaCl extender without DMSO [19].

The effects of cryoprotectant (type and concentration), cooling, thawing and their interactions are the major focus of most studies in sperm cryopreservation. The classification of sperm agglutination presented in this study enables visualization of the effects of these parameters and their interactions. For sperm from diploid and tetraploid oysters,

DMSO concentrations above 12% were found to prevent agglutination (a level of zero) in samples with sperm concentrations ranging from 2.5×10^7 to 5×10^9 cells mL^{-1}. Similarly, samples with sperm concentrations of 5×10^7 cells per mL or lower did not show visible agglutination regardless of the DMSO concentration. Various cryoprotectants at the same concentration were found to have different effects on the level of agglutination. The present study showed that methanol was associated with lower levels of agglutination. Glycerol was found to cause sperm agglutination in previous studies with Pacific oyster [5] and common carp [29]. A higher agglutination level was observed for glycerol when compared with DMSO at the concentrations of 5% and 10% (Dong, unpublished data). Cooling and especially thawing at slower rates were found to favor the formation of high levels of agglutination. For example, thawing at room temperature in the air consistently yielded high levels of agglutination. In addition, sperm from diploid and tetraploid oysters responded differently in agglutination level at various combined treatments of DMSO concentration and thawing method (Fig. 4). Besides the factors evaluated in this study, other factors such as extender composition may also affect the level of sperm agglutination. For example, sodium citrate was considered to cause agglutination of thawed semen from Atlantic croaker as it occurred in samples diluted in 3% sodium citrate extender with 15% DMSO, while no agglutination was observed for samples diluted in other extenders with the addition of 15% DMSO [19]. The fact that sperm agglutination level varied with different treatment factors or their combinations confirmed the importance of protocol optimization for the multiple factors involved in sperm cryopreservation.

For better-studied species, inconsistency among various reports is common and is often probably related to cryoprotectant concentration rather than the type of cryoprotectant. This is especially true for sperm cryopreservation in Pacific oysters where DMSO has been the main cryoprotectant used in all studies, and the concentration of DMSO considered to be optimal has varied from 5% to 20% among studies [1,11]. In the present study, differences in sperm concentration were found to affect the level of sperm agglutination for samples after thawing. It is likely that the lack of standardized initial sperm concentration leads to variation in results, especially in relation to the percent of cryoprotectant that offers best protection. This is highly relevant given the fact that most cryopreservation studies in all aquatic species have not standardized sperm concentration prior to freezing, instead, using methods such as dilution ratios based on volume. For example, in the most frequently studied group of aquatic organisms, salmonid fishes, examination of 27 published reports (between 1978 and 2003) revealed the use of dilution ratios (mainly 1:3 of sperm-to-extender) for all studies without control of sperm concentration. For the most frequently studied invertebrate, oysters, a recent review identified the failure of sperm concentration standardization in 75% of the 16 peer-reviewed journal articles [11]. These studies in particular were characterized by inconsistent or conflicting results. However, in contrast, in two separate studies of *C. gigas* comparing DMSO concentrations of 5, 10, and 15% [28] and 5, 8, and 10% (Dong, unpublished data) each indicated the optimal DMSO concentration to be 5% when the same sperm concentration 1×10^9 cells mL^{-1}) was used for freezing.

In the present study, samples frozen at a high sperm concentration (e.g., $>1 \times 10^8$ cells mL^{-1}) with low cryoprotectant concentrations (e.g., 2% DMSO) or without addition of cryoprotectant were each found to form the highest level of sperm agglutination (5; noodles) when thawed at suboptimized conditions such as at room temperature. However, microscopic examination revealed that these noodles were of two different types. Agglutination produced without addition of cryoprotectant was found to be associated with the formation of large open areas and sperm that exhibited morphological damage. On the contrary, cross sections of agglutination formed with addition of cryoprotectant at low concentrations showed sperm heads that retained their round shapes, and the presence of few large open areas. This observation provides direct evidence of cryoprotective action for maintenance of sperm morphology during freezing and thawing. Micrographic differences of the cross sections from these two types of noodles (formed with and without cryoprotectant) also suggested that variations in other treatment factors such as cooling rate, or types of cryoprotectants could also produce differences in the cross sections, and thus could be detected by using the same technique, but future studies are required to confirm this.

The micrographs presented in this study are analogous to those obtained with freeze fracture electron micrographs of cryopreserved human sperm (http://www.asymptote.co.uk/gallery/spermcryopreservation), in which a cross fracture of the straw (0.25-ml) followed by deep etching revealed the structure of ice (etched areas) and of the freeze-concentrated materials including sperm (non-etched material). The etched areas in these micrographs would correspond to the open areas in this study, suggesting the presence of ice crystal formation during cryopreservation processes, or more likely due to the recrystallization resulting from slow thawing (RT). Freeze fracture is the only technique currently available that enables visual observation of samples within the micro-environment of the straw, and the samples observed are in the frozen state when using this technique. Sperm agglutination, specifically noodle formation, helps to retain the spatial micro-structure of samples within the straw (Fig. 1B) even after thawing, and would provide a new tool to observe the straw micro-environment and infer the processes that occur during cryopreservation. Morphological appearances obtained with the cross sectioning of "noodles" would represent the final outcome of the freezing and thawing process. Future studies should evaluate the possibility to quantify the morphologic differences using image analysis. Sperm agglutination could thus be an important tool to understand the

cryopreservation process within the micro-environment of the straw. However, molluscan (acrosomal) sperm may be necessary to take advantage of this new research opportunity.

Although the present study clearly demonstrated the effects of cryoprotectant (type and concentration), cooling, thawing, and sperm concentration on the formation and level of sperm agglutination for thawed sperm from oysters, the exact mechanism of how sperm agglutinate remains unclear. In mammals, serum-induced head-to-head sperm agglutination has been observed under a variety of conditions [23,25,27,32,33], however, this phenomenon only occurs between viable sperm [25], and biocolloidal relationships between serum proteins and specialized regions of the cell membrane were proposed to be the cause [7]. In aquatic species, early studies addressing development of fertilization theory revealed the role of the acrosome reaction in sperm agglutination for starfish, sea urchin, and bivalves upon exposing sperm to egg-water and other stimuli [e.g.,8–10,24]. Therefore, agglutination in oyster sperm after thawing is likely due at least in part to progression of the acrosomal reaction upon thawing because unlike most species of fish (sturgeons being a notable exception) oyster sperm possess a functional acrosome [6,17]. However, agglutination has been observed for non-acrosomal fish sperm, for example, the Atlantic croaker [19]. In these cases it is likely that the physical processes of freezing and thawing cause the sperm cell membrane to rupture, especially when there is no cryoprotectant or a low concentration of cryoprotectant, and the ruptured sperm release cytoplasmic agents (e.g. peptides, or enzymes) that agglutinate sperm. Future studies are required to uncover the range of mechanisms of sperm agglutination in aquatic species. In addition, the classification for sperm agglutination presented in this study was based on a macroscopic scale with visual observation. Homogeneous suspensions (i.e., described as having no agglutination) might reveal some level of sperm agglutination if systematic observations were made microscopically.

It is especially noteworthy that high levels of agglutination did not necessarily lead to low fertilization. The present study also showed lower percentages of non-agglutinated sperm available with the higher levels of agglutination. However, fertilization as high as 96% was observed for thawed samples with the agglutination level of 5 from diploids and 48% from tetraploid oysters. Thus, sperm agglutination was found to have no negative effect on percent fertilization in this study. This was in agreement with a recent report for sperm of common carp, in which "the jelly-like agglutination" observed after thawing in samples frozen with sugar-based extenders did not reduce fertilization and hatching rate [20]. In the present study, high post-thaw motility (>30%) was observed for the non-agglutinated portion of samples with an agglutination level of 5 after crushing. Similar to this, motile sperm (5–15%) were also found in "coagulated" sperm samples from the common carp [29]. Apparently, sperm viability was relatively high in samples with high agglutination levels considering the low percent of non-agglutinated sperm available after thawing. Thus, in sub-optimal conditions, some sperm cells appear to gain protection from the formation of agglutination, which may also explain why some sperm, although at a very low percentage, survived freezing in samples even without addition of any cryoprotectant [1].

Results presented in this study call attention to the requirement to standardize sperm concentrations prior to cryopreservation; otherwise reporting of cryoprotectant concentration or molarity offers little value and can be misleading. It remains to be determined if the processes underlying sperm agglutination during cryopreservation observed in samples from diploid and tetraploid oysters are the same as that observed in other aquatic species. Unlike most anacrosomal fish sperm, the formation of sperm agglutination in oyster sperm is likely produced by the acrosome reaction, and could provide an important tool to understand the cryopreservation process within the micro-environment of the straw. In those species for which sperm do not readily agglutinate, sub-optimal conditions could yield similar micro-environment patterns, however they would be disrupted after thawing and simply yield a mixture of damaged and undamaged sperm in suspension. Therefore, increased understanding of sperm agglutination in oysters could benefit sperm cryopreservation in other species and reveal fundamental mechanisms to improve our understanding of the cryobiology of freezing and thawing.

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